TOXICOLOGICAL AND CLINICAL INVESTIGATIONS OF METAMIZOLE-ASSOCIATED NEUTROPENIA

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„La meilleure façon de ne pas avancer est de suivre une idée fixe."

— Jacques Prévert

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SUMMARY

Metamizole is a non-opioid analgesic, antipyretic, and spasmolytic prodrug, which is widely prescribed in certain countries due to its good efficacy and low gastrointestinal toxicity. Despite the favorable safety profile overall, metamizole has been banned in several countries due to reports of metamizole-associated neutropenia, a severe and potentially fatal decrease of circulating neutrophil granulocytes. In Switzerland and Germany, metamizole use has increased over the last fifteen years even though it has been restricted to narrow indications.

The aim of this PhD project was to elucidate the underlying toxicological mechanisms of metamizole-associated neutropenia. The gained knowledge could lead to a better understanding of the cellular mechanisms of metamizole-associated neutropenia and improve the safety of metamizole treatment. Hence, this thesis is composed of toxicological *in vitro* investigations of direct metamizole metabolite toxicity on neutrophils and neutrophil progenitor cells as well as of clinical investigations composed of an observational case-control study.

For the *in vitro* part of this thesis, I investigated the cytotoxicity of metamizole and its main metabolites on the promyelocytic cell line HL60 in comparison with mature neutrophils. To form potentially cytotoxic secondary metabolites, I combined the metamizole metabolites with components of the neutrophil antioxidative system. Furthermore, I assessed potential formation of cytotoxic metabolites after combination of metamizole metabolites with various iron compounds found in neutrophils and blood. None of the tested metamizole metabolites was toxic in any cell line. The main metamizole metabolite N-methyl-4-aminoantipyrine (MAA) even reduced cytotoxicity of the myeloperoxidase substrate hydrogen peroxide at low concentrations (< 50 μM), but increased cytotoxicity at a concentration of 100 μM hydrogen peroxide. In contrast, neutrophil granulocytes were resistant to any tested hydrogen peroxide concentration and MAA. Furthermore, MAA did not increase the toxicity of the iron compounds lactoferrin, hemoglobin or methemoglobin in HL60 cells. However, the hemoglobin degradation product hemin was toxic on HL60 cells and cytotoxicity was increased by MAA. The radical scavengers N-acetylcysteine and glutathione as well as the iron chelator ethylenediaminetetraacetic acid (EDTA) were able to reduce the toxicity of hemin and MAA-hemin. The interaction between the trivalent iron ion of hemin and MAA was displayed in the absorption spectrum of hemin. The spectrum changed concentration-dependently after addition of MAA, suggesting an interaction between hemin iron and MAA. The corresponding NMR of the combination MAA and hemin revealed the formation of a stable MAA reaction product with a reaction pathway involving the

formation of an electrophilic intermediate. Hence, the main metamizole metabolite MAA increased the cytotoxicity of hemin by a reaction involving the formation of an electrophilic metabolite, which elicits apoptosis in promyelocytic HL60 cells but not in mature neutrophil granulocytes. These results suggest that the cellular antioxidative defense system and/or heme-metabolizing capacity changes during neutrophil differentiation, rendering mature cells less susceptible to the reactive MAA intermediate. Thus, I assessed the toxicity of hemin and MAA on differentiating HL60 cells that were differentiated into mature neutrophils over 5 days. After 3 days differentiation, the cell population was predominantly metamyelocytic and resistant against MAA-hemin, whereas hemin alone was still cytotoxic. At 5 days of differentiation, when the cell population consisted mainly of mature neutrophils, hemin was not toxic anymore. These results were compared with immature myeloid cells from umbilical cord blood, representing early neutrophil precursor cells, which were differentiated over 14 days into the neutrophil lineage. Similarly to promyelocytic HL60 cells, MAA-hemin was more toxic than hemin alone on immature myeloid cells from umbilical cord blood. However, no cytotoxicity was observed on freshly isolated human neutrophils. During differentiation of HL60 cells, the protein expression of enzymes responsible for hemin metabolism increased. Inhibition of the heme-metabolizing enzymes heme oxygenase-1 or cytochrome P450 reductase increased hemin and MAA-hemin toxicity in undifferentiated HL60 cells. In differentiated HL60 cells, only hemin was cytotoxic. Furthermore, protein expression of enzymes involved in defense against superoxide radicals and hydrogen peroxide degradation increased with HL60 cell differentiation. Accordingly, the cellular glutathione pool, which represents the non-enzymatic antioxidative defense system, increased in parallel with HL60 cell differentiation. Hence, the resistance of differentiated HL60 cells is associated with the development of heme metabolism and of the antioxidative defense system including the cellular glutathione pool.

The observational case-control study aimed to identify possible risk factors for the development of neutropenia associated with metamizole use. Therefore, 48 patients with metamizole-associated neutropenia treated at the University Hospitals Basel and Bern (2005-2017) were characterized and compared with 39 tolerant controls who took metamizole for at least 28 days perpetually without developing neutropenia. Neutropenia patients were subdivided into inpatient or outpatient cases who had developed neutropenia after metamizole treatment in the hospital or at home, respectively. Hence, outpatient cases were compared in more detail with tolerant control patients who had also received metamizole in an outpatient treatment setting. Due to the similar treatment circumstances, it was possible to analyze risk factors in a regression-based model. The main finding of this study was the increased frequency of acute infections among neutropenia patients before neutropenia diagnosis compared to tolerant control patients. There was no

association between the development of neutropenia after metamizole treatment and non-myelotoxic and non-immunosuppressive co-medication ($p=0.6627$), history of drug allergy ($p=0.1304$), or preexisting auto-immune diseases ($p=0.2313$). Thus, acute infections may increase the risk to develop neutropenia during metamizole treatment. But since it cannot be distinguished for all case patients, whether this is a consequence of or a risk factor for neutropenia, further investigation is needed.

In conclusion, the main metamizole metabolite MAA can form an electrophilic intermediate in presence of hemin and potentially also in presence of other highly oxidative compounds. Thus, weak antioxidative defense, low heme-metabolizing capacity, or increased generation of free heme or other highly oxidative compounds, as it might occur during infections, may render cells more susceptible to MAA toxicity and therefore facilitate neutropenia development.

ABBREVIATIONS

AA	4-aminoantipyrine
AAA	N-acetyl-4-aminoantipyrine
ADR	Adverse drug reaction
ATP	Adenosine triphosphate
COX	Cyclooxygenase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAA	N-formyl-4-aminoantipyrine
G-CSF	Granulocyte-colony stimulating factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IC_{50}	Half maximal inhibitory concentration
MAA	N-methyl-4-aminoantipyrine
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
NSAID	Nonsteroidal anti-inflammatory drug
ROS	Reactive oxygen species
SOD	Superoxide dismutase
STEAP	Six transmembrane epithelial antigen of the prostate

1. INTRODUCTION

1.1. Metamizole

1.1.1. History and background

Metamizole (dipyrone) was first synthesized by the German company Hoechst AG in 1920 and was available over-the-counter in many countries worldwide until the 1970s (Nikolova et al. 2012). Due to several reports of cases with fatal agranulocytosis, metamizole was withdrawn from the market in certain countries (e.g. Australia 1965, Norway 1976, USA 1977, Denmark 1979) whereas its use has been restricted to second-line treatment and reclassified as prescription drug in other countries (e.g. Germany 1987, Spain 1989, Switzerland 1992) (UnitedNations 2005). To evaluate the actual risk of metamizole-associated neutropenia, several studies have been performed. The International Agranulocytosis and Aplastic Anemia Study (IAAAS), a population-based case-control study was conducted in the 1980s to assess the risk-benefit profile of various analgesics (IAAAS 1986). The study included agranulocytosis cases and controls from several European countries, resulting in an estimated excess risk for any exposure to metamizole in a one-week period of 1.1 per million. Strikingly, the incidences varied depending on the country, which was reflected in later studies investigating metamizole-associated neutropenia risk. This resulted in locally different incidence rates. In Sweden, metamizole had been banned in 1974 due to agranulocytosis reports and was reintroduced to the market in 1995 based on the IAAA study. After several new agranulocytosis cases, metamizole has again been withdrawn from the Swedish market in 1999 (UnitedNations 2005). A local Swedish study reported an incidence rate of approximately 1:1500 prescriptions, which were based on eight community cases and over 10'000 prescriptions in the period of 1995 to 1999 (Hedenmalm and Spigset 2002). In contrary, a Spanish study including several hematology units in the greater area of Barcelona, reported an incidence of 0.56 cases per million inhabitants and year (Ibáñez et al. 2005). These results are in line with a recent study from Switzerland, where an incidence of around 1 case per million person-days of use was found for metamizole use between 2006 and 2012 (Blaser et al. 2015). The apparent differences between the studies might derive from the different study designs and inclusion criteria used in these studies. Additionally, a genetic predisposition could account for the observed regional differences.

1.1.2. Properties

Chemically, metamizole is a pyrazole consisting of an antipyrine substituted at C-4 by a methyl(sulfomethyl)amino group (EMBL-EBI 2018). Metamizole is a prodrug, which after application is immediately demethylated in the gut or mesenteric and portal circulation to its main metabolite N-methyl-4-aminoantipyrine (MAA). MAA is the main active metamizole derivative with a high oral bioavailability of >80% and elimination half-life of 3.3 hours (Documed 2017; Ergun et al. 2004). MAA can either be demethylated to the less active metabolite 4-aminoantipyrine (AA) or formylated to the inactive metabolite N-formyl-4-aminoantipyrine (FAA) (Levy et al. 1995). Demethylation of MAA is catalyzed by a combination of CYP3A4, 2B6, 2C8, and 2C9. Additionally, myeloperoxidase (MPO), which is contained in neutrophils, is able to demethylate MAA in presence of hydrogen peroxide. The demethylation capacity of neutrophils and MPO containing neutrophil precursor cells was found to be considerably larger than the hepatic capacity and sufficient to account for the entire demethylation of MAA in humans (Bachmann et al. 2018). The demethylation product AA can be further acetylated to the inactive metabolite N-acetyl-4-aminoantipyrine (AAA) (Geisslinger et al. 1996). All four metabolites are excreted predominantly in the urine (Zylber-Katz et al. 1992). Besides these four main metabolites, additional minor metabolites have been described and some metabolites are still unknown (Rogosch et al. 2012; Volz and Kellner 1980).

1.1.3. Mechanism of action

Metamizole is used in human and veterinary medicine due to its analgesic, antipyretic and spasmolytic effects (Blaser et al. 2015; Jasiecka et al. 2014; Sanchez et al. 2002). Although still under debate, it is likely that the mode of action includes several different mechanisms.

1.1.3.1. Analgesic effect

Metamizole has been shown to inhibit cyclooxygenase (COX) and thus decreases prostaglandin synthesis (Campos et al. 1999; Chandrasekharan et al. 2002; Hinz et al. 2007; Pierre et al. 2007). Whereas some studies showed equipotent inhibition of COX-1 and COX-2 by metamizole, others demonstrated lower IC_{50} values for COX-2 inhibition compared to COX-1 (Campos et al. 1999; Hinz et al. 2007; Pierre et al. 2007). Additionally, COX-3 inhibition by metamizole has been shown to be more potent than COX-1 and COX-2 inhibition (Chandrasekharan et al. 2002). The underlying mechanism of COX inhibition has been investigated intensively. It has been suggested that the main metamizole metabolite MAA targets the initiation of the catalytic reaction of COX-1 and COX-2 by reducing the higher oxidation states of COX (Pierre et al. 2007). In addition, MAA and AA can form metabolites with the COX substrate arachidonic acid that were

tested positive for COX-1 and COX-2 inhibition and cannabis receptor binding (Rogosch et al. 2012). The latter would lead to analgesic and spasmolytic effects through the endocannabinoid system. Various additional modes of action have been proposed, including interactions with glutamate-dependent transmission and a modulating effect of glutamate-induced hyperalgesia (Beirith et al. 1998; Siebel et al. 2004), potential release of noradrenaline and therefore interaction with the adrenergic system (Silva et al. 2015), and activation of ATP-sensitive potassium channels as well as large- and small-conductance calcium-activated potassium channels (Alves and Duarte 2002; Ortiz et al. 2003).

1.1.3.2. Spasmolytic effect

The spasmolytic effect of metamizole is assumed to affect the vascular smooth muscles. Proposed mechanisms are metamizole affecting ATP sensitive potassium channels by angiotensinogen II inhibition resulting in vasodilatation (Valenzuela et al. 2005) or inhibition of intracellular calcium release by direct inhibition of phospholipase C or impairment of phospholipase C activation by G protein-coupled receptor (Gulmez et al. 2006).

1.1.3.3. Antipyretic effect

Metamizole exerts a strong antipyretic effect, whose mechanism is still not understood. It has been assumed that metamizole acts centrally on the hypothalamic heat-regulating center (Nikolova et al. 2012). Furthermore, COX inhibition and thus decreasing prostaglandin E_2 synthesis in the CNS has been proposed as mechanism of the antipyretic effect. Recent studies showed inhibition of prostaglandin E_2 synthesis in the hypothalamus by metamizole or its metabolites MAA and AA in parallel with reduction of fever (Kanashiro et al. 2009; Malvar Ddo et al. 2014). Additionally, metamizole and MAA have been linked to prostaglandin-independent antipyretic effects but with still unknown mechanism (Malvar Ddo et al. 2014; Malvar Ddo et al. 2011). Accordingly, metamizole has been shown to reduce fever induced by lipopolysaccharides, TNF-α, interleukin-1β, interleukin-6, and the release of corticotrophin releasing hormone from the hypothalamus (de Souza et al. 2002).

1.1.3.4. Effect on platelet aggregation

The effect of metamizole itself on platelet aggregation and thromboxane A2 synthesis as well as potential interactions with other platelet aggregating drugs has been studied intensively. Several studies showed effective inhibition of platelet aggregation and thromboxane A2 formation for 6 hours in patients treated with metamizole (Graff et al. 2007; Papp et al. 2014). Various case-control studies evaluated the risk of upper gastrointestinal bleedings and most of them reported a significantly increased risk associated with metamizole, which was generally lower than for nonsteroidal anti-inflammatory drugs (NSAIDs) but higher compared to paracetamol (Andrade

et al. 2016). Accordingly, the mortality associated with gastrointestinal complications related to metamizole was lower than for NSAIDs (Andrade et al. 1998). However, metamizole reduced the inhibitory effect of low-dose acetyl salicylic acid on platelet aggregation, potentially by a competitive interaction between the two drugs (Papp et al. 2014). This unwanted effect of metamizole on acetyl salicylic acid platelet aggregation inhibition can be circumvented by metamizole intake after acetyl salicylic acid (Polzin et al. 2015). Furthermore, arachidonic acid-induced platelet aggregation was inhibited by metamizole, maybe by exhausting the arachidonic acid pool due to metabolite formation of metamizole with arachidonic acid (Pfrepper et al. 2019; Rogosch et al. 2012).

1.1.4. Advantages of metamizole

The proposed mechanisms of metamizole action might also explain the observed advantages of metamizole compared to NSAIDs. The described predominant COX-2 *versus* COX-1 inhibition would explain the good gastrointestinal tolerability of metamizole. The low risk for renal impairment might be based on the vascular smooth muscle relaxing effect improving renal blood circulation. Additionally, a meta-analysis of epidemiologic studies of serious adverse drug effects associated with various analgesics revealed that the absolute mortality risk associated with metamizole is over 20-fold lower than for NSAIDs (Andrade et al. 2016). Hence, metamizole represents a valuable reserve medication for patients with renal impairment, hypertension, increased bleeding risk, or intolerance for NSAIDs. Additionally, it has been shown that metamizole increases the analgesic effect of morphine also in presence of morphine tolerance (Hernandez-Delgadillo and Cruz 2004). Furthermore, the combination of metamizole and tramadol lead to synergistic analgesic effects, but only at dosages resulting in over 50% pain reduction (Poveda et al. 2003). Hence, dose reduction of opiates by combination with metamizole could reduce the risk for opiate adverse drug reactions (ADR) or addiction development.

1.1.5. Toxicity beyond hematology

Although high metamizole dosage is not a risk factor for metamizole-associated neutropenia, toxicity in other organs due to overdose has been reported. A review of metamizole overdoses reported predominantly mild gastrointestinal toxicity without any hematological ADR (Bentur and Cohen 2004). Accordingly, transient renal insufficiency and kidney injury has been described after metamizole overdose (Abu-Kishk et al. 2010; Berruti et al. 1998; Hassan et al. 2011; Peces and Pedrajas 2004; Stueber et al. 2017). The mechanism of kidney injury by metamizole might me similar as for NSAIDs by inhibition of the prostaglandin synthesis (Zapater et al. 2015).

Furthermore, it has been shown that the metamizole metabolite MAA is demethylated partly in the liver where also further metabolism might take place, whereby the formed and potentially toxic metabolites could cause liver injury. A study showed that metamizole was toxic for monocyte-derived hepatocyte-like cells from patients who had experienced idiosyncratic drug-induced liver injury after metamizole treatment (Benesic et al. 2018). The mechanism of metamizole-induced liver toxicity is unknown and the incidence of metamizole-induced liver injury is very low (Andrade et al. 2016).

1.2. Neutrophil granulocytes

The cellular immune system consists of leukocytes, which can be subdivided into granulocytes (neutrophils, basophils, and eosinophils), lymphocytes (T-cells, B-cells, and natural killer cells), and monocytes. Granulocytes can be distinguished from the mononucleated lymphocytes and monocytes by their lobulated nucleus and the granules that have given the name to these cells (Borregaard and Cowland 1997). Based on the staining properties of their granules, granulocytes can be classified as eosinophils, which contain acidophilic granules, as basophils, which contain granules susceptible to basic dyes, and as neutrophils, whose granules react minimally to staining (Chan et al. 2010). Neutrophils represent the most abundant granulocytes and also the main part of leukocytes in the blood (Rosales 2018).

Neutrophils originate from hematopoietic stem cells as initial precursors, which are slowly dividing and capable of self-renewal (Orkin and Zon 2008). Hematopoietic stem cells lose their self-renewing potential while developing into multipotent precursor cells that can give rise to all blood cell lineages (Gorgens et al. 2013). These multipotent precursors differentiate into granulocyte–monocyte progenitors in response to extracellular stimuli such as granulocyte-colony stimulating factor (G-CSF) and cytokines, and intracellular regulators, such as transcription factors. Subsequently, the cells commit to the neutrophil lineage by turning into myeloblasts (Lieschke et al. 1994; von Vietinghoff and Ley 2008). By transition from myeloblast to promyelocte stage, granulopoiesis is initiated and progresses onward the differentiation (Borregaard and Cowland 1997). Promyelocytes still have a round nucleus but are larger than myeloblasts and are characterized by the presence of azurophilic or primary granules. These peroxidase-positive granules are defined by their MPO content (Borregaard and Cowland 1997; Kjeldsen et al. 1993). Following the neutrophil maturation process the cells differentiate into myelocytes, which are characterized by appearance of specific or secondary peroxidase-negative granules, which are defined by their lactoferrin content (Borregaard and Cowland 1997; von Vietinghoff and Ley 2008). Next in the maturation sequence are metamyelocytes, which are

characterized by their kidney-shaped nucleus (Mora-Jensen et al. 2011; von Vietinghoff and Ley 2008). Metamyelocytes differentiate into band neutrophils, characterized by their band-shaped nucleus and the appearance of gelatinase or tertiary granules, which are defined by their gelatinase content (Borregaard and Cowland 1997; Cowland and Borregaard 2016). Band neutrophils turn into fully mature neutrophils with a segmented nucleus and the presence of secretory vesicles, which are rich in various receptors (Cowland and Borregaard 2016).

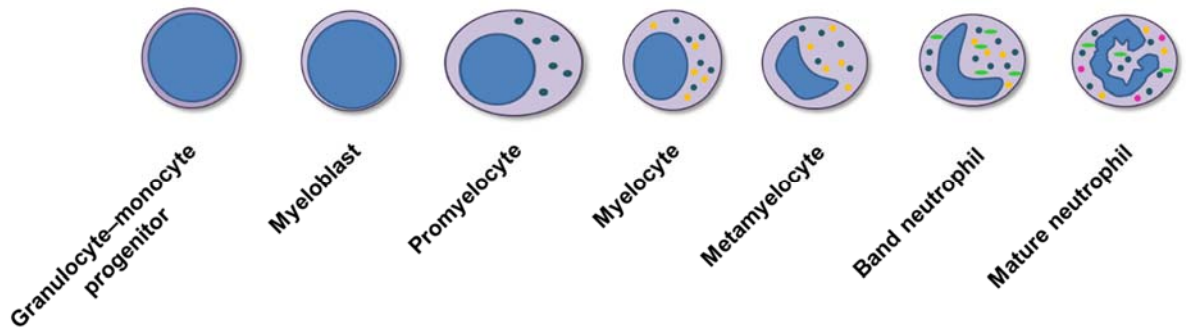

Figure 1 Neutrophil differentiation

During the differentiation process, the cells are located in the bone marrow until the mature neutrophil stage at which the cells are able to enter the blood stream (Bainton et al. 1971). Once released into circulation, neutrophils exert their primary function in innate host defense by various methods. By contact and recognition of a pathogen, phagocytosis is induced and the pathogens are engulfed by the neutrophil plasma membrane into a newly formed vacuole, the phagosome. Next, the phagosome fuses with granules to form the phagolysosome (Murphy and Weaver 2017). At the same time, the neutrophil undergoes respiratory burst by which the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex assembles at the plasma or phagosomal membrane due to neutrophil activation (Nauseef 2004). NADPH oxidase converts oxygen to superoxide, which is further converted to the MPO substrate hydrogen peroxide by superoxide dismutases (SOD). MPO is a heme-containing enzyme located in the azurophilic granules of neutrophils (Fiedler et al. 2000; Oren and Taylor 1995). MPO released from granules after fusion with the phagosome uses hydrogen peroxide formed by NADPH oxidase to catalyze the production of the antimicrobial hypochlorous acid (Bardoel et al. 2014). Hence, the phagolysosome contains hydrogen peroxide, hypochlorous acid and degrading enzymes to destroy the ingested pathogens (Rosales 2018). Neutrophils can also fuse their granules with the plasma membrane to release their content and attack surrounding pathogens. Further, neutrophils may produce extracellular traps (NET) consisting of DNA fibers and proteins from the granules to fend invading microorganisms even after neutrophil cell death

(Bardoel et al. 2014; Rosales 2018). In contrast to lymphocytes and monocytes, neutrophils have a short lifespan of less than 24 hours in circulation, for which reason any disturbance in myelopoiesis shortly ends in a lack of circulating neutrophils and risk of neutropenia (McCracken and Allen 2014).

1.3. Metamizole-associated neutropenia

Despite the good efficacy and gastrointestinal tolerability, metamizole has been banned in several countries (Sweden, USA, India) due to reports of metamizole-associated neutropenia (Blaser et al. 2015; Hedenmalm and Spigset 2002). Neutropenia is a decrease of circulating neutrophils below 1.5 x 10^9 cells/L and therefore represents a severe impairment of the innate immune system.

A decrease of the neutrophil count to less than 1.5 x 10^9/L (neutropenia), or less than 0.5 x 10^9/L (severe neutropenia or agranulocytosis) increases the susceptibility of the organism to infections. The first clinical manifestations often are inflammatory mucosal lesions, sore throat with or without fever, which is followed by infections such as tonsillitis or pneumonia and in severe cases sepsis and/or death. Depending on severity of the neutropenia and accompanying infections, patients are treated with G-CSF and antibiotics and by removal of all potentially neutropenia-causing drugs. Despite any treatment efforts, neutropenia is a serious disease with a fatality rate of approximately 5% (Andres and Maloisel 2008).

The mechanisms of metamizole-induced neutropenia are still under debate and there are no effective strategies to predict in whom it is likely to occur, nor to prevent this severe ADR (Andres and Maloisel 2008; Garbe 2007). The chemical structure of metamizole is closely related to aminopyrine, a drug which was withdrawn from the market after it had been associated with agranulocytosis in the 1930s (Huguley 1964). Investigations of the underlying mechanism of aminopyrine-induced agranulocytosis showed leucocyte agglutination with serum of previously sensitized patients in the presence of aminopyrine. Additionally, an observed increase in symptom-severity upon re-exposure as well as fever and rash in some patients suggest an immunologic mechanism of metamizole-induced neutropenia (Moeschlin 1955; Moeschlin and Wagner 1952). On the other hand, certain characteristics such as the rapid onset within a day after the first few doses in some patients without known previous exposure and the observation of bi- and pancytopenia are more compatible with direct toxicity of metamizole or its metabolites on circulating cells and/or their precursors in the bone marrow. This theory is in agreement with descriptions of bone marrow smears in patients with metamizole-associated agranulocytosis, showing a stop at the promyelocyte stage in the maturation of granulocytes (Kummer et al. 2006).

This observation indicates that the causing agent acts within the bone marrow by destroying immature and therefore potentially more sensitive cells. Hence, peripheral destruction can be excluded as the major mechanism, since in that case an increased proliferation of the entire cell lineage would be expected due to a feedback mechanism. Additionally, the unique neutrophil characteristic to form highly reactive compounds such as hydrogen peroxide and hypochlorous acid might contribute to the formation of potentially cytotoxic metamizole metabolites in the bone marrow.

Besides external influences, the patient's genetic predisposition can have a major impact on the risk to develop an ADR. Hence, pharmacogenetics testing for markers of ADR risk is used in the USA for several drugs (e.g. carbamazepine, abacavir, azathioprine) to decrease the ADRs (Lesko and Zineh 2010). For various ADRs (e.g. drug-induced liver injury, Steven-Johnson syndrome/toxic epidermal necrolysis) strong associations with variants in the human leukocyte antigen (HLA) region have been described (Daly et al. 2009; Hung et al. 2006; Mallal et al. 2002). The identification of genetic variants associated with increased ADR risk might help to prevent life-threatening ADRs and could also lead to a better understanding of the underlying mechanisms of these reactions. Since patients experiencing metamizole-associated neutropenia represent a very heterogeneous population with no clinical or demographic predictive factors, a genetic basis for metamizole-associated neutropenia is likely.

1.4. Risk factors

There are currently no predictable risk factors for metamizole-associated neutropenia. This ADR has been observed in infants up to elderly without any dose-dependency, which raises the urge to find predictive factors to minimize the number of affected patients. With this aim, several studies have been performed, reporting a higher rate of metamizole-associated neutropenia among elderly people and women (Blaser et al. 2015; Maj and Lis 2002). This might be exposure-related due to the higher analgesic use among elderly and the more frequent physician visits and therefore higher prescription rate for women. No association has been described for dosage or treatment duration and neutropenia occurrence (Andersohn et al. 2007; Andrade et al. 2016; Blaser et al. 2015).

Co-medication with myelotoxic or immunosuppressive drugs might potentiate the risk of neutropenia during metamizole treatment. Treatment with methotrexate combined with metamizole has been shown to be a risk factor for fatal neutropenia (Blaser et al. 2015; Stammschulte et al. 2015). Also non-myelotoxic and non-immunosuppressive drugs can elicit

neutropenia and might increase the risk of metamizole-associated neutropenia when combined (Andersohn et al. 2007). There are no reports about reduced metamizole metabolism and therefore increased metamizole plasma levels due to other drugs. However, since metamizole-associated neutropenia is not dose-dependent, reduced metamizole clearance can be excluded as potential risk factor.

Comorbidities affecting the neutrophil count might amplify possible metamizole-induced neutrophil loss. Especially immune depriving illnesses but also viral, bacterial, or fungal infections are known to reduce the neutrophil count in the circulation and bone marrow (Boxer and Dale 2002; Celkan and Koç 2015; Galani and Andreakos 2015). It has been shown for HIV and its equivalent in rhesus macaques, SIV, to induce neutrophil apoptosis leading to neutropenia (Elbim et al. 2009; Salmen et al. 2007). Accordingly, influenza A virus infection accelerates neutrophil apoptosis and the rate and extent of apoptosis was enhanced by an additional bacterial infection (Colamussi et al. 1999). Infected cells may be more sensitive to drugs and metabolites than healthy cells. This theory is supported by the observation that peripheral blood mononuclear cells infected with HIV or SF-162 virus were more susceptible to sulfamethoxazole hydroxylamine metabolite toxicity (Levy 1997). This observation is in line with the observation of an increased prevalence of hepatitis C infections in patients who developed metamizole-associated leukopenia (Blaser et al. 2017).

1.5. Heme metabolism

It has been shown that the metamizole metabolites MAA and AA can form stable complexes with heme and that MAA is able to reduce Fe^{3+} to Fe^{2+} (Pierre et al. 2007). Accordingly, in the subsequent original research, the interaction of MAA and the hemoglobin degradation product hemin is of central interest, for which reason a short overview about cellular heme metabolism will complete this introduction.

When old erythrocytes are removed from the circulation, the contained hemoglobin is degraded and the iron is recycled, avoiding any free heme generation (Chung et al. 2012; Kumar and Bandyopadhyay 2005). Hemoglobin degradation mostly takes place in spleen, liver, bone marrow, and macrophages and the accruing heme or its oxidized form hemin are further degraded by heme-oxygenase to iron, biliverdin, and carbon monoxide (Pimstone et al. 1971; Wagner et al. 1962). Released iron is either stored as ferritin-bond iron (Fe^{3+}) or exported in reduced form (Fe^{2+}) by the iron exporter protein ferroportin (Hentze et al. 2010). Exported iron is immediately bound by transferrin as ferric iron (Fe^{3+}), which is taken up by cells over the transferrin receptor.

After binding of transferrin, the transferrin receptor complex is internalized through endocytosis (Schultz et al. 2010). In the emerging endosome, ferric iron and transferrin dissociate from the transferrin receptor due to the low pH and the iron is reduced to Fe^{2+} by STEAP metalloreductases. Ferrous iron (Fe^{2+}) is then exported via the divalent metal transporter 1 to the cytosol, where it enters the cellular labile iron pool. A further iron acquisition pathway is apical absorption of dietary iron (Fe^{3+}) after reduction by duodenal cytochrome b. Macrophages can also acquire heme-iron as heme-hemopexin complex or hemoglobin-haptoglobin complex or by phagocytosis of erythrocytes (Boretti et al. 2014; Hvidberg et al. 2005). Iron from the labile iron pool is utilized for direct incorporation into iron proteins or is transported into the mitochondrial intermembrane space by mitoferrin for synthesis of hemo-proteins or iron-sulfur cluster containing proteins (Chung et al. 2012; Hentze et al. 2010; Schultz et al. 2010). Heme is formed through an enzymatic cascade in the cytosol and the mitochondria (Hentze et al. 2010; Schultz et al. 2010). First, protoporphyrin IX precursor is synthesized in the mitochondrial matrix and exported to the cytosol, where it is converted to coproporphyrinogen III. Next, coproporphyrinogen III is transported into the mitochondrial intermembrane space and converted to protoporphyrin IX (Severance and Hamza 2009). Then iron is incorporated into protoporphyrin IX by ferrochelatase (Dailey et al. 2000; Lange et al. 1999). Unused iron can be either exported via ferroportin and loaded to transferrin or stored as ferritin-iron (Fe^{3+}). The amount of iron present in the labile iron pool depends on iron uptake, utilization, storage, and export rates, which are tightly regulated to avoid iron excess or deficiency (Hentze et al. 2010).

1.6. References

Abu-Kishk I, Goldman M, Mordish Y, et al. (2010) Transient renal insufficiency following dipyrone overdose. Archives of disease in childhood 95(3):233-4 doi:10.1136/adc.2009.171793

Alves D, Duarte I (2002) Involvement of ATP-sensitive K(+) channels in the peripheral antinociceptive effect induced by dipyrone. European journal of pharmacology 444(1-2):47-52

Andersohn F, Konzen C, Garbe E (2007) Systematic review: agranulocytosis induced by nonchemotherapy drugs. Ann Intern Med 146(9):657-665

Andrade S, Bartels DB, Lange R, et al. (2016) Safety of metamizole: a systematic review of the literature. Journal of clinical pharmacy and therapeutics 41(5):459-77 doi:10.1111/jcpt.12422

Andrade SE, Martinez C, Walker AM (1998) Comparative safety evaluation of non-narcotic analgesics. Journal of clinical epidemiology 51(12):1357-65

Andres E, Maloisel F (2008) Idiosyncratic drug-induced agranulocytosis or acute neutropenia. Current opinion in hematology 15(1):15-21 doi:10.1097/MOH.0b013e3282f15fb9

Bachmann F, Duthaler U, Rudin D, et al. (2018) N-demethylation of N-methyl-4-aminoantipyrine, the main metabolite of metamizole. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 120:172-180 doi:https://doi.org/10.1016/j.ejps.2018.05.003

Bainton DF, Ullyot JL, Farquhar MG (1971) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. The Journal of experimental medicine 134(4):907-34

Bardoel BW, Kenny EF, Sollberger G, et al. (2014) The balancing act of neutrophils. Cell host & microbe 15(5):526-36 doi:10.1016/j.chom.2014.04.011

Beirith A, Santos AR, Rodrigues AL, et al. (1998) Spinal and supraspinal antinociceptive action of dipyrone in formalin, capsaicin and glutamate tests. Study of the mechanism of action. European journal of pharmacology 345(3):233-45

Benesic A, Rotter I, Dragoi D, et al. (2018) Development and Validation of a Test to Identify Drugs That Cause Idiosyncratic Drug-Induced Liver Injury. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 16(9):1488-1494.e5 doi:10.1016/j.cgh.2018.04.049

Bentur Y, Cohen O (2004) Dipyrone overdose. Journal of toxicology Clinical toxicology 42(3):261-5

Berruti V, Salvidio G, Saffioti S, et al. (1998) Noramidopyrine (Metamizol) and acute interstitial nephritis. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 13(8):2110-2

Blaser L, Hassna H, Hofmann S, et al. (2017) Leucopenia associated with metamizole: a case-control study. Swiss medical weekly 147:w14438 doi:smw.2017.14438

Blaser LS, Tramonti A, Egger P, et al. (2015) Hematological safety of metamizole: retrospective analysis of WHO and Swiss spontaneous safety reports. European journal of clinical pharmacology 71(2):209-17 doi:10.1007/s00228-014-1781-z

Boretti FS, Baek JH, Palmer AF, et al. (2014) Modeling hemoglobin and hemoglobin:haptoglobin complex clearance in a non-rodent species-pharmacokinetic and therapeutic implications. Frontiers in physiology 5:385-385 doi:10.3389/fphys.2014.00385

Borregaard N, Cowland JB (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 89(10):3503-21

Boxer L, Dale DC (2002) Neutropenia: causes and consequences. Seminars in hematology 39(2):75-81

Campos C, de Gregorio R, Garcia-Nieto R, et al. (1999) Regulation of cyclooxygenase activity by metamizol. European journal of pharmacology 378(3):339-47

Celkan T, Koç BŞ (2015) Approach to the patient with neutropenia in childhood. Turk pediatri arsivi 50(3):136-144 doi:10.5152/TurkPediatriArs.2015.2295

Chan YK, Tsai MH, Huang DC, et al. (2010) Leukocyte nucleus segmentation and nucleus lobe counting. BMC bioinformatics 11:558 doi:10.1186/1471-2105-11-558

Chandrasekharan NV, Dai H, Roos KL, et al. (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proceedings of the National Academy of Sciences of the United States of America 99(21):13926-31 doi:10.1073/pnas.162468699

Chung J, Chen C, Paw BH (2012) Heme metabolism and erythropoiesis. Current opinion in hematology 19(3):156-62 doi:10.1097/MOH.0b013e328351c48b

Colamussi ML, White MR, Crouch E, et al. (1999) Influenza A virus accelerates neutrophil apoptosis and markedly potentiates apoptotic effects of bacteria. Blood 93(7):2395-403

Cowland JB, Borregaard N (2016) Granulopoiesis and granules of human neutrophils. Immunological reviews 273(1):11-28 doi:10.1111/imr.12440

Dailey HA, Dailey TA, Wu CK, et al. (2000) Ferrochelatase at the millennium: structures, mechanisms and [2Fe-2S] clusters. Cellular and molecular life sciences : CMLS 57(13-14):1909-26

Daly AK, Donaldson PT, Bhatnagar P, et al. (2009) HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. Nature genetics 41(7):816-819 doi:10.1038/ng.379

de Souza GEP, Cardoso RA, Melo MCC, et al. (2002) A comparative study of the antipyretic effects of indomethacin and dipyrone in rats. Inflammation Research 51(1):24-32 doi:10.1007/PL00000278

Documed (2017) Swiss product information online. In: Available via HCI Solutions AG. http://www.compendium.ch Accessed 30 Jan 2019

Elbim C, Monceaux V, Francois S, et al. (2009) Increased neutrophil apoptosis in chronically SIV-infected macaques. Retrovirology 6:29 doi:10.1186/1742-4690-6-29

EMBL-EBI (2018) Chemical Entities of Biological Interest (ChEBI). In. https://www.ebi.ac.uk/chebi/searchId.do?chebiId=CHEBI:62088 Accessed 27 Mar 2019

Ergun H, Frattarelli DA, Aranda JV (2004) Characterization of the role of physicochemical factors on the hydrolysis of dipyrone. Journal of pharmaceutical and biomedical analysis 35(3):479-87 doi:10.1016/j.jpba.2004.02.004

Fiedler TJ, Davey CA, Fenna RE (2000) X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 A resolution. The Journal of biological chemistry 275(16):11964-71

Galani IE, Andreakos E (2015) Neutrophils in viral infections: Current concepts and caveats. Journal of leukocyte biology 98(4):557-64 doi:10.1189/jlb.4VMR1114-555R

Garbe E (2007) Non-chemotherapy drug-induced agranulocytosis. Expert opinion on drug safety 6(3):323-335 doi:10.1517/14740338.6.3.323

Geisslinger G, Böcker R, Levy M (1996) High-Performance Liquid Chromatographic Analysis of Dipyrone Metabolites to Study Their Formation in Human Liver Microsomes. Pharmaceutical research 13(8):1272-1275 doi:10.1023/A:1016088925786

Gorgens A, Radtke S, Horn PA, et al. (2013) New relationships of human hematopoietic lineages facilitate detection of multipotent hematopoietic stem and progenitor cells. Cell cycle (Georgetown, Tex) 12(22):3478-82 doi:10.4161/cc.26900

Graff J, Arabmotlagh M, Cheung R, et al. (2007) Effects of parecoxib and dipyrone on platelet aggregation in patients undergoing meniscectomy: a double-blind, randomized, parallel-group study. Clinical therapeutics 29(3):438-47

Gulmez SE, Gurdal H, Tulunay FC (2006) Airway smooth muscle relaxations induced by dipyrone. Pharmacology 78(4):202-8 doi:10.1159/000096688

Hassan K, Khazim K, Hassan F, et al. (2011) Acute kidney injury associated with metamizole sodium ingestion. Renal failure 33(5):544-7 doi:10.3109/0886022x.2011.569107

Hedenmalm K, Spigset O (2002) Agranulocytosis and other blood dyscrasias associated with dipyrone (metamizole). European journal of clinical pharmacology 58(4):265-274 doi:10.1007/s00228-002-0465-2

Hentze MW, Muckenthaler MU, Galy B, et al. (2010) Two to tango: regulation of Mammalian iron metabolism. Cell 142(1):24-38 doi:10.1016/j.cell.2010.06.028

Hernandez-Delgadillo GP, Cruz SL (2004) Dipyrone potentiates morphine-induced antinociception in dipyrone-treated and morphine-tolerant rats. European journal of pharmacology 502(1-2):67-73 doi:10.1016/j.ejphar.2004.08.032

Hinz B, Cheremina O, Bachmakov J, et al. (2007) Dipyrone elicits substantial inhibition of peripheral cyclooxygenases in humans: new insights into the pharmacology of an old analgesic. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 21(10):2343-2351 doi:10.1096/fj.06-8061com

Huguley CM, Jr. (1964) Agranulocytosis Induced by Dipyrone, a Hazardous Antipyretic and Analgesic. Jama 189:938-41

Hung SI, Chung WH, Jee SH, et al. (2006) Genetic susceptibility to carbamazepine-induced cutaneous adverse drug reactions. Pharmacogenetics and genomics 16(4):297-306 doi:10.1097/01.fpc.0000199500.46842.4a

Hvidberg V, Maniecki MB, Jacobsen C, et al. (2005) Identification of the receptor scavenging hemopexin-heme complexes. Blood 106(7):2572-9 doi:10.1182/blood-2005-03-1185

IAAAS (1986) Risks of agranulocytosis and aplastic anemia. A first report of their relation to drug use with special reference to analgesics. The International Agranulocytosis and Aplastic Anemia Study. Jama 256(13):1749-57

Ibáñez L, Vidal X, Ballarín E, et al. (2005) Agranulocytosis associated with dipyrone (metamizol). European journal of clinical pharmacology 60(11):821-829 doi:10.1007/s00228-004-0836-y

Jasiecka A, Maslanka T, Jaroszewski JJ (2014) Pharmacological characteristics of metamizole. Polish journal of veterinary sciences 17(1):207-14

Kanashiro A, Pessini AC, Machado RR, et al. (2009) Characterization and pharmacological evaluation of febrile response on zymosan-induced arthritis in rats. American journal of physiology Regulatory, integrative and comparative physiology 296(5):R1631-40 doi:10.1152/ajpregu.90527.2008

Kjeldsen L, Bainton DF, Sengelov H, et al. (1993) Structural and functional heterogeneity among peroxidase-negative granules in human neutrophils: identification of a distinct gelatinase-containing granule subset by combined immunocytochemistry and subcellular fractionation. Blood 82(10):3183-91

Kumar S, Bandyopadhyay U (2005) Free heme toxicity and its detoxification systems in human. Toxicology letters 157(3):175-88 doi:10.1016/j.toxlet.2005.03.004

Kummer O, Haschke M, Tuchscherer D, et al. (2006) [Agranulocytosis in a patient treated with metamizole and clopidogrel]. Praxis (Bern 1994) 95(45):1743-5; quiz 1746-7

Lange H, Kispal G, Lill R (1999) Mechanism of iron transport to the site of heme synthesis inside yeast mitochondria. The Journal of biological chemistry 274(27):18989-96

Lesko LJ, Zineh I (2010) DNA, drugs and chariots: on a decade of pharmacogenomics at the US FDA. Pharmacogenomics 11(4):507-512 doi:10.2217/pgs.10.16

Levy M (1997) Role of viral infections in the induction of adverse drug reactions. Drug safety 16(1):1-8 doi:10.2165/00002018-199716010-00001

Levy M, Zylber-Katz E, Rosenkranz B (1995) Clinical pharmacokinetics of dipyrone and its metabolites. Clinical pharmacokinetics 28(3):216-34 doi:10.2165/00003088-199528030-00004

Lieschke GJ, Grail D, Hodgson G, et al. (1994) Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood 84(6):1737-46

Maj S, Lis Y (2002) The incidence of metamizole sodium-induced agranulocytosis in Poland. The Journal of international medical research 30(5):488-95 doi:10.1177/147323000203000504

Mallal S, Nolan D, Witt C, et al. (2002) Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. Lancet (London, England) 359(9308):727-732

Malvar Ddo C, Aguiar FA, Vaz Ade L, et al. (2014) Dipyrone metabolite 4-MAA induces hypothermia and inhibits PGE2 -dependent and -independent fever while 4-AA only blocks PGE2 -dependent fever. British journal of pharmacology 171(15):3666-79 doi:10.1111/bph.12717

Malvar Ddo C, Soares DM, Fabricio AS, et al. (2011) The antipyretic effect of dipyrone is unrelated to inhibition of PGE(2) synthesis in the hypothalamus. British journal of pharmacology 162(6):1401-9 doi:10.1111/j.1476-5381.2010.01150.x

McCracken JM, Allen L-AH (2014) Regulation of human neutrophil apoptosis and lifespan in health and disease. Journal of cell death 7:15-23 doi:10.4137/JCD.S11038

Moeschlin S (1955) Immunological granulocytopenia and agranulocytosis; clinical aspects. Le Sang 26(1):32-51

Moeschlin S, Wagner K (1952) [Agranulocytosis due to the occurrence of leukocyte-agglutinins; pyramidon and cold agglutinins]. Acta haematologica 8(1-2):29-41

Mora-Jensen H, Jendholm J, Fossum A, et al. (2011) Technical advance: immunophenotypical characterization of human neutrophil differentiation. Journal of leukocyte biology 90(3):629-34 doi:10.1189/jlb.0311123

Murphy K, Weaver C (2017) Janeway's immunobiology, 9th edition edn. New York, NY : Garland Science/Taylor & Francis Group, LLC, United States

Nauseef WM (2004) Assembly of the phagocyte NADPH oxidase. Histochemistry and cell biology 122(4):277-91 doi:10.1007/s00418-004-0679-8

Nikolova I, Tencheva J, Voinikov J, et al. (2012) Metamizole: A Review Profile of a Well-Known "Forgotten" Drug. Part I: Pharmaceutical and Nonclinical Profile. Biotechnology & Biotechnological Equipment 26(6):3329-3337 doi:10.5504/BBEQ.2012.0089

Oren A, Taylor JM (1995) The subcellular localization of defensins and myeloperoxidase in human neutrophils: immunocytochemical evidence for azurophil granule heterogeneity. The Journal of laboratory and clinical medicine 125(3):340-7

Orkin SH, Zon LI (2008) SnapShot: hematopoiesis. Cell 132(4):712 doi:10.1016/j.cell.2008.02.013

Ortiz MI, Castaneda-Hernandez G, Granados-Soto V (2003) Possible involvement of potassium channels in peripheral antinociception induced by metamizol: lack of participation of ATP-sensitive K+ channels. Pharmacology, biochemistry, and behavior 74(2):465-70

Papp J, Sandor B, Vamos Z, et al. (2014) Antiplatelet effect of acetylsalicylic acid, metamizole and their combination - in vitro and in vivo comparisons. Clinical hemorheology and microcirculation 56(1):1-12 doi:10.3233/ch-2012-1636

Peces R, Pedrajas A (2004) Non-oliguric acute renal failure and abortion induced by metamizol overdose. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 19(10):2683-5 doi:10.1093/ndt/gfh372

Pfrepper C, Deters S, Metze M, et al. (2019) Metamizole inhibits arachidonic acid-induced platelet aggregation after surgery and impairs the effect of aspirin in hospitalized patients. European journal of clinical pharmacology doi:10.1007/s00228-019-02646-9

Pierre SC, Schmidt R, Brenneis C, et al. (2007) Inhibition of cyclooxygenases by dipyrone. British journal of pharmacology 151(4):494-503 doi:10.1038/sj.bjp.0707239

Pimstone NR, Tenhunen R, Seitz PT, et al. (1971) The enzymatic degradation of hemoglobin to bile pigments by macrophages. The Journal of experimental medicine 133(6):1264-81

Polzin A, Richter S, Schror K, et al. (2015) Prevention of dipyrone (metamizole) induced inhibition of aspirin antiplatelet effects. Thrombosis and haemostasis 114(1):87-95 doi:10.1160/th14-11-0922

Poveda R, Planas E, Pol O, et al. (2003) Interaction between metamizol and tramadol in a model of acute visceral pain in rats. European journal of pain (London, England) 7(5):439-48 doi:10.1016/s1090-3801(03)00003-x

Rogosch T, Sinning C, Podlewski A, et al. (2012) Novel bioactive metabolites of dipyrone (metamizol). Bioorg Med Chem 20(1):101-7 doi:10.1016/j.bmc.2011.11.028

Rosales C (2018) Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? Frontiers in physiology 9:113-113 doi:10.3389/fphys.2018.00113

Salmen S, Montes H, Soyano A, et al. (2007) Mechanisms of neutrophil death in human immunodeficiency virus-infected patients: role of reactive oxygen species, caspases and

map kinase pathways. Clinical and experimental immunology 150(3):539-45 doi:10.1111/j.1365-2249.2007.03524.x

Sanchez S, Alarcon de la Lastra C, Ortiz P, et al. (2002) Gastrointestinal tolerability of metamizol, acetaminophen, and diclofenac in subchronic treatment in rats. Digestive diseases and sciences 47(12):2791-8

Schultz IJ, Chen C, Paw BH, et al. (2010) Iron and porphyrin trafficking in heme biogenesis. The Journal of biological chemistry 285(35):26753-9 doi:10.1074/jbc.R110.119503

Severance S, Hamza I (2009) Trafficking of heme and porphyrins in metazoa. Chemical reviews 109(10):4596-616 doi:10.1021/cr9001116

Siebel JS, Beirith A, Calixto JB (2004) Evidence for the involvement of metabotropic glutamatergic, neurokinin 1 receptor pathways and protein kinase C in the antinociceptive effect of dipyrone in mice. Brain research 1003(1-2):61-7 doi:10.1016/j.brainres.2003.11.075

Silva LC, Miranda e Castor MG, Souza TC, et al. (2015) NSAIDs induce peripheral antinociception by interaction with the adrenergic system. Life sciences 130:7-11 doi:10.1016/j.lfs.2015.03.011

Stammschulte T, Ludwig WD, Muhlbauer B, et al. (2015) Metamizole (dipyrone)-associated agranulocytosis. An analysis of German spontaneous reports 1990-2012. European journal of clinical pharmacology 71(9):1129-38 doi:10.1007/s00228-015-1895-y

Stueber T, Buessecker L, Leffler A, et al. (2017) The use of dipyrone in the ICU is associated with acute kidney injury: A retrospective cohort analysis. European journal of anaesthesiology 34(10):673-680 doi:10.1097/eja.0000000000000627

UnitedNations UNDoEaSA (2005) Consolidated List of Products - Whose Consumption and/or Sale Have Been Banned, Withdrawn, Severely Restricted or Not Approved by Governments. In: Affairs UNDoEaS (ed). New York, p 598

Valenzuela F, Garcia-Saiso S, Lemini C, et al. (2005) Metamizol acts as an ATP sensitive potassium channel opener to inhibit the contracting response induced by angiotensin II but not to norepinephrine in rat thoracic aorta smooth muscle. Vascular pharmacology 43(2):120-7 doi:10.1016/j.vph.2005.05.003

Volz M, Kellner HM (1980) Kinetics and metabolism of pyrazolones (propyphenazone, aminopyrine and dipyrone). British journal of clinical pharmacology 10 Suppl 2(Suppl 2):299S-308S

von Vietinghoff S, Ley K (2008) Homeostatic regulation of blood neutrophil counts. Journal of immunology (Baltimore, Md : 1950) 181(8):5183-8

Wagner HN, Jr., Razzak MA, Gaertner RA, et al. (1962) Removal of erythrocytes from the circulation. Archives of internal medicine 110:90-7

Zapater P, Llanos L, Barquero C, et al. (2015) Acute effects of dipyrone on renal function in patients with cirrhosis: a randomized controlled trial. Basic & clinical pharmacology & toxicology 116(3):257-63 doi:10.1111/bcpt.12312

Zylber-Katz E, Granit L, Levy M (1992) Formation and excretion of dipyrone metabolites in man. European journal of clinical pharmacology 42(2):187-191 doi:10.1007/BF00278482

2. RATIONALE AND MOTIVATION

Although metamizole is an analgesic with several advantages compared to NSAIDs, it has been banned in several countries or been restricted to narrow indications as in Switzerland due to the rare occurrence of metamizole-associated neutropenia (UnitedNations 2005). Removing medications from the market due to severe ADRs is not an adequate solution regarding the need for effective alternative analgesics to NSAIDs. Patients intolerant to NSAIDs are left with fewer therapeutic options or need to switch to opiates, which may be inappropriate for the indication. A more preferable approach would be to elucidate the mechanisms of metamizole-associated neutropenia to identify potential risk factors. Hence, patients at risk could be monitored or excluded from metamizole treatment. The need for an alternative treatment option to NSAIDs has led to a continuous increase in metamizole prescriptions in recent years accompanied by a growing number of neutropenia reports (Blaser et al. 2015). The increasing use of metamizole in parallel with the uncertainty of its safety for the individual patient emphasizes the need to investigate the underlying mechanisms of this severe ADR. Despite the long history of metamizole use and awareness of its potential to induce neutropenia, the underlying mechanisms are still not understood. Immune-mediated mechanisms have been discussed as well as direct cytotoxic effects of metamizole or its metabolites, whereas the immune-mediated theory seems to be more accepted and widespread (Curtis 2014; Hamerschlak and Cavalcanti 2005; Uetrecht 1996). The four main metamizole metabolites reach sufficiently high plasma and potentially also bone marrow concentrations to be hemotoxic (Blaser et al., unpublished data). Bone marrow biopsies of patients with metamizole-associated neutropenia consistently showed a stop at the promyelocyte differentiation stage in neutrophil maturation (Isik et al. 2014; Pfersdorff et al. 2011). This observation indicates that potential cytotoxic metamizole metabolites affect the immature cells in the bone marrow and not mature cells in the circulation. Potentially cytotoxic metabolites would be expected to be formed either in the liver or in the neutrophils itself. It has been shown for the thienopyridine derivatives ticlopidin and clopidogrel to be metabolized by liver microsomes to metabolites, which were cytotoxic for myeloid progenitor cells. Additionally, MPO of neutrophils is able to convert ticlopidine and the main metabolite clopidogrel carboxylate to myelotoxic metabolites (Maseneni et al. 2012).

The current work is part of a collaboration between pharmacology & toxicology, immunology, and genetics groups to investigate the underlying mechanisms of metamizole-associated neutropenia. This thesis covers the toxicological *in vitro* investigations of direct metamizole

metabolite toxicity on neutrophils and neutrophil progenitor cells as well as clinical investigations composed of an observational case-control study. In the first part of this thesis, I will focus on the toxicological *in vitro* investigations and in the second part, I will present the results of the clinical data assessment of the observational case-control study.

The *in vitro* investigations were based on previous experiments showing no cytotoxicity of metamizole and its main metabolites on mature neutrophils and the observation that bone marrow biopsies of neutropenia patients showed a maturation stop at the promyelocytic stage. Hence, I planned to investigate the cytotoxicity of metamizole and its main metabolites on the promyelocytic cell line HL60 and myeloid progenitor cells from umbilical cord blood in comparison with mature neutrophils. To form potentially cytotoxic secondary metabolites, I combined the metamizole metabolites with components of the neutrophil antioxidative system: MPO or horseradish peroxidase, hydrogen peroxide, and hypochloric acid. I further assessed potential formation of cytotoxic metabolites after combination of metamizole metabolites with various iron compounds found in neutrophils and blood.

In a second *in vitro* project, I aimed to investigate the difference between HL60 cells and mature neutrophils leading to the observed higher resistance of mature cells. To assess potential cellular changes, I differentiated promyelocytic HL60 cells into mature neutrophils and investigated the change in protein expression of heme-metabolizing enzymes and enzymes involved in the first line antioxidative defense as well as the cellular glutathione pool.

The observational case-control study was based on a previous retrospective analysis of the hematological safety of metamizole using reports from the WHO Global Database and the Swiss Pharmacovigilance Database (Blaser et al. 2015). The main risk factors for fatal outcome found in this analysis were female gender, higher age, and co-medication with methotrexate, an immunosuppressive drug known to cause neutropenia. Interestingly, about a third of all neutropenia cases occurred within a latency time of less than 7 days, questioning the concept of an immunological reaction as underlying mechanism. In contrast to this previous retrospective analysis, all included patients were interviewed personally to get a more complete image of the cases, enabling a more detailed risk analysis. Additionally, only cases without any myelotoxic or immunosuppressive co-medication were included. This allowed us to focus on the influence of daily co-medication, which has also been reported to influence the neutrophil count as well as other potentially involved risk factors (Andersohn et al. 2007). Case patients of this study were compared to controls, which are expected to be tolerant to metamizole. This was accomplished by inclusion of control patients, who had been under continuous metamizole treatment for at least 28 days without any hematological adverse events. According to previous studies, the risk

was very low that these patients would develop metamizole-associated neutropenia after this treatment period (Blaser et al. 2015). This study design enabled me to compare tolerant control patients, who received metamizole in an outpatient treatment setting, with a subgroup of all included neutropenia cases, who had developed neutropenia after metamizole treatment at home. Since these two groups had similar treatment circumstances, it was possible to analyze risk factors in a regression-based model.

Since metamizole has been used as pain killer over almost 100 years and its potential to induce neutropenia has been described extensively but no mechanism has been identified so far, it is likely that several factors might influence the development of metamizole-associated neutropenia.

2.1. References

Andersohn F, Konzen C, Garbe E (2007) Systematic review: agranulocytosis induced by nonchemotherapy drugs. Ann Intern Med 146(9):657-665

Blaser LS, Tramonti A, Egger P, et al. (2015) Hematological safety of metamizole: retrospective analysis of WHO and Swiss spontaneous safety reports. European journal of clinical pharmacology 71(2):209-17 doi:10.1007/s00228-014-1781-z

Curtis BR (2014) Drug-induced immune neutropenia/agranulocytosis. Immunohematology 30(2):95-101

Hamerschlak N, Cavalcanti AB (2005) Neutropenia, agranulocytosis and dipyrone. Sao Paulo Medical Journal 123:247-249

Isik M, Kaya Z, Belen FB, et al. (2014) Life-threatening agranulocytosis, anemia, and plasmacytosis after dipyrone use for fever in a child. Journal of pediatric hematology/oncology 36(1):e46-8 doi:10.1097/MPH.0b013e31826e7d9d

Maseneni S, Donzelli M, Taegtmeyer AB, et al. (2012) Toxicity of clopidogrel and ticlopidine on human myeloid progenitor cells: importance of metabolites. Toxicology 299(2-3):139-45 doi:10.1016/j.tox.2012.05.017

Pfersdorff M, Spes J, Kraus MR (2011) [17-year-old patient with neutropenia and fever during therapy with analgesics]. Deutsche medizinische Wochenschrift (1946) 136(8):365-8 doi:10.1055/s-0031-1272537

Uetrecht JP (1996) Reactive metabolites and agranulocytosis. European journal of haematology Supplementum 60:83-88

UnitedNations UNDoEaSA (2005) Consolidated List of Products - Whose Consumption and/or Sale Have Been Banned, Withdrawn, Severely Restricted or Not Approved by Governments. In: Affairs UNDoEaS (ed). New York, p 598

3. TOXICOLOGIGAL *IN VITRO* INVESTIGATIONS

3.1. Non-immunological toxicological mechanisms of metamizole-associated neutropenia in HL60 cells

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Non-immunological toxicological mechanisms of metamizole-associated neutropenia in HL60 cells

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ABSTRACT

Metamizole is an analgesic and antipyretic, but can cause neutropenia and agranulocytosis. We investigated the toxicity of the metabolites N-methyl-4-aminoantipyrine (MAA), 4-aminoantipyrine (AA), N-formyl-4-aminoantipyrine (FAA) and N-acetyl-4-aminoantipyrine (AAA) on neutrophil granulocytes and on HL60 cells (granulocyte precursor cell line). MAA, FAA, AA, and AAA (up to 100 μM) alone were not toxic for HL60 cells or granulocytes. In the presence of the myeloperoxidase substrate H_2O_2, MAA reduced cytotoxicity for HL60 cells at low concentrations (< 50 μM), but increased cytotoxicity at 100 μM H_2O_2. Neutrophil granulocytes were resistant to H_2O_2 and MAA. Fe^{2+} and Fe^{3+} were not toxic to HL60 cells, irrespective of the presence of H_2O_2 and MAA. Similarly, MAA did not increase the toxicity of lactoferrin, hemoglobin or methemoglobin for HL60 cells. Hemin (hemoglobin degradation product containing a porphyrin ring and Fe^{3+}) was toxic on HL60 cells and cytotoxicity was increased by MAA. EDTA, N-acetylcystein and glutathione prevented the toxicity of hemin and hemin/MAA. The absorption spectrum of hemin changed concentration-dependently after addition of MAA, suggesting an interaction between Fe^{3+} and MAA. NMR revealed the formation of a stable MAA reaction product with a reaction pathway involving the formation of an electrophilic intermediate. In conclusion, MAA, the principle metabolite of metamizole, increased cytotoxicity of hemin by a reaction involving the formation of an electrophilic metabolite. Accordingly, cytotoxicity of MAA/hemin could be prevented by the iron chelator EDTA and by the electron donors NAC and glutathione. Situations with increased production of hemin may represent a risk factor for metamizole-associated granulocytopenia.

1. Introduction

Metamizole is a non-opioid analgesic, antipyretic, and spasmolytic prodrug, which is widely used in certain countries due to its good efficacy and low gastrointestinal toxicity [1,2]. It is rapidly converted non-enzymatically to the active drug N-methyl-4-aminoantipyrine (MAA) in the gut and in the mesenteric and portal circulation (Fig. 1). MAA has a good oral bioavailability and is converted enzymatically to the 4-aminoantipyrine (AA) which is also physiologically active [3]. The majority of AA is acetylated to N-acetyl-4-aminoantipyrine (AAA) and a smaller part is formylated to N-formyl-4-aminoantipyrine (FAA) [4–6]. Although metamizole has a favourable safety profile overall, susceptible patients may experience neutropenia or agranulocytosis, a severe and potentially fatal decrease of circulating neutrophil granulocytes [1,7]. The four major metabolites MAA, AA, AAA and FAA reach sufficiently high plasma (and presumably also bone marrow) concentrations to potentially be hematotoxic [8]. The mechanisms underlying metamizole-induced neutropenia are poorly understood, and there are no effective strategies to predict in whom neutropenia is likely to occur, nor to prevent this life-threatening adverse drug reaction [9,10]. Bone marrow biopsies of affected patients showed a stop at the myelocyte stage in granulocyte maturation [11], indicating that the toxicity of metamizole affects the bone marrow and not peripheral granulocytes. An HLA-linked toxicity has been proposed in one study, suggesting an immunological mechanism [12]. On the other hand, the absence of immunological features in affected patients and the onset

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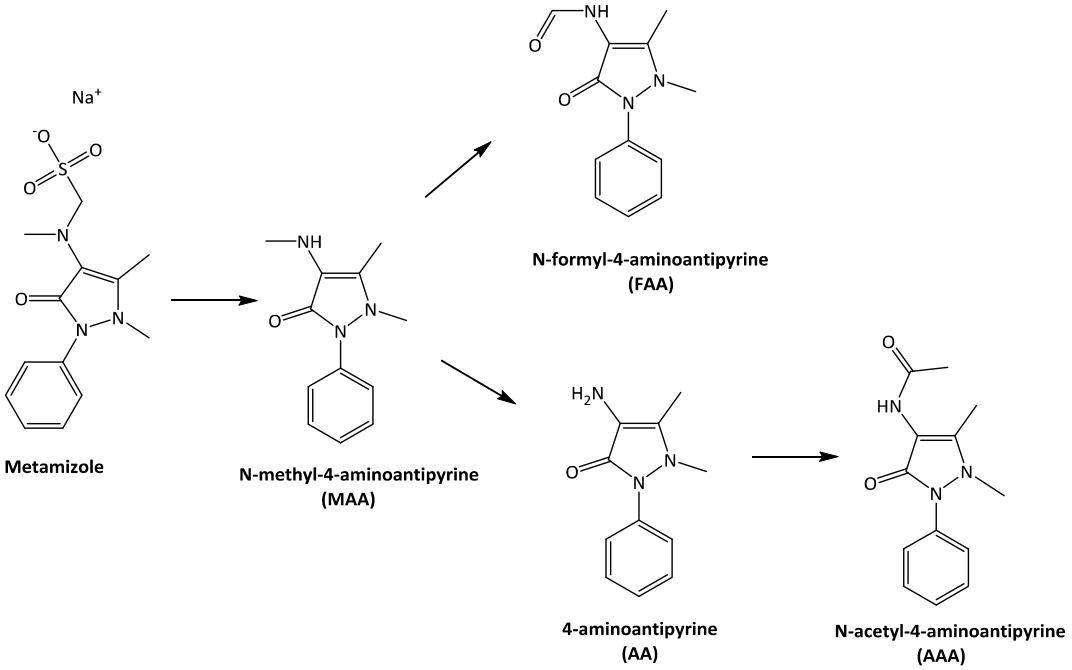

Fig. 1. Metabolism of metamizole. Metamizole is rapidly converted non-enzymatically in the intestinal tract to the active metabolite N-methyl-4-aminoantipyrine (MAA). MAA has a good oral bioavailability and can be converted enzymatically to 4-aminoantipyrine (AA) or formylated to N-formyl-4-aminoantipyrine (FAA). The majority of AA is acetylated to N-acetyl-4-aminoantipyrine (AAA).

within a few days after start of therapy in some previously unexposed patients are compatible with direct metabolic toxicity on bone marrow granulocyte precursors.

Uetrecht et al. have shown that aminopyrine (N,N-dimethyl-4-aminoantipyrine), which is structurally closely related to MAA, can form reactive metabolites after oxidation by hypochlorite [13]. Hypochlorite can be produced by myeloperoxidase, a heme-containing enzyme detectable in granulocyte maturation starting from promyelocytes, which are direct myelocyte precursors [14]. Myeloperoxidase could therefore be involved in myelotoxicity associated with MAA. Myeloperoxidase is also present in circulating neutrophil granulocytes and in HL60 cells [15,16], a human promyeloid cell line. The main function of myeloperoxidase is to destroy phagocytosed microorganisms by generating reactive intermediates within the phagosome [17,18]. The generation of reactive intermediates needs the presence of hydrogen peroxide (H_2O_2) and an anion such as chloride·H_2O_2 arises mainly from the respiratory burst produced by phagocyte NADPH oxidase [19]. In the presence of H_2O_2, myeloperoxidase catalyzes the formation of reactive intermediates such as hypochlorous acid (HOCl), which can destroy phagocytosed microorganisms [20]. Importantly, H_2O_2 is also a substrate for the Fenton reaction, which is dependent on Fe^{2+} and produces reactive hydroxyl radicals [21,22]. Since iron in the form of free iron (mainly Fe^{3+}), ferritin (Fe^{3+}) or complexed in heme (as Fe^{3+} or Fe^{2+}) is abundant in bone marrow [23], an iron-mediated mechanism is a possibility for explaining the bone marrow toxicity of MAA. In favour of an iron-associated mechanism, Pierre et al. have shown that MAA and AA (but not FAA and AAA) react with heme-bound Fe^{3+} [24].

In the current study, we investigated the possibility of direct (non-immunological) toxicity of metamizole and its metabolites (MAA, FAA, AA, and AAA) on circulating neutrophil granulocytes and on the human granulocyte precursor cell line HL60. For that, we investigated the possible involvement of myeloperoxidase, the Fenton reaction and of heme-bound iron in the cytotoxicity of MAA. The studies show that MAA reacts with Fe^{3+} in hemin, leading to the formation of electrophilic MAA metabolites that are cytotoxic.

2. Materials and methods

2.1. Chemicals and cell culture reagents

We purchased metamizole, N-methyl-4-aminoantipyrine, N-formyl-4-aminoantipyrine, 4-aminoantipyrine, and N-acetyl-4-aminoantipyrine, natrium chloride (NaCl), Tris-HCl, $Fe(NO_3)_3$, nitrilotriacetic acid (NTA), doxycycline, lactoferrin, iron chloride (Fe^{3+}), iron sulfate (Fe^{2+}), sodium hydroxide (NaOH), hemoglobin, methemoglobin, hemin, glutathione (GSH), N-acetyl-cysteine (NAC), and ethylenediaminetetraacetic acid (EDTA) from Sigma–Aldrich (Buchs, Switzerland). We obtained RPMI 1640 medium, fetal bovine serum (FBS), and phosphate buffered saline (PBS) from GIBCO (Lucerne, Switzerland), dextran 500 from Roth AG (Arlesheim, Switzerland), Ficoll-Paque from GE Healthcare (Glattbrugg, Switzerland) and BD Pharm Lyse lysing buffer as well as the 96-well cell culture plates from BD Biosciences (Franklin Lakes, NJ, USA).

2.2. Promyelocytic HL60 cells

We maintained HL60 cells (CCL-240, lot number 7703261, ATCC, Wesel, Germany) in RPMI medium supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. For all subsequent described assays, HL60 cells from passages 11 to 25 were used.

2.3. Mature neutrophil granulocytes

We isolated the neutrophil granulocytes freshly from human whole blood obtained from the local blood donation center Basel (Switzerland). Since the donors remained anonymous, the study did not require approval from the local Ethics Committee. We isolated the neutrophils from the blood by a modification of the method described by Klebanoff et al. [25]. Briefly, we diluted 20 mL blood with 25 mL phosphate-buffered saline (PBS), carefully layered it over 15 mL Ficoll-Paque and centrifuged it at 200*g* for 20 min. We discarded the supernatant and mixed the pellet with 4% dextran in 0.9% NaCl solution and allowed the erythrocytes to settle for 30 min. We washed the

supernatant layer with PBS and lysed the remaining erythrocytes with BD Pharm Lyse lysing buffer. Subsequently, we washed the cells twice with PBS and resuspended them in RPMI medium containing 10% fetal bovine serum (FBS). Viability was assessed by using trypan blue analysis and always exceeded > 90%. We stained some samples with CD66b antibody (BioLegend, San Diego, CA, USA) and confirmed by flow cytometry analysis > 92% content of neutrophil granulocytes.

2.4. Effect of metamizole and metamizole metabolites on plasma membrane integrity

For the experiments, we seeded 2×10^5 HL60 cells or 1×10^6 freshly isolated neutrophils in 1 mL RPMI containing 10% FBS in 2 mL Eppendorf tubes. The stock solutions of test compounds (metamizole, MAA, FAA, AA, and AAA) were prepared in DMSO (Sigma–Aldrich, Buchs, Switzerland). We added the test compounds to the cell-suspension at a concentration of 1–100 μM for metamizole, FAA, AA, AAA and 1–200 μM for MAA. We chose these concentrations based on available pharmacokinetic data in humans [4]. Subsequently, we added 50 μL of each suspension-mixture in triplicate to a 96-well plate and incubated the plate for 24 and 48 h at 37 °C, 5% CO_2. The DMSO concentration did not exceed 0.1% in all incubations, including control incubations, as this DMSO concentration is not cytotoxic [26]. We used Triton X (Sigma–Aldrich, Buchs, Switzerland) at a final concentration of 0.1% as a positive control for plasma membrane toxicity. We performed all experiments in triplicate and repeated them at least three times using different cell isolations.

To assess a possible loss of plasma membrane integrity, reflected by the release of adenylate kinase, we used the firefly luciferase system (ToxiLight® BioAssay Kit, Lonza, Basel, Switzerland). After incubation for 24 and 48 h in presence of test compounds, we added 50 μL assay buffer to 50 μL cell suspension from treated cells and measured luminescence after 5 min of incubation with a Tecan Infinite pro 200 microplate reader (Tecan, Männedorf, Switzerland).

2.5. Determination of the cellular ATP content after incubation with metamizole and metamizole metabolites

We incubated HL60 cells and neutrophil granulocytes with test compounds as described before. To assess the ATP content, which reflects the cellular energy metabolism, we used the CellTiter-Glo® luminescent assay (Promega Corporation, Madison, USA). After incubation for 24 and 48 h in presence of test compounds, we added 50 μL assay buffer to 50 μL cell suspension from treated cells and measured luminescence after 10 min incubation with a Tecan Infinite pro 200 microplate reader (Tecan, Männedorf, Switzerland).

2.6. Determination of apoptosis and necrosis

To determine the percentage of dead cells (including necrotic cells and cells in late apoptosis), we used propidium iodide (PI) (Molecular probes, Oregon, USA), a red fluorescent dye incapable to permeate intact or early apoptotic cells, but able to stain permeable necrotic cells by binding to nucleic acid. After the incubation of HL60 cells with 100 μM MAA and co-incubation with different concentrations (10–100 μM) of H_2O_2 (Sigma–Aldrich, Buchs, Switzerland) for 24 h, the cells were centrifuged at 500*g* for 5 min and washed with PBS before PI staining (Molecular probes, Oregon, USA) at a final concentration of 10 μg/mL. After 15 min incubation in the dark, we analyzed the cells with a CytoFLEX flow cytometer (Beckmann Coulter, Indianapolis, USA) and assessed the data using FlowJo software 10.08 (Tree Star, Ashland, OR, USA).

To determine the percentage of early apoptotic cells, we used Annexin V, a Ca^{2+}-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (PS). PS is located on the inner cytoplasmic surface of intact cell membranes. In apoptotic cells, PS is translocated to the outer leaflet of the plasma membrane, where Annexin V binds to it. After the incubation of HL60 cells with 100 μM MAA and different concentrations (10–100 μM) of H_2O_2 for 24 h, the cells were centrifuged at 500*g* for 5 min and washed with PBS before Alexa Fluor 488® Annexin V staining (Molecular probes, Oregon, USA). After 15 min incubation in the dark, we analyzed the cells with a CytoFLEX flow cytometer (Beckmann Coulter, Indianapolis, USA) assessed the data using FlowJo software 10.08 (Tree Star, Ashland, OR, USA).

2.7. Incubation of metamizole metabolites with H_2O_2

We prepared 2×10^5 HL60 cell suspensions or 1×10^6 freshly isolated neutrophils and added 100 μM MAA as described above. To simulate the neutrophil oxidation system, we also used H_2O_2 in PBS pH 7.4 at final concentrations of 10–100 μM. One hundred μM was the only concentration of H_2O_2 in HL60 cells, where the addition of MAA increased the cytotoxicity. For neutrophil granulocytes, we therefore used only this concentration. For that, 10^6 freshly isolated neutrophils were co-incubated with 100 μM H_2O_2 and MAA as described above. We then assessed membrane integrity and ATP content as described previously.

2.8. Cytotoxicity of MAA in the presence of HOCl

We prepared 2×10^5 HL60 cells in 2 mL Eppendorf tubes and added 100 μM MAA as described above. We then immediately added 100 μM HOCl (Sigma–Aldrich, Buchs, Switzerland), which would be the highest reachable concentration when 100 μM hydrogen peroxide is converted to HOCl by myeloperoxidase. We incubated the cells and assessed membrane toxicity and ATP content as outlined before.

2.9. Cytotoxicity of MAA in the presence of H_2O_2 and free iron

We prepared 2×10^5 HL60 cells in 2 mL Eppendorf tubes and added 100 μM MAA as described above. Before adding 10–100 μM of H_2O_2, we pre-incubated the reaction solution with 50 μL of ferrous iron ($FeSO_4$) at a final concentration of 12.5 μM. Then, we incubated the cells for 24 h and assessed membrane toxicity, ATP content and percentage of apoptotic and necrotic cells as outlined previously.

2.10. Preparation of apo- and hololactoferrin

To obtain apolactoferrin, 4 mg/mL lactoferrin was dissolved in MilliQ water and dialyzed intensively against 0.1 M citric acid/citrate buffer (pH 3.0) containing 0.1 M NaCl for 24 h using a dialysis membrane with a molecular weight cutoff of 10'000 Da (Slide-A-Lyzer® Dialysis Cassette, Thermo Scientific, USA). Afterwards, we dialyzed the obtained apolactoferrin solution against MilliQ water for 24 h.

To obtain hololactoferrin, we dissolved 4 mg/mL lactoferrin in 10 mM Tris-HCl buffer (pH 7.2) containing 74 mM NaCl. We then added freshly prepared Fe nitrilotriacetic acid (NTA) solution consisting of 9.9 mM $Fe(NO_3)_3$ and 8.5 mM NTA in water and adjusted the pH to 7.0 with 5 M sodium bicarbonate solution. To achieve the highest possible iron saturation, we used an iron to lactoferrin molar ratio of 4:1 [27].

To determine the iron saturation of the different lactoferrin varieties, we measured the absorption spectrophotometrically at 280 nm and 465 nm [28]. We determined an iron content of 15.5%, 63%, and 87% for apolactoferrin, lactoferrin, and hololactoferrin, respectively.

2.11. Cytotoxicity of MAA in the presence of lactoferrin or different free iron compounds

For the cytotoxicity assays we prepared 2×10^5 HL60 cells in 2 mL Eppendorf tubes and added 100 μM MAA as described above as well as 4 μg/mL lactoferrin, or apo- or hololactoferrin or 12.5 μM $FeSO_4$ or $FeCl_3$. Then, we incubated the cells for 24 h and assessed membrane

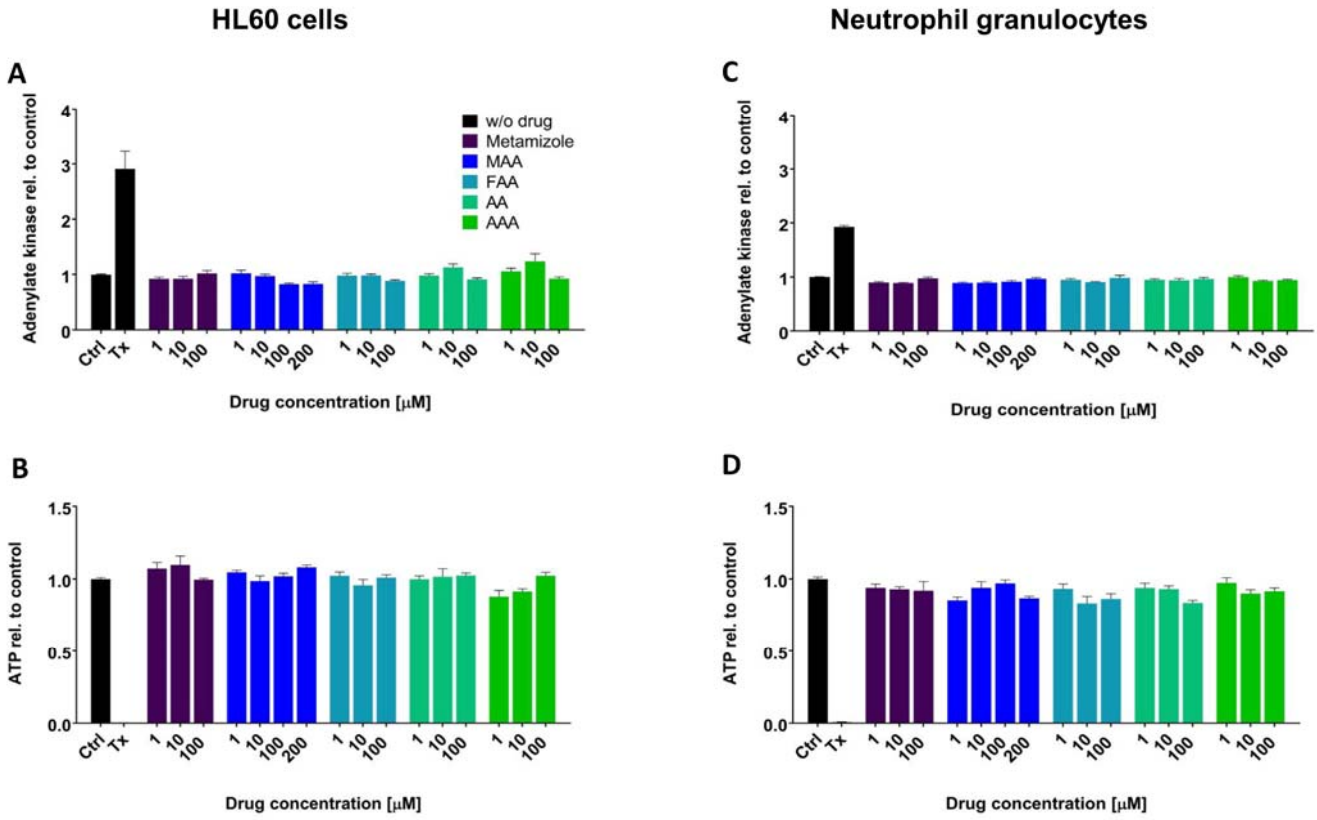

Fig. 2. Effect of N-methyl-4-aminoantipyrine (MAA), 4-aminoantipyrine (AA), N-formyl-4-aminoantipyrine (FAA) and N-acetyl-4-aminoantipyrine (AAA) on cytotoxicity for HL60 cells and freshly isolated neutrophil granulocytes. Cells were treated with the toxicants for 24 h. Membrane integrity was determined as the release of adenylate kinase into the supernatant of HL60 cells (A) and neutrophil granulocytes (C). The cellular ATP content was determined in HL60 cells (B) and neutrophil granulocytes (D). There were no statistically significant differences between treatments and controls in HL60 cells or neutrophil granulocytes. Ctrl: control, Tx: 0.1% Triton X.

toxicity and ATP content as outlined previously.

2.12. Cytotoxicity of MAA in the presence of hemoglobin, methemoglobin or hemin

We prepared 2×10^5 HL60 cells in 2 mL Eppendorf tubes and added 100 μM MAA and 500 μg/mL hemoglobin, methemoglobin or 12.5 μM hemin. Similarly, we prepared 1×10^6 neutrophil granulocytes in 2 mL Eppendorf tubes and added 100 μM MAA and 12.5 and 25 μM hemin. Since hemin is not readily soluble in PBS, we first dissolved it in 10 mM NaOH as a 1 mM stock solution and then diluted it to the final concentration. We also added an equivalent concentration of NaOH to the vehicle control. Then, we incubated the cells and assessed membrane toxicity, ATP content and Annexin V/PI staining as outlined previously.

To chelate the added iron of hemin, we added 100 μM EDTA [29] to the reaction solution 15 min before addition of hemin. Further, to scavenge possible radicals 1 mM glutathione (GSH) or 1 mM N-acetylcysteine (NAC) (final concentrations) were added to the reaction solution in the same way as EDTA mentioned before.

2.13. Activation of pro-apoptotic caspase-3/7

We prepared 2×10^5 HL60 cells in 2 mL Eppendorf tubes and added 100 μM MAA and 12.5 μM hemin as mentioned before. To assess the activity of caspase-3/7, which plays a key effector role in apoptosis, we used the Caspase-Glo® 3/7 luminescent assay (Promega Corporation, Madison, USA). After incubation for 24 h in presence of the test compounds, we added 50 μL assay buffer to 50 μL cell suspension from treated cells and measured luminescence after 60 min of incubation with a Tecan Infinite pro 200 microplate reader (Tecan, Männedorf, Switzerland).

2.14. Interaction of MAA with hemin

We performed a spectrophotometric titration to assess whether MAA has an effect on hemin absorption. The chromophoric character of hemin makes it possible to follow a possible reaction via absorption spectroscopy. Absorption spectra were measured using a Varian-Cary 5000 spectrophotometer. Hemin disodium salt (0.35 mM) was dissolved in D_2O and an absorption spectrum was recorded in the range 200–700 nm. MAA was added to the solution in steps of 0.5 equivalents up to 4 equivalents. An absorption spectrum was recorded after each addition of MAA.

2.15. NMR kinetic

Hemin (0.5 mM) was dissolved in D_2O in a 5 mL round-bottomed flask. After the addition of MAA (2 mM), the solution was heated to 37 °C. The flask was equipped with a septum containing a needle to guarantee diffusion of atmospheric oxygen. After defined time points, ^1H and ^{13}C NMR spectra were recorded at room temperature using a Bruker Advance III-500 NMR spectrometer. Stability of MAA in D_2O was confirmed by NMR kinetic runs on a solution of MAA in D_2O kept at 37 °C over the course of one week (data not shown).

2.16. Data analysis

Data are presented as the mean ± SEM from at least three independent experiments. We measured each value in triplicate. We used GraphPad Prism software (GraphPad Software Inc., San Diego, CA,

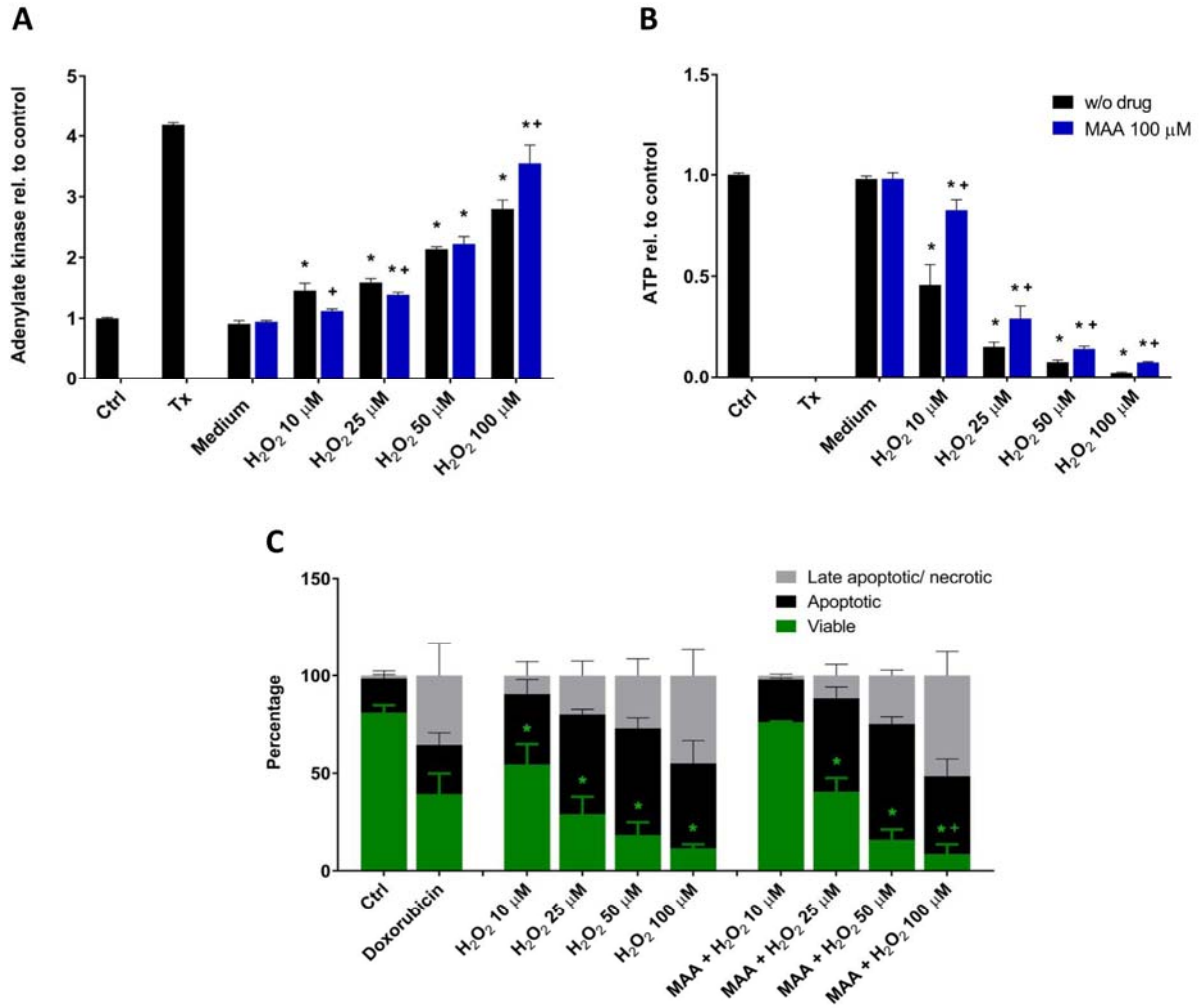

Fig. 3. Effect of H_2O_2 and MAA on HL60 cells. Cells were treated with different H_2O_2 concentrations in the absence or presence of 100 µM MAA for 24 h. (A) Effect on adenylate kinase release (marker of membrane integrity), (B) effect on intracellular ATP concentration and (C) effect on cell death by apoptosis or necrosis. Apoptosis and necrosis were assessed by staining with Annexin V and propidium iodide, respectively. *p < 0.05 vs. control incubations (Ctrl), $^{+}$p < 0.05 vs. incubations w/o MAA of the same treatment group. Ctrl: control, Tx: 0.1% Triton X, MAA: N-methyl-4-aminoantipyrine.

USA) for statistical analyses. Differences between many groups were tested by one-way ANOVA followed by Bonferroni's multiple comparison test to localize significant results in the ANOVA. Differences within two groups were tested by an unpaired *t*-test. A $p < 0.05$ was considered to be a significant difference.

3. Results

3.1. Plasma membrane toxicity and ATP depletion by metamizole and metamizole metabolites in HL60 cells and neutrophil granulocytes

Freshly isolated human granulocytes and HL60 cells, a granulocyte precursor cell line expressing myeloperoxidase [15], were used as cell models for assessing plasma membrane toxicity of metamizole and metamizole metabolites. Metamizole (up to 100 µM), N-methyl-4-aminoantipyrine (MAA, up to 200 µM), 4-aminoantipyrine (AA), N-acetyl-4-aminoantipyrine (AAA) and N-formyl-4-aminoantipyrine (FAA) (all up to 100 µM) showed neither toxicity for HL60 cells (Fig. 2A and B), nor for freshly isolated human granulocytes after 24 h incubation (Fig. 2C and D) or 48 h incubation (data not shown).

3.2. Possible role of MPO in MAA associated plasma membrane toxicity in HL60 cells

Membrane toxicity of MAA and AA could be related to myeloperoxidase activity, which is expressed in HL60 cells [14,15]. In the presence of chloride and H_2O_2, MPO can form hypochlorite, which is used by granulocytes for destroying bacteria [18,20] and which could react with MAA or AA and form toxic metabolites [13]. We therefore assessed the toxicity of MAA and AA in the presence of H_2O_2, the substrate of MPO, in the presence of chloride.

As shown in Fig. 3A, 100 µM MAA did not impair membrane integrity of HL60 cells, whereas H_2O_2 showed a concentration-dependent toxicity, reaching significance at 10 µM. Up to 25 µM H_2O_2, MAA significantly prevented the toxicity of H_2O_2, but at 100 µM, MAA rendered H_2O_2 more toxic. The cellular ATP content dropped H_2O_2 concentration-dependently to less than 10% of control values for 100 µM H_2O_2, whereas 100 µM MAA did not significantly reduce the cellular ATP content (Fig. 3B). MAA partially prevented the H_2O_2-associated drop in the cellular ATP content for all H_2O_2 concentrations investigated. The assessment of the apoptotic and necrotic cell fractions showed a H_2O_2 concentration-dependent increase predominantly in necrotic cells (Fig. 3C). The apoptotic cell fraction increased up to 25 µM H_2O_2, whereas at higher H_2O_2 concentrations only the necrotic fraction

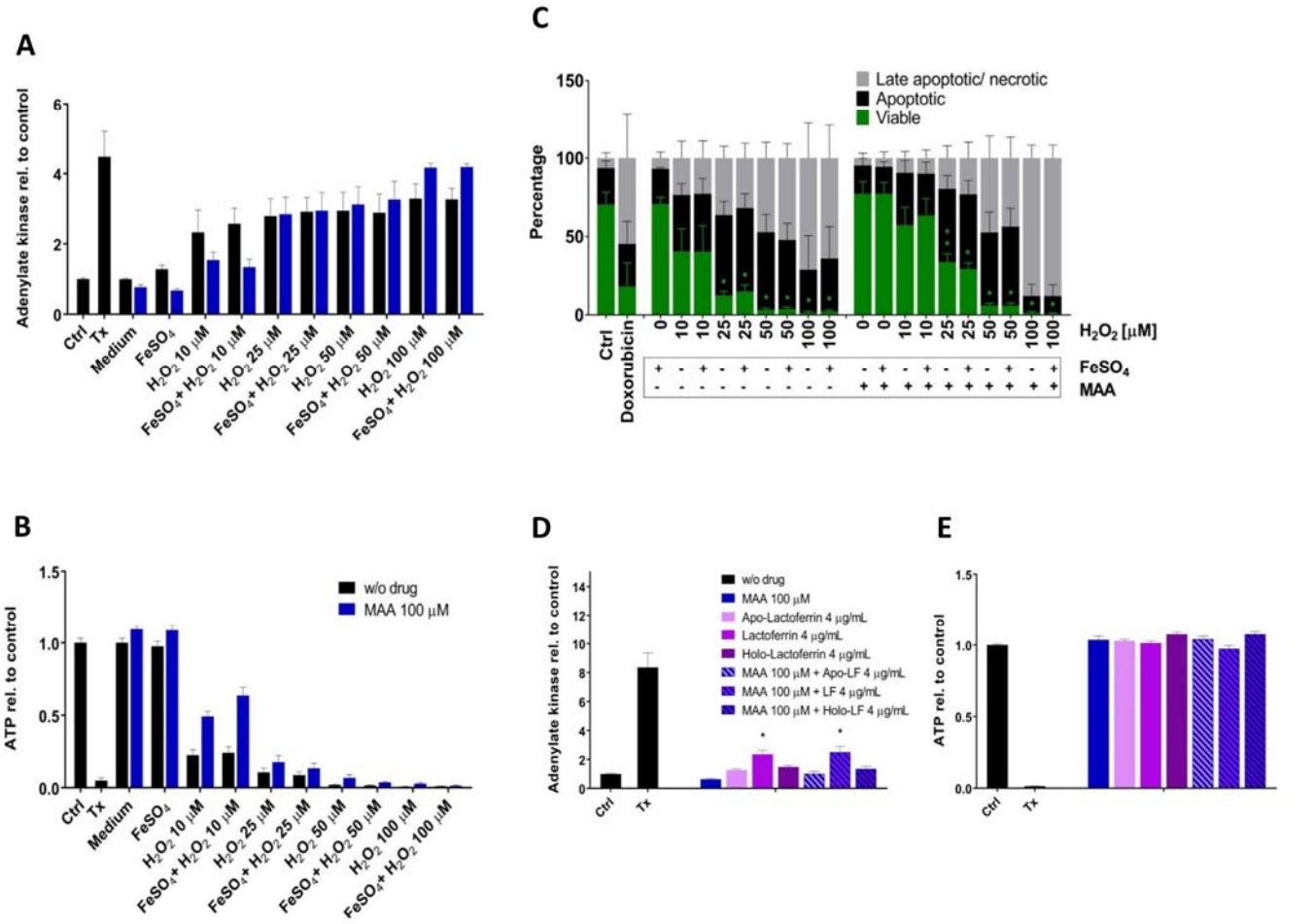

Fig. 4. Effect of $FeSO_4$, H_2O_2, lactoferrin and MAA on HL60 cells. Cells were treated with $FeSO_4$ (12.5 µM) and different H_2O_2 concentrations in the absence or presence of 100 µM MAA for 24 h. Apoptosis and necrosis were assessed by staining with Annexin V and propidium iodide, respectively. (A) Effect of $FeSO_4$ (12.5 µM) and H_2O_2 on adenylate kinase release (marker of membrane integrity), (B) effect of $FeSO_4$ (12.5 µM) and H_2O_2 on intracellular ATP concentration and (C) effect on cell death by apoptosis or necrosis. Apo-lactoferrin and holo-lactoferrin were prepared as described in Methods. Cells were treated with the different lactoferrins in the presence or the absence of MAA for 24 h. (D) Effect of lactoferrin on adenylate kinase release (marker of membrane integrity), (E) effect of lactoferrin on intracellular ATP concentration. $^*p < 0.05$ vs. control incubations (Ctrl). $^*p < 0.05$ vs. control incubations (Ctrl), $^+p < 0.05$ vs. incubations w/o MAA of the same treatment group. Ctrl: control, Tx: 0.1% Triton X, MAA: N-methyl-4-aminoantipyrine.

further increased. Similar to the findings for membrane toxicity, MAA was protective up to 25 µM H_2O_2 but increased the necrotic cell fraction at 100 µM H_2O_2. Similar results regarding membrane toxicity and cellular ATP content were found for the incubation of 100 µM AA with 100 µM H_2O_2, whereas FAA and AAA had no effect on H_2O_2 cytotoxicity (data not shown).

Interestingly, freshly isolated neutrophils were much more resistant to the toxic effects of H_2O_2 and MAA (data not shown). H_2O_2 alone was not membrane-toxic and did not deplete the cellular ATP pool. Furthermore, the addition of MAA (up to 200 µM) did not affect membrane toxicity or cellular ATP depletion in the presence of H_2O_2.

In order to test directly the possibility that hypochlorite could mediate the toxicity of MAA or AA, we incubated HL60 cells with up to 200 µM MAA or AA in the presence of 100 µM NaClO (data not shown). Under these conditions, we did not observe membrane toxicity or cellular ATP depletion, excluding a role of hypochlorite in MAA-associated toxicity.

We also performed experiments in the presence of the MPO inhibitor PF1335 [30], which did not change the effect of 100 µM H_2O_2 in the absence or presence of 100 µM MAA on membrane integrity and ATP content of HL60 cells (results not shown).

These results indicated that myeloperoxidase has no important role in the toxicity of MAA and AA. We therefore concentrated on a possible role of the Fenton reaction or other iron-related mechanisms for myelotoxicity of MAA and AA. Iron compounds are common in the human body and are known to be reactive and to be able to form cytotoxic metabolites from organic compounds [22].

3.3. Cytotoxicity of MAA in the presence of different free iron compounds

In a next step, we assessed the cytotoxicity of the iron compounds $FeSO_4$ and $FeCl_3$, which contain Fe^{2+} and Fe^{3+} respectively. These iron compounds provide free iron ions in aqueous solution and MAA may react with them to give cytotoxic metabolites. However, that neither the Fe^{2+} and Fe^{3+} ions were associated with membrane toxicity or decreased cellular ATP levels of HL60 cells in the presence of MAA (data not shown).

Next, we investigated the possibility that MAA could increase cytotoxicity of the combination Fe^{2+} and H_2O_2. As shown in Fig. 4, cytotoxicity of H_2O_2 was not increased in the presence of Fe^{2+}. Similar to incubations containing only H_2O_2 (shown in Fig. 3), the addition of MAA decreased membrane toxicity (up to 10 µM H_2O_2), ATP depletion and cytotoxicity in incubations containing Fe^{2+} and H_2O_2.

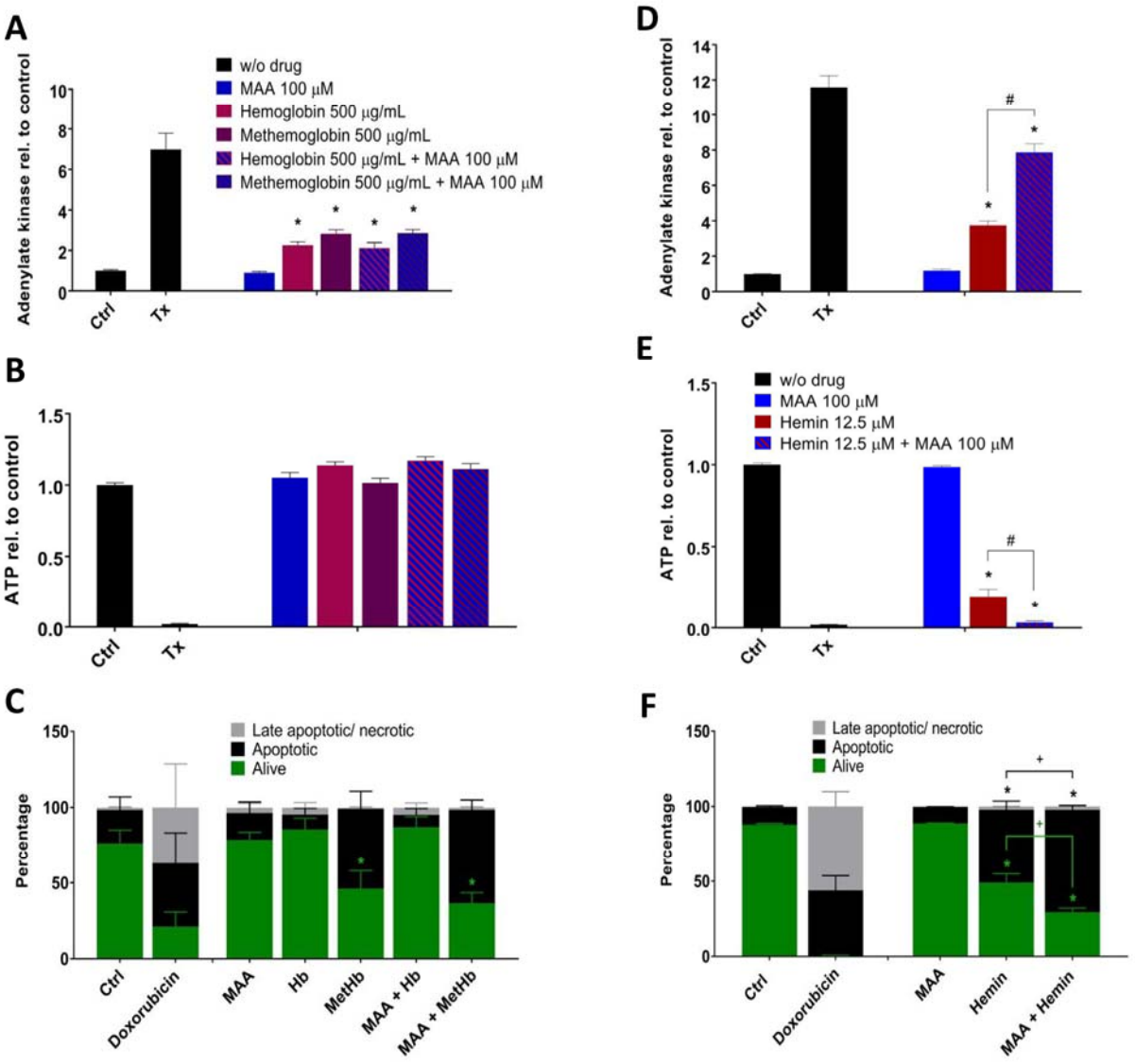

Fig. 5. Effect of hemoglobin, methemoglobin, hemin and MAA on HL60 cells. Cells were treated with hemoglobin, methemoglobin or hemin in the absence or presence of 100 µM MAA for 24 h. (A) Effect on adenylate kinase release (marker of membrane integrity) by hemoglobin and methemoglobin, (B) effect on intracellular ATP concentration by hemoglobin and methemoglobin and (C) effect on cell death by apoptosis or necrosis by hemoglobin and methemoglobin. (D) Effect on adenylate kinase release (marker of membrane integrity) by hemin, (E) effect on intracellular ATP concentration by hemin and (F) effect on cell death by apoptosis or necrosis by hemin. Apoptosis and necrosis were assessed by staining with Annexin V and propidium iodide, respectively. *$p < 0.05$ vs. control incubations (Ctrl), $^{+}p < 0.05$ vs. incubations w/o MAA of the same treatment group. Ctrl: control, Tx: 0.1% Triton X, MAA: N-methyl-4-aminoantipyrine.

3.4. Cytotoxicity of MAA in the presence of different forms of lactoferrin

Lactoferrin, a protein containing Fe^{3+}, is found in secondary granules of neutrophils [31,32] and, to a lower extent, in blood and plasma [33]. During inflammation, the plasma lactoferrin concentration as well as the amount on the neutrophil surface increase due to neutrophil degranulation [31,32]. MAA and AA may react with lactoferrin and form cytotoxic metabolites. In order to test this hypothesis, we incubated HL60 cells and MAA with lactoferrins of different iron saturation and assessed membrane toxicity and ATP content of the cells. As shown in Fig. 4D and E, none of the different lactoferrin forms was more cytotoxic (membrane damage and ATP content) when incubated with MAA. Lactoferrin itself slightly impaired membrane integrity, but had no effect on the cellular ATP content. Since we chose a 2–3 fold higher lactoferrin concentration than the highest concentrations observed in human plasma [33], we considered it as unlikely that lactoferrin contributed to metamizole-induced neutropenia. This may be due to the fact that the iron in lactoferrin is well embedded and protected against environmental effects, making reactions with MAA and cytotoxic metabolite formation unlikely.

3.5. Cytotoxicity of MAA in the presence of hemoglobin, methemoglobin or hemin

Hemoglobin is another physiologically occurring compound containing iron. It is contained in erythrocyte precursors in bone marrow (in the vicinity of granulocyte precursors) and in mature erythrocytes in the blood. Both hemoglobin (containing Fe^{2+}) and its oxidized form methemoglobin (containing Fe^{3+}) represent an iron source that could possibly react with MAA to form cytotoxic intermediates. Hemoglobin consists of four subunits, each with a heme group containing an iron in its center [34]. Hemoglobin can be degraded to heme (Fe^{2+}) or the oxidized form hemin (Fe^{3+}) by macrophages in liver, bone marrow and spleen [35]. As shown in Fig. 5A and B, hemoglobin, and even more methemoglobin, affected the membrane integrity, but did not decrease the ATP content of HL60 cells. The addition of MAA did not lead to a

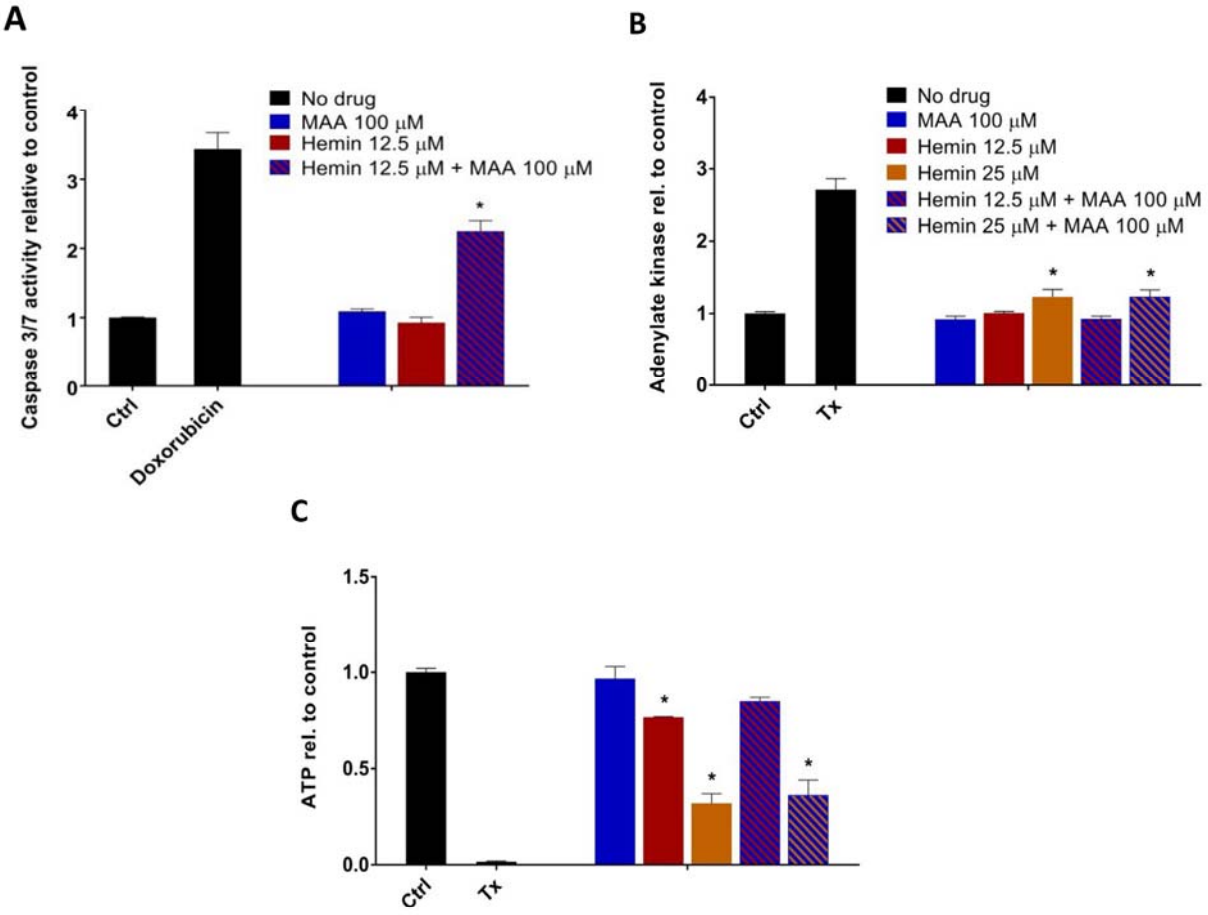

Fig. 6. Effect of hemin and MAA on caspase 3 activity in HL60 cells as well as effect on cytotoxicity in neutrophil granulocytes. (A) HL60 cells were treated with 12.5 μM hemin in the absence or presence of 100 μM MAA for 24 h. Caspase 3 activity was measured using luminescence as described in Methods. Doxorubicin was used as a positive control. (B-C) Freshly isolated neutrophil granulocytes were treated with 12.5 or 25 μM hemin in the absence or presence of 100 μM MAA for 24 h. (B) Effect on adenylate kinase release (marker of membrane integrity), (C) effect on intracellular ATP concentration. *$p < 0.05$ vs. control incubations (Ctrl), Ctrl: control, Tx: 0.1% Triton X, MAA: N-methyl-4-aminoantipyrine.

significant increase in the observed membrane toxicity or a decrease in the cellular ATP content. These findings were confirmed by the staining for apoptotic and necrotic cells, where only methemoglobin showed a significant higher percentage of apoptotic (but not necrotic) cells than control incubations (Fig. 5C). The addition of MAA did not significantly increase the percentage of apoptotic or necrotic cells in the presence of hemoglobin or methemoglobin.

Since hemoglobin and methemoglobin represent large molecules wherein the iron ions are embedded, it is uncertain whether MAA was able to get in contact with the iron ions. We therefore also assessed the cytotoxicity of hemin, an early breakdown product of hemoglobin. Hemin consists of Fe^{3+} within a protoporphyrin ring but, in contrast to hemoglobin, without the globin chains [36]. The iron is therefore probably more accessible for external molecules. As shown in Fig. 5D, hemin alone affected membrane integrity and this toxicity was significantly increased by MAA. Similarly, hemin alone reduced the ATP content of HL60 cells and this effect was accentuated in the presence of MAA (Fig. 5E). These results were confirmed by staining for apoptotic and necrotic cells, where the percentage of apoptotic cells was significantly increased by MAA and hemin compared to hemin alone (Fig. 5F). In order to confirm these results, we determined the activity of the pro-apoptotic caspases 3 and 7. After 24 h of incubation, the combination of hemin and MAA significantly increased the activity of caspase 3 in comparison to MAA or hemin alone (Fig. 6A), confirming the results shown in Fig. 5E.

Interestingly, freshly isolated neutrophils were much more resistant to the toxic effects of hemin and MAA (Fig. 6B and C). The hemin concentration (12.5 μM) that induced membrane toxicity in HL60 cells was not cytotoxic and depleted the cellular ATP pool only slightly. Furthermore, the addition of MAA did not increase plasma membrane toxicity or cellular ATP depletion in the presence of hemin. In contrary, MAA attenuated the drop in ATP in presence of 12.5 μM hemin.

3.6. Effect of EDTA on plasma membrane toxicity of MAA in HL60 cells

The next step was to expose HL60 cells to EDTA in order to chelate iron. In incubations containing MAA and hemin, EDTA reduced the plasma membrane toxicity of MAA and hemin to almost control levels. EDTA also attenuated the ATP depletion associated with MAA and hemin. These findings confirmed the important role of iron in the toxicity of hemin and MAA (data not shown).

3.7. Prevention of the plasma membrane toxicity of MAA with antioxidants

A possible explanation of the toxicity of MAA associated with hemin is the formation of reactive metabolites (radicals) from MAA [22]. We investigated this possibility by the addition of antioxidants to the incubations, which can trap electrophilic reactive metabolites such as radicals. As shown in Fig. 7A and B, the addition of NAC efficiently prevented plasma membrane toxicity and ATP depletion associated with hemin or the combination of hemin and MAA, suggesting that the formation of reactive intermediates was responsible for the observed

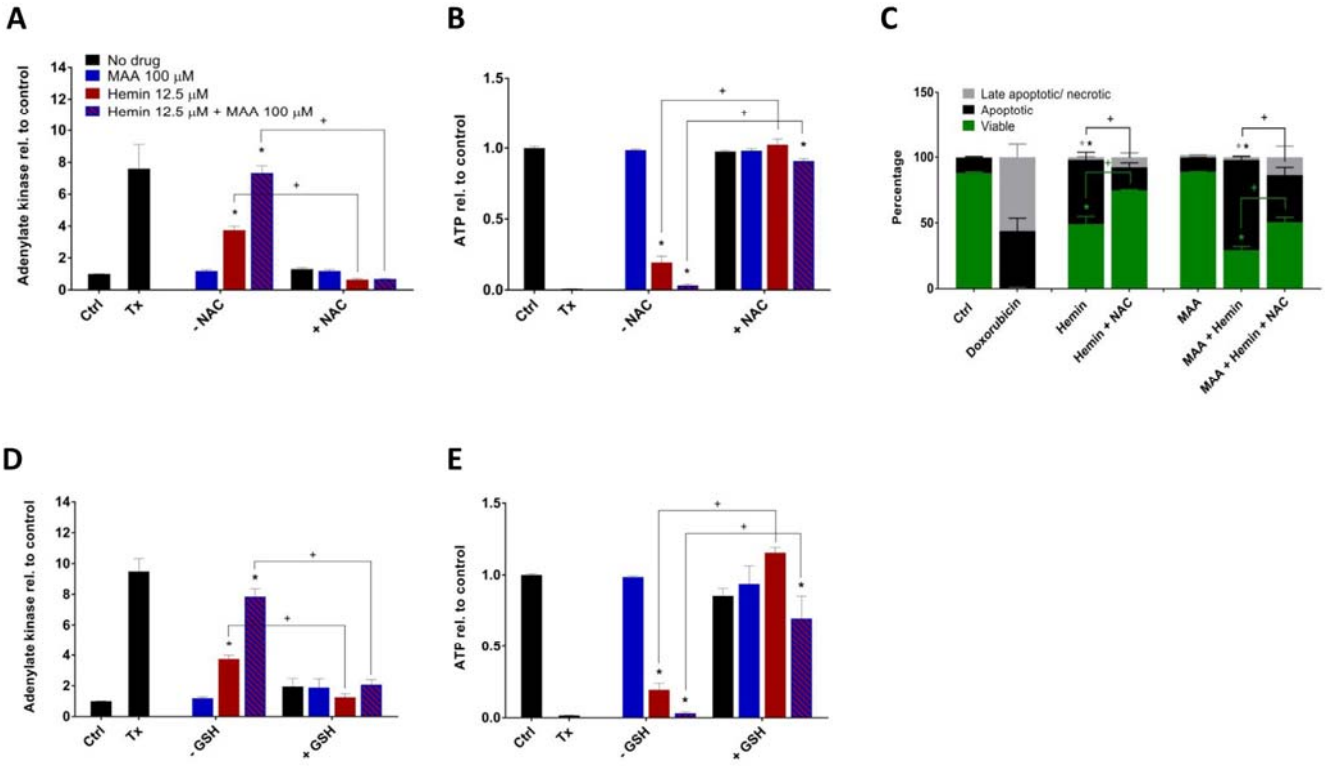

Fig. 7. Effect of N-acetylcysteine (NAC), glutathione (GSH), and MAA on the effect of hemin on HL60 cells. Cells were treated with hemin in absence or presence of NAC or MAA for 24 h. (A) Effect on adenylate kinase release (marker of membrane integrity), (B) effect on intracellular ATP concentration and (C) effect on cell death by apoptosis or necrosis. Apoptosis and necrosis were assessed by staining with Annexin V and propidium iodide, respectively. Cells were treated with hemin in absence or presence of GSH or MAA for 24 h. (D) Effect on adenylate kinase release (marker of membrane integrity), (E) effect on intracellular ATP concentration. $^{*}p < 0.05$ vs. control incubations (Ctrl), $^{+}p < 0.05$ vs. incubations w/o NAC or GSH of the same treatment group. Ctrl: control, Tx: 0.1% Triton X, MAA: N-methyl-4-aminoantipyrine.

toxicity. As shown in Fig. 7C, NAC also increased the percentage of viable and reduced the percentage of apoptotic cells when co-incubated with hemin or the combination hemin and MAA compared to the respective incubations without NAC. As shown in Fig. 7D and 7E, glutathione also reduced membrane toxicity and ATP depletion when co-incubated with hemin or the combination hemin and MAA compared to the respective incubations without glutathione.

These results confirm the iron-associated formation of reactive metabolites as a mechanism for the toxicity of MAA on HL60 cells.

3.8. Spectrophotometric monitoring of the interaction of MAA with hemin

It is known that MAA interacts with Fe^{3+} ions in aqueous solution through an electron exchange that reduces Fe^{3+} to Fe^{2+}. The reaction of MAA with $FeCl_3$ has been used as an analytical method to detect the presence of MAA. The amount of analyte has been assessed from both absorption of the Fe^{2+}-(MAA) complex [37] and of $[Fe(phen)_3]^{2+}$ after reduction of Fe^{3+} to Fe^{2+} by MAA [38].

Once iron is coordinated by a rigid square-planar ligand such as hemin, it is not available to form a complex with either MAA or 1,10-phenanthroline. If Fe^{3+} is reduced to Fe^{2+} in hemin by MAA, the reduction process should result in different spectroscopic properties of the porphyrin core. Keeping this in mind, we performed a spectrophotometric titration to assess the effect of MAA on hemin absorption. As shown in Fig. 8A, the addition of small amounts of MAA resulted in a decrease and redshift of the hemin absorption bands. In the region between 450 and 650 nm two bands remained, a spectroscopic signal of a metallated porphyrin core. In comparison, the spectrum of non-metallated protoporphyrin contains four bands in this region [39,40,38,37].

The absorption spectrum indicated that the electronic properties of the hemin ring were changing, possibly due to a change in the oxidation state of the aromatic ring or of the coordinated metal centre.

3.9. Product identification by NMR

After having confirmed an interaction between MAA and hemin, we moved on to the identification of the product resulting from this reaction. NMR spectroscopy was the analytical method of choice, since it allows structural elucidation at both the proton and carbon level. We therefore incubated MAA and hemin in D_2O at 37 °C for 192 h. From ^{1}H NMR spectra obtained at different time points, it is evident that MAA converted to a single reaction product in less than 95 h under our experimental conditions (Fig. 8B). The product did not undergo further transformations when incubated under these conditions for another four days.

Fig. 8C and D show a comparison of the ^{1}H and ^{13}C NMR spectra of the starting material MAA, the demethylated derivative AA and the reaction product detected under our experimental conditions. The signals have been assigned through 2D NMR experiments (COSY, NOESY, HMBC and HMQC) (Fig. 9A-C). The spectra show that the reaction product was not AA and allowed us to propose a possible structure of the reaction product (Fig. 8C).

A possible reaction sequence leading to the proposed product is given in Fig. 9E. Finally, we simulated the ^{13}C NMR spectrum of the proposed product using the ChemBioDraw Ultra 14.0 software, which yielded a good match with the experimental spectrum (Fig. 9D).

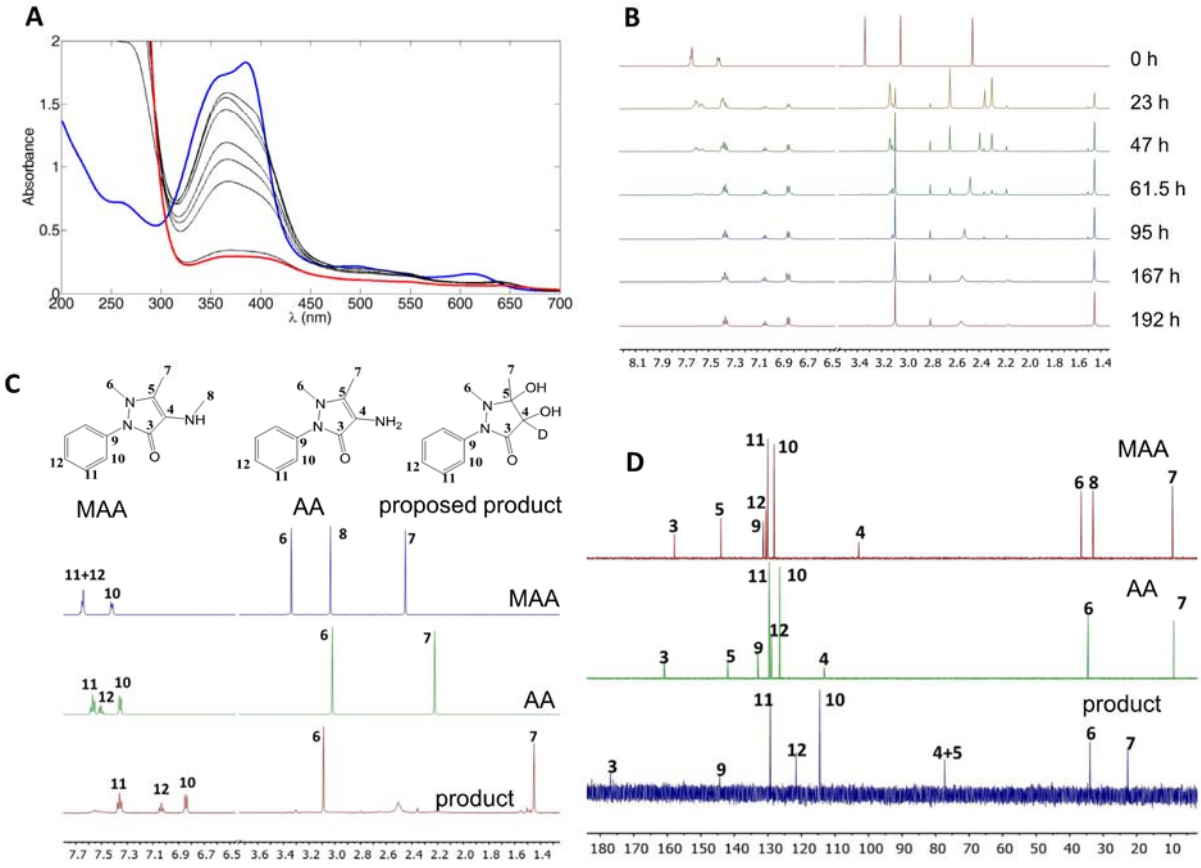

Fig. 8. Reaction of MAA with hemin. (A) Hemin was dissolved in D_2O and an absorption spectrum was recorded in the range of 200 to 700 nm. MAA was added to the solution in steps of 0.5 equivalents up to 4 equivalents. The blue line represents the spectrum of MAA alone and the red line hemin in the presence of 4 equivalents MAA. (B) Hemin was dissolved in D_2O and 4 equivalents of MAA added. The solution was kept at 37 °C and 1H and 13C NMR spectra were recorded at room temperature at the time points indicated. (C) Comparison of the 1H NMR spectra obtained from the starting material MAA, the demethylated derivative AA and the proposed reaction product (D) Comparison of the 13C NMR spectra obtained from the starting material MAA, the demethylated derivative AA and the proposed reaction product. MAA: N-methyl-4-aminoantipyrine, AA: 4-aminoantipyrine. The title of the ordinates in Fig. B, C and D is intensity without units.

4. Discussion

The current study shows that MAA, AA, FAA and AAA alone were not toxic for HL60 cells, that MAA did not significantly increase the toxicity of Fe^{2+}, Fe^{3+}, hemoglobin or lactoferrin and that MAA increased the toxicity of H_2O_2 only at high concentrations (100 µM). On the other hand, MAA reduced Fe^{3+} to Fe^{2+} in hemin by a reaction producing reactive intermediates which may explain the increased cytotoxicity of hemin by MAA.

After having shown that MAA, AA, AAA and FAA are not toxic on HL60 cells and freshly isolated granulocytes up to 200 µM, we investigated the possibility that active and possibly cytotoxic metabolites could be formed via substrates and/or products of the myeloperoxidase reaction. Utrecht et al. have shown previously that toxic products can be formed from aminopyrine (dimethylaminoantipyrine) by hypochlorite, which is a reaction product of MPO [13]. We therefore tested the effect of MAA on HL60 cells and granulocytes at different H_2O_2 concentrations. At low H_2O_2 concentrations (< 50 µM), MAA was protective for membrane toxicity and ATP depletion by H_2O_2, and only at the highest H_2O_2 concentration tested (100 µM), we observed an increased cytotoxicity associated with MAA. In plasma, H_2O_2 concentrations are dependent on race and gender and vary between 1 and 5 µM [41,42]. In patients with inflammatory diseases, the H_2O_2 concentration in plasma can reach 50 µM and up to 100 µM in activated phagocytes [42,43]. We could therefore not completely exclude the possibility that MAA in combination with high H_2O_2 concentrations could become cytotoxic for cells in bone marrow. On the other hand, taking also into account the lack of toxicity on HL60 cells for the combination 100 µM hypochlorite and MAA, led us to the conclusion that substrates and/or reaction products of MPO are an unlikely cause for MAA-associated myelotoxicity.

Next, we studied the possibility that the Fenton reaction could play a role in MAA-associated myelotoxicity. The Fenton reaction describes the oxidation of organic substrates in the presence of iron salts and H_2O_2 [22]. It has recently been described by Giri and Golder that metamizole can be degraded by a Fenton reaction [44]. Although Giri and Golder used different conditions compared to the current study, e.g. a cell-free system, high H_2O_2 concentrations (22.5 mM) and an acid milieu (50 mM H_2SO_4), they demonstrated that metamizole can be degraded in the presence of Fe^{2+} and H_2O_2 via several steps eventually leading to the production of hydroxyl radicals. In the current study, aqueous Fe^{2+} and Fe^{3+} were not cytotoxic for HL60 cells, irrespective of the absence or presence of MAA. In addition, Fe^{2+} did not increase the toxicity of H_2O_2, and, similar to incubations containing only H_2O_2, MAA was protective in the presence of Fe^{2+} and H_2O_2 combinations at H_2O_2 concentrations < 100 µM. These results suggested that the Fenton reaction played no important role in the myelotoxicity of MAA. Pierre et al. have described a change in the absorption spectrum of MAA in the presence of Fe^{2+} (or Fe^{3+}) and H_2O_2, suggesting that MAA can react with Fe^{2+} (or an intermediate of the Fenton reaction) under these conditions [24]. However, in the study of Pierre et al., the change in the absorption spectrum of MAA in the presence of Fe^{2+} and H_2O_2 was much smaller than the change observed in the presence complexed iron such as hemin. Taking into account the study of Pierre et al. [24] and

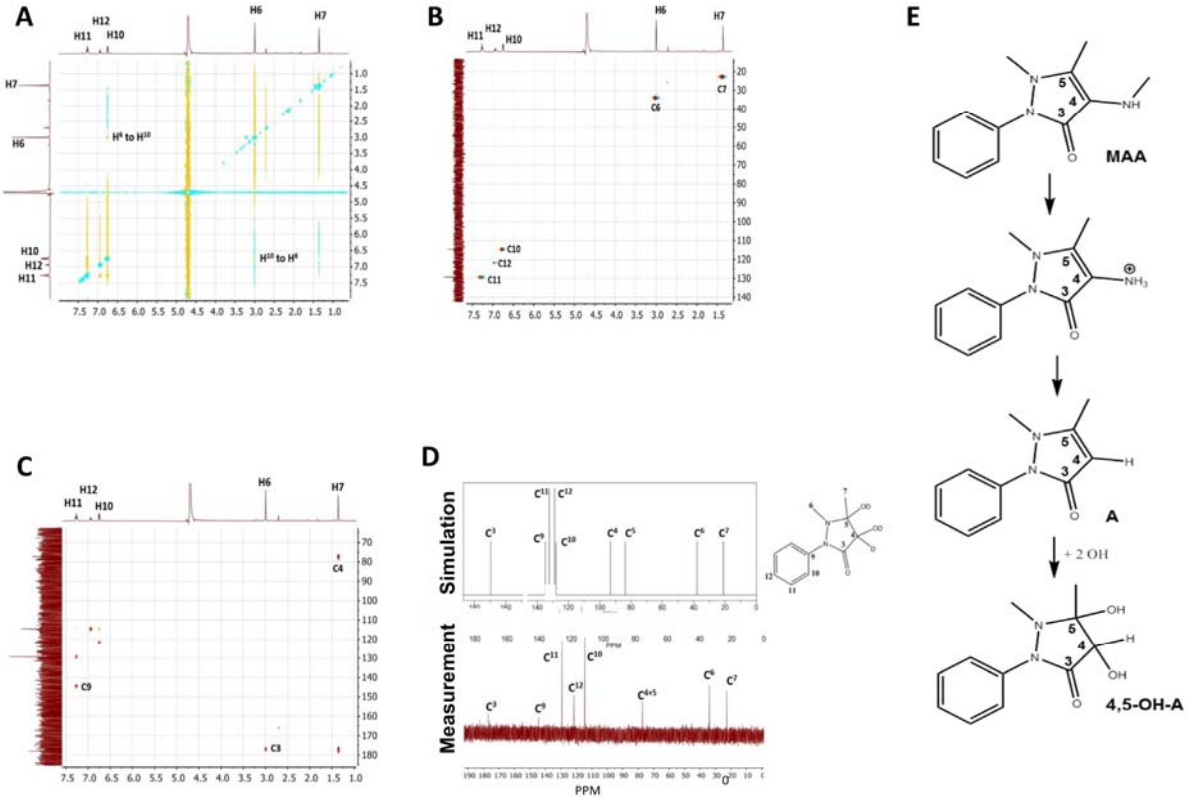

Fig. 9. Reaction pathway and comparison of the reaction product with simulated 13C NMR spectrum. (A) 500 MHz NOESY spectrum of a D_2O solution of the reaction product (295 K). δ = 4.79 ppm residual solvent peak. (B) HMQC spectrum (^1H, 500 MHz; ^{13}C, 126 MHz) of a D_2O solution of the reaction product (295 K). δ = 4.79 ppm residual solvent peak. Hydrogen-bearing carbons are assigned. (C) HMBC spectrum (^1H, 500 MHz; ^{13}C, 126 MHz) of a D_2O solution of the reaction product (295 K). δ = 4.79 ppm residual solvent peak. Quaternary carbons are assigned. (D) The simulated ^{13}C NMR spectrum of the proposed product (using ChemBioDraw Ultra 14.0 software) yielded a good match with the real spectrum. (E) The proposed reaction pathway is based on the publication by Giri and Golder (44). This pathway includes an electrophilic intermediate, which may be trapped by electron donors such as NAC and glutathione and which may be cytotoxic. MAA: N-methyl-4-aminoantipyrine, A: antipyrine, 4,5-OH-A: 4,5-dihydroxyantipyrine.

the results of the current study we can conclude that MAA can be involved in Fenton reactions, but that such reactions are not associated in toxicity on HL60 cells. We therefore also excluded Fenton reactions as a likely possibility of cytotoxicity associated with MAA.

As shown by Pierre et al. [24], MAA and AA (but not FAA and AAA) can react with complexed iron. We therefore studied possible interactions of MAA with lactoferrin, hemoglobin and methemoglobin. Lactoferrin is a serin protease containing Fe^{3+} which is expressed in promyelocytes during the development of neutrophil granulocytes [31,32]. It is stored in secondary granules of neutrophils and released on the granulocyte surface and into the blood during infections. In the current study, lactoferrin but not apo- or holo-lactoferrin was slightly membrane-toxic on HL60 cells, but this toxicity was not increased by MAA. We therefore excluded the possibility that cytotoxicity of MAA was associated with lactoferrin. Similarly, hemoglobin and methemoglobin were slightly membrane-toxic on HL60 cells, but did not decrease the cellular ATP content. Sine MAA did not increase the toxicity of hemoglobin and methemoglobin; we excluded also possibility that hemoglobin or methemoglobin were associated with cytotoxicity of MAA.

Next, we studied a possible interaction with hemin. Hemin is the degradation product of hemoglobin and contains Fe^{3+} in a porphyrin ring [45]. Hemin can be taken up by cells and can react with H_2O_2 to produce cytotoxic radicals. Hemin itself was membrane-toxic and decreased the ATP content of HL60 cells and this toxicity was significantly increased by the addition of MAA. Further experiments showed that the toxicity of hemin and the combination hemin and MAA could almost completely be prevented by EDTA and by antioxidants such as NAC and glutathione. These findings indicated that Fe^{3+} and radical formation plaid a role in the toxicity of hemin and of the combination hemin and MAA. In spectrophotometric experiments, we could confirm an interaction between MAA and hemin. NMR studies allowed us then to identify a final reaction product and, based on the publication by Giri and Golder [44], to propose a reaction sequence leading to the stable reaction product. This sequence starts with N-demethylation and yields a reactive electrophilic intermediate that may be responsible for the cytotoxicity of the combination MAA and hemin. The formation of an electrophilic intermediate is in agreement with the finding in the current study that the antioxidants (or electron donors) NAC and glutathione were able to prevent the toxicity of MAA and hemin.

The current study suggests that the availability of hemin, e.g. after bleeding or after hemolysis represents a risk factor for MAA-associated myelotoxicity. In the study of Blaser et al. [7], patients with pre-existing hypersensitivity reactions, concomitant drugs known to be associated with leukopenia, hepatitis C infection and pre-existing hematological diseases were overrepresented in patients with metamizole-associated leukopenia compared to control persons, but only a minority of the patients was carrier of such factors. While immunological factors may trigger metamizole-associated granulocytopenia in some patients, non-immunological factors may trigger this adverse reaction in others. Increased breakdown of erythrocytes with abundant hemin may be one of them.

In conclusion, MAA, the principle metabolite of metamizole, clearly increased the cytotoxicity of hemin by a reaction involving the formation of an electrophilic metabolite. Toxicity could be prevented by electron donors such as NAC and glutathione. Situations with increased production of hemin such as extravascular hemolysis may be a risk factor for metamizole-associated granulocytopenia.

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Conflict of interest

None of the authors has a conflict of interest regarding this study.

References

[1] L.S. Blaser, A. Tramonti, P. Egger, M. Haschke, S. Krahenbuhl, A.E. Ratz Bravo, Hematological safety of metamizole: retrospective analysis of WHO and Swiss spontaneous safety reports, Eur. J. Clin. Pharmacol. 71 (2) (2015) 209–217.
[2] S. Sánchez, C. De La Lastra, P. Ortiz, V. Motilva, M.J. Martín, Gastrointestinal tolerability of metamizol, acetaminophen, and diclofenac in subchronic treatment in rats, Dig. Dis. Sci. 47 (12) (2002) 2791–2798.
[3] F. Bachmann, U. Duthaler, D. Rudin, S. Krahenbuhl, M. Haschke, N-demethylation of N-methyl-4-aminoantipyrine, the main metabolite of metamizole, Eur. J. Pharm. Sci. 120 (2018) 172–180.
[4] G. Heinemeyer, H.J. Gramm, I. Roots, R. Dennhardt, W. Simgen, The kinetics of metamizol and its metabolites in critical-care patients with acute renal dysfunction, Eur. J. Clin. Pharmacol. 45 (5) (1993) 445–450.
[5] C. Martinez, I. Andreu, G. Amo, M.A. Miranda, G. Esguevillas, M.J. Torres, et al., Gender and functional CYP2C and NAT2 polymorphisms determine the metabolic profile of metamizole, Biochem. Pharmacol. 92 (3) (2014) 457–466.
[6] J.A. Agundez, C. Martinez, J. Benitez, Metabolism of aminopyrine and derivatives in man: in vivo study of monomorphic and polymorphic metabolic pathways, Xenobiotica 25 (4) (1995) 417–427.
[7] L. Blaser, H. Hassna, S. Hofmann, A. Holbro, M. Haschke, A. Ratz Bravo, et al., Leucopenia associated with metamizole: a case-control study Swiss medical weekly, 2017, 147, w14438.
[8] M. Levy, D. Flusser, E. Zylber-Katz, L. Granit, Plasma kinetics of dipyrone metabolites in rapid and slow acetylators, Eur. J. Clin. Pharmacol. 27 (4) (1984) 453–458.
[9] E. Andres, F. Maloisel, Idiosyncratic drug-induced agranulocytosis or acute neutropenia, Curr. Opin. Hematol. 15 (1) (2008) 15–21.
[10] E. Garbe, Non-chemotherapy drug-induced agranulocytosis, Expert Opin. Drug Saf. 6 (3) (2007) 323–335.
[11] O. Kummer, M. Haschke, D. Tuchscherer, M. Lampert, F. Martius, S. Krahenbuhl, Agranulocytosis in a patient treated with metamizole and clopidogrel, Praxis (Bern 1994) 95 (45) (2006) 1743–1745 quiz 6-7.
[12] V. Vlahov, N. Bacracheva, D. Tontcheva, E. Naumova, M. Mavrudieva, P. Ilieva, et al., Genetic factors and risk of agranulocytosis from metamizol, Pharmacogenetics 6 (1) (1996) 67–72.
[13] J.P. Uetrecht, H.M. Ma, E. MacKnight, R. McClelland, Oxidation of aminopyrine by hypochlorite to a reactive dication: possible implications for aminopyrine-induced agranulocytosis, Chem. Res. Toxicol. 8 (2) (1995) 226–233.
[14] T. Tsuruta, K. Tani, A. Hoshika, S. Asano, Myeloperoxidase gene expression and regulation by myeloid cell growth factors in normal and leukemic cells, . Leuk. Lymphoma 32 (3-4) (1999) 257–267.
[15] S. Maseneni, M. Donzelli, A.B. Taegtmeyer, K. Brecht, S. Krahenbuhl, Toxicity of clopidogrel and ticlopidine on human myeloid progenitor cells: importance of metabolites, Toxicology 299 (2–3) (2012) 139–145.
[16] M. Yamada, M. Mori, T. Sugimura, Purification and characterization of small molecular weight myeloperoxidase from human promyelocytic leukemia HL-60 cells, Biochemistry 20 (4) (1981) 766–771.
[17] Y. Dang, G.M. Lowe, S.W. Edwards, D.W. Galvani, The effects of GM-CSF on myeloperoxidase release in normal and myelodysplastic neutrophils, Leuk. Res. 17 (12) (1993) 1037–1044.
[18] B.S. van der Veen, M.P. de Winther, P. Heeringa, Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease, Antioxid. Redox Signal. 11 (11) (2009) 2899–2937.
[19] J.M. Lü, P.H. Lin, Q. Yao, C. Chen, Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems, J. Cell Mol. Med. 14 (4) (2010) 840–860.
[20] C.C. Winterbourn, Biological reactivity and biomarkers of the neutrophil oxidant, hypochlorous acid, Toxicology (2002) 181–182 223-7.
[21] J.M. Gutteridge, Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides, FEBS Lett. 201 (2) (1986) 291–295.
[22] C.C. Winterbourn, Toxicity of iron and hydrogen peroxide: the Fenton reaction, Toxicol. Lett. 82-83 (1995) 969-74.
[23] P. Lipinski, J.C. Drapier, L. Oliveira, H. Retmanska, B. Sochanowicz, M. Kruszewski, Intracellular iron status as a hallmark of mammalian cell susceptibility to oxidative stress: a study of L5178Y mouse lymphoma cell lines differentially sensitive to H(2)O(2), Blood 95 (9) (2000) 2960–2966.
[24] S.C. Pierre, R. Schmidt, C. Brenneis, M. Michaelis, G. Geisslinger, K. Scholich, Inhibition of cyclooxygenases by dipyrone, Br. J. Pharmacol. 151 (4) (2007) 494–503.
[25] S.J. Klebanoff, S. Olszowski, W.C. Van Voorhis, J.A. Ledbetter, A.M. Waltersdorph, K.G. Schlechte, Effects of gamma-interferon on human neutrophils: protection from deterioration on storage, Blood 80 (1) (1992) 225–234.
[26] K.M. Waldhauser, M. Torok, H.R. Ha, U. Thomet, D. Konrad, K. Brecht, et al., Hepatocellular toxicity and pharmacological effect of amiodarone and amiodarone derivatives, J. Pharmacol. Experimental Therapeutics 319 (3) (2006) 1413–1423.
[27] P.H. van Berkel, M.E. Geerts, H.A. van Veen, P.M. Kooiman, F.R. Pieper, H.A. de Boer, et al., Glycosylated and unglycosylated human lactoferrins both bind iron and show identical affinities towards human lysozyme and bacterial lipopolysaccharide, but differ in their susceptibilities towards tryptic proteolysis, Biochem. J. 312 (Pt 1) (1995) 107–114.
[28] S. Hashizume, K. Kuroda, H. Murakami, Cell culture assay of biological activity of lactoferrin and transferrin, in: D. Barnes, D.A. Sirbasku, (Eds.), Methods in Enzymology, 147: Academic Press, 1987, pp. 302–314.
[29] Q. Al-Anbaky, Z. Al-Karakooly, S.P. Kilaparty, M. Agrawal, Y.M. Albkuri, A.B. RanguMagar, et al., Cytotoxicity of manganese (III) complex in human breast adenocarcinoma cell line is mediated by the generation of reactive oxygen species followed by mitochondrial damage, Int. J. Toxicol. 35 (6) (2016) 672–682.
[30] W. Zheng, R. Warner, R. Ruggeri, C. Su, C. Cortes, A. Skoura, et al., PF-1355, a mechanism-based myeloperoxidase inhibitor, prevents immune complex vasculitis and anti-glomerular basement membrane glomerulonephritis, J. Pharmacol. Experimental Therapeutics 353 (2) (2015) 288–298.
[31] M.E. Drago-Serrano, R. Campos-Rodriguez, J.C. Carrero, M. de la Garza, Lactoferrin: balancing ups and downs of inflammation due to microbial infections, Int. J. Mol. Sci. 18 (3) (2017).
[32] T.G. Kanyshkova, V.N. Buneva, G.A. Nevinsky, Lactoferrin and its biological functions, Biochem. Biokhimiia 66 (1) (2001) 1–7.
[33] T. Olofsson, I. Olsson, P. Venge, Myeloperoxidase and lactoferrin of blood neutrophils and plasma in chronic granulocytic leukaemia, Scandinavian J. Haematol. 18 (2) (1977) 113–120.
[34] A.J. Marengo-Rowe, Structure-function relations of human hemoglobins, Proceedings (Baylor University Medical Center) 19 (3) (2006) 239–245.
[35] N.R. Pimstone, R. Tenhunen, P.T. Seitz, H.S. Marver, R. Schmid, The enzymatic degradation of hemoglobin to bile pigments by macrophages, J. Exp. Med. 133 (6) (1971) 1264–1281.
[36] S. Kumar, U. Bandyopadhyay, Free heme toxicity and its detoxification systems in human, Toxicol. Lett. 157 (3) (2005) 175–188.
[37] W.T. Suarez, O.D. Pessoa-Neto, F.C. Vicentini, B.C. Janegitz, R.C. Faria, O. Fatibello-Filho, Flow injection spectrophotometric determination of dipyrone in pharmaceutical formulations using Fe(III) as reagent, Anal. Lett. 44 (3) (2011) 340–348.
[38] S.Z. Qureshi, A. Saeed, S. Haque, Some observations on a simple method for the determination of novalgin in drug formulations with iron(III)-1,10-phenanthroline, Microchem. J. 41 (3) (1990) 362–365.
[39] Documed. 'Arzneimittel-Kompendium Der Schweiz: Fachinformation Novalgin®'. 2016.
[40] E. Andres, F. Maloisel, J.E. Kurtz, G. Kaltenbach, M. Alt, J.C. Weber, et al., Modern management of non-chemotherapy drug-induced agranulocytosis: a monocentric cohort study of 90 cases and review of the literature, Eur. J. Intern. Med. 13 (5) (2002) 324–328.
[41] F. Lacy, M.T. Kailasam, D.T. O'Connor, G.W. Schmid-Schonbein, R.J. Parmer, Plasma hydrogen peroxide production in human essential hypertension: role of heredity, gender, and ethnicity, Hypertension (Dallas, Tex: 1979) 36 (5) (2000) 878–884.
[42] H.J. Forman, A. Bernardo, K.J. Davies, What is the concentration of hydrogen peroxide in blood and plasma? Arch. Biochem. Biophys. 603 (2016) 48–53.
[43] W. Droge, Free radicals in the physiological control of cell function, Physiol. Rev. 82 (1) (2002) 47–95.
[44] A.S. Giri, A.K. Golder, Decomposition of drug mixture in Fenton and photo-Fenton processes: comparison to singly treatment, evolution of inorganic ions and toxicity assay, Chemosphere 127 (2015) 254–261.
[45] S.R. Robinson, T.N. Dang, R. Dringen, G.M. Bishop, Hemin toxicity: a preventable source of brain damage following hemorrhagic stroke, Redox Report 14 (6) (2009) 228–235.

3.2. Toxicity of metamizole on differentiating HL60 cells and human neutrophil granulocytes

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Toxicity of metamizole on differentiating HL60 cells and human neutrophil granulocytes

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ABSTRACT

Metamizole is an analgesic and antipyretic with a superior analgesic efficacy than paracetamol. Since metamizole can cause neutropenia and agranulocytosis, it is currently used in only few countries. In a previous study, we have shown that *N*-methyl-4-aminoantipyrine (MAA), the active metamizole metabolite, reacts with hemin and forms an electrophilic metabolite that is toxic for HL60 cells, but not for mature neutrophil granulocytes. In the current study, we investigated the toxicity of hemin (12.5 μM) and MAA (100 μM) on differentiating HL60 cells. In undifferentiated HL60 cells, hemin decreased the viability and this effect was significantly increased by MAA. Similarly, hemin/MAA was more toxic than hemin alone on human cord blood cells. At 3 days (metamyelocyte stage) and 5 days of differentiation (mature neutrophils), hemin/MAA was not toxic on HL60 cells, whereas hemin alone was still toxic. No toxicity was observed on freshly isolated human neutrophils. The protein expression of enzymes responsible for hemin metabolism increased with HL60 cell differentiation. Inhibition of heme oxygenase-1 or cytochrome P450 reductase increased the toxicity of hemin and hemin/MAA in undifferentiated, but only for hemin in differentiated HL60 cells. Similar to the enzymes involved in hemin metabolism, the protein expression of enzymes involved in antioxidative defense and the cellular glutathione pool increased with HL60 cell differentiation. In conclusion, HL60 cells become resistant to the toxicity of hemin/MAA and partly also of hemin during their differentiation. This resistance is associated with the development of heme metabolism and of the antioxidative defense system including the cellular glutathione pool.

1. Introduction

Metamizole is a non-opioid analgesic, antipyretic, and spasmolytic prodrug often prescribed in certain countries due to its good efficacy and low gastrointestinal toxicity (Blaser et al., 2015; Sánchez et al., 2002). After oral application, it is rapidly converted non-enzymatically to the active drug *N*-methyl-4-aminoantipyrine (MAA) in the gut and in the mesenteric and portal circulation. Despite the favorable safety profile of metamizole, susceptible patients may experience neutropenia or agranulocytosis, a severe and potentially fatal decrease of circulating neutrophil granulocytes (Blaser et al., 2017, 2015). The mechanisms underlying metamizole-induced neutropenia are poorly understood, and there are currently no strategies to predict in whom neutropenia is likely to occur, nor to prevent this life-threatening adverse drug reaction (Andres and Maloisel, 2008; Garbe, 2007). Bone marrow biopsies of affected patients showed a stop at the promyelocyte and myelocyte stage in granulocyte maturation (Kummer et al., 2006), demonstrating that the effect of metamizole on neutrophil granulocytes occurs in the bone marrow and not in the peripheral circulation. This is in agreement with findings of a previous study (Rudin et al., 2019) showing that the main metamizole metabolite MAA reacts with the hemoglobin break down product hemin to a reactive intermediate that is cytotoxic for promyelocytic HL60 cells, but not for mature neutrophil granulocytes. This suggests that the cellular heme metabolism and/or antioxidative capacity changes during granulocyte differentiation, rendering the cells less susceptible to the reactive MAA intermediate. Enzymes involved in heme metabolism are heme oxygenase-1 (HO-1) that degrades heme to biliverdin, which is then further metabolized to bilirubin by biliverdin reductase (BVR) (Baranano et al., 2002; Pimstone et al., 1971; Tenhunen et al., 1968). Both enzymes need electrons transferred from NADPH by cytochrome P450 reductase (CYPOR) (Emerson and LeVine, 2000; Takao et al., 2017). These enzymes, which are involved in heme

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metabolism, may affect the resistance development of differentiating myeloid cells towards heme and heme combined with MAA.

In addition, neutrophils develop their antioxidative defense system during myeloid maturation (Ballinger et al., 1994; Bardoel et al., 2014). According to Ighodaro and Akinloye, the first line antioxidative defense of most mammalian cells consists of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) (Ighodaro and Akinloye, 2018). These enzymes interrupt the formation of radicals before any damage occurs. The second line antioxidative defense scavenges or neutralizes radicals formed mainly by electron donation. Within this group, glutathione (GSH) represents the most abundant cellular radical scavenger, which is able to neutralize a wide variety of reactive molecules (Galano and Alvarez-Idaboy, 2011; Ighodaro and Akinloye, 2018). Both the first and second line antioxidative defense system may change its activity during myeloid differentiation, which could influence the cellular susceptibility towards reactive molecules.

In the current study, we assessed the susceptibility of undifferentiated and differentiating HL60 cells and isolated human neutrophils to reactive MAA intermediates and related the findings to the activity of heme metabolism and the antioxidative defense during cell maturation. The current study shows that already after 3 days of differentiation, when undifferentiated HL60 cells have reached metamyelocyte stage, cells are significantly more resistant against the toxicity of MAA added to hemin. The corresponding mRNA and protein expression generally showed increases for heme metabolizing and antioxidative enzymes. This increase in the capacity for heme metabolism and antioxidative defense can explain the resistance of mature neutrophils towards MAA in the presence of hemin.

2. Materials and methods

2.1. Chemicals and reagents

N-methyl-4-aminoantipyrine (MAA), hemin, doxorubicine, staurosporine, diphenyleneiodonium (DPI), all-*trans*-Retinoic acid (ATRA), sodium hydroxide (NaOH) and sodium chloride (NaCl), were purchased from Sigma–Aldrich (Buchs, Switzerland). Zinc (II) protoporphyrin IX (ZnPP) was obtained from Enzo Life Sciences AG (Lausen, Switzerland). Dextran 500 was purchased from Roth AG (Arlesheim, Switzerland). All other chemicals used were obtained at the highest quality from different suppliers.

2.2. CFU-GM assay

Frozen aliquots of cord blood cells were kindly provided by Dr. Swarna Maseneni, University of Basel. The Ethics Committee of Nordwest- and Zentralschweiz had approved the study protocol for the isolation of human mononuclear cells (hMNCs). Immature myeloid cells from umbilical cord blood were used as model for neutrophil precursors in the bone marrow. To conduct the CFU-GM assay, the standard operating procedure established by Pessina et al. (Pessina et al., 2001) was used. Briefly, frozen aliquots of cord blood cells were thawed at 37 °C and washed twice with IMDM medium containing 20% FBS (Gibco, Lucerne, Switzerland). The cell viability was assessed by trypan blue exclusion and always exceeded 90%. To confirm the relationship between seeded cells and colonies obtained, an internal linearity control was performed. Therefore, 2.5×10^4, 5×10^4, 7.5×10^4, and 10×10^4 cells per plate were seeded. For cytotoxicity assays, 5×10^4 cells were seeded in duplicate in 35 mm petri dishes containing MethoCult™ H4534 Classic without EPO methylcellulose medium (Stemcell™ Technologies, Vancouver, Canada). The cells were incubated with 100 µM MAA, 12.5 µM hemin, or 100 µM MAA + 12.5 µM hemin to assess cytotoxicity. The used MAA concentration of 100 µM complies plasma concentrations during common pharmacological treatment with metamizole (Levy et al., 1984). Control incubations contained 1 µM staurosporine as positive control and 0.1% DMSO (Sigma–Aldrich, Buchs, Switzerland) as negative control. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 14 days, the colonies obtained were counted using an inverted microscope considering clusters containing 40 or more cells as a colony.

2.3. Differentiation of HL60 cells

HL60 cells (CCL-240, lot number 7703261, ATCC, Wesel, Germany) were maintained in RPMI medium (Gibco, Lucerne, Switzerland) supplemented with 10% FBS (Gibco, Lucerne, Switzerland) at 37 °C in a humidified atmosphere containing 5% CO_2. HL60 cells are immature human neutrophils on the promyelocyte maturation stage, which represents a maturation stage that can be affected in patients with metamizole-associated neutropenia (Kummer et al., 2006). HL60 cells can be differentiated into mature neutrophils, which have similar morphologic and functional characteristics as mature neutrophils isolated from human blood (Breitman et al., 1980; Collins et al., 1978).To induce differentiation, 3.5×10^5 HL60 cells were prepared in a T-25 seeding flask (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and 1.25% DMSO and 1 µM ATRA were added.

To monitor the differentiation progress, the percentage of CD66a positive cells was determined every 24 h. Therefore, 2×10^5 cells per well were added to a 96 v-well plate (Corning, New York, USA), centrifuged at 500 × g for 5 min and resuspended in 30 µL staining buffer containing fluorescent dye conjugated monoclonal antibody CD66a-PerCP/Cy5.5 1 µg (Biolegend, London, UK). As a control, unstained cells in staining buffer (Biolegend, London, UK) were used and cells stained with PerCP/Cy5.5 Mouse IgG2b, κ isotype control antibody (Biolegend, London, UK). The cells were incubated for 30 min at 4 °C in the dark followed by two washes with PBS (Gibco, Lucerne, Switzerland) containing 0.1% BSA. Subsequently, the cells were resuspended in (180 µL) PBS for analysis by flow cytometry with a CytoFLEX flow cytometer (Beckmann Coulter, Indianapolis, USA) and the data was assessed using FlowJo software 10.08 (Tree Star, Ashland, OR, USA).

2.4. Cytotoxicity assays

HL60 cells were allowed to differentiate over 5 days as mentioned before. Every day of differentiation, samples of differentiating HL60 cells were prepared in RPMI medium without FBS in a 24-well plate (BD Biosciences, Franklin Lakes, New Jersey, USA) at a density of 2×10^5 cells/mL. The stock solution of MAA was prepared in DMSO and added to the cell solution at a final concentration of 100 µM. Triton X (Sigma–Aldrich, Buchs, Switzerland) was used at a final concentration of 0.1% as a positive control for plasma membrane toxicity. Last, 50 µL of hemin was added to the reaction solution at a final concentration of 12.5 µM. Since hemin is not readily soluble in PBS, it was first dissolved in 10 mM NaOH as a 1 mM stock solution and then diluted to the final concentration. An equivalent concentration of NaOH was added to the vehicle control.

The concentrations of MAA and hemin were chosen based on previously obtained results (Rudin et al., 2019). Subsequently, 50 µL of each suspension-mixture was added in triplicate to a 96-well plate (BD Biosciences, Franklin Lakes, New Jersey, USA) and incubated for 24 h at 37 °C, 5% CO_2. The DMSO concentration did not exceed 0.1% in all incubations, including control incubations, as this DMSO concentration is not cytotoxic (Waldhauser et al., 2006). All experiments were performed in triplicates and repeated at least three times using different cell isolations.

To assess a possible loss of plasma membrane integrity, which is reflected by the release of adenylate kinase, the firefly luciferase system (ToxiLight® BioAssay Kit, Lonza, Basel, Switzerland) was used. After incubation for 24 h in presence of test compounds, 50 µL assay buffer was added to 50 µL cell suspension from treated cells and luminescence was measured after 5 min of incubation with a Tecan Infinite pro

200 microplate reader (Tecan, Männedorf, Switzerland).

2.5. Determination of the cellular ATP content

HL60 cells were differentiated and treated with test compounds as outlined above. To assess the cellular ATP content as marker for the cellular energy metabolism, the CellTiter-Glo® luminescent assay (Promega Corporation, Madison, USA) was used. After incubation for 24 h in presence of test compounds, 50 µL assay buffer was added to 50 µL cell suspension from treated cells and luminescence was measured after 10 min of incubation with a Tecan Infinite pro 200 microplate reader (Tecan, Männedorf, Switzerland).

2.6. Isolation of neutrophil granulocytes

Neutrophil granulocytes were isolated freshly from human whole blood obtained from the local blood donation center Basel (Switzerland). Since the donors remained anonymous, the study did not require approval from the local Ethics Committee. The neutrophils were isolated from the blood by a modification of the method described by Klebanoff et al. (1992). Briefly, 20 mL blood was diluted with 25 mL PBS and carefully layered over 15 mL Ficoll-Paque (GE Healthcare, Glattbrugg, Switzerland) and centrifuged at 200*g* for 20 min. Next, the pellet was mixed with 4% dextran in 0.9% NaCl solution and to allow the erythrocytes to settle for 30 min. The supernatant layer was washed with PBS and the remaining erythrocytes were lysed with BD Pharm Lyse lysing buffer (BD Biosciences, Franklin Lakes, NJ, USA). Subsequently, we washed the cells twice with PBS and resuspended them in RPMI medium containing 10% FBS. Viability was assessed by using trypan blue analysis and always exceeded > 90%.

2.7. Determination of apoptosis and necrosis

To determine the percentage of dead cells (including necrotic cells and cells in late apoptosis), we used propidium iodide (PI) (Molecular probes, Oregon, USA), a red fluorescent dye incapable to permeate intact or early apoptotic cells, but able to stain permeable necrotic cells by binding to nucleic acid. After incubation of HL60 cells with 100 µM MAA and 12.5 µM hemin for 24 h, the cells were centrifuged at 500 *g* for 5 min and washed with PBS before PI staining (Molecular probes, Oregon, USA) at a final concentration of 10 µg/mL. After 15 min incubation in the dark, we analyzed the cells with a CytoFLEX flow cytometer (Beckmann Coulter, Indianapolis, USA) and assessed the data using FlowJo software 10.08 (Tree Star, Ashland, OR, USA). Cells without MAA or hemin treatment were used as control.

To determine the percentage of early apoptotic cells, we used Annexin V, a Ca^{2+}-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (PS). PS is located on the inner cytoplasmic surface of intact cell membranes. In apoptotic cells, PS is translocated to the outer leaflet of the plasma membrane, where Annexin V binds to it. After the incubation of HL60 cells with 100 µM MAA and 12.5 µM hemin for 24 h, the cells were centrifuged at 500*g* for 5 min and washed with PBS before Alexa Fluor 488® Annexin V staining (Molecular probes, Oregon, USA). After 15 min incubation in the dark, we analyzed the cells with a CytoFLEX flow cytometer (Beckmann Coulter, Indianapolis, USA) assessed the data using FlowJo software 10.08 (Tree Star, Ashland, OR, USA). Cells without MAA or hemin treatment were used as control.

2.8. Inhibition of cytochrome P450 reductase by diphenyleneiodonium (DPI)

To investigate whether CYPOR is involved in MAA and hemin cytotoxicity, the CYPOR inhibitor diphenyleneiodonium (DPI) was used (McGuire et al., 1998). For the experiments, HL60 cells were pre-incubated with 20 µM DPI for 10 min, washed twice, and then incubated with MAA and hemin as described before.

2.9. Inhibition of heme oxygenase by zinc (II) protoporphyrin IX (ZnPP)

HO-1 degrades the pro-oxidant heme/hemin to biliverdin (Abraham and Kappas, 2008). To investigate the role of HO-1 in MAA/hemin cytotoxicity, the HO-1 inhibitor ZnPP was used. HL60 cells were pre-incubated with 10 µM ZnPP for 24 h, washed twice and recovered for 24 h, and incubated with MAA and hemin as outlined above.

2.10. Inhibition of heme oxygenase by montelukast

To assess the impact of biliverdin reductase on MAA/hemin toxicity, HL60 cells were co-incubated with MAA and/or hemin and the biliverdin reductase inhibitor montelukast without pre-incubation. The stock solution of montelukast was prepared in DMSO and added to the cell solution at a final concentration of 1 µM and incubated with MAA and hemin as outlined above. One µM montelukast was the highest, non-cytotoxic concentration tested in HL60 cells (data not shown). Treatment with MAA/hemin and cytotoxicity assays were performed as described before.

2.11. Quantitative real-time PCR

HL60 cells were allowed to differentiate over 5 days and neutrophil granulocytes isolated from whole blood as mentioned before. mRNA was extracted from differentiating HL60 cells and freshly isolated neutrophils and purified using the Qiagen RNeasy mini extraction kit (Qiagen, Hombrechtikon, Switzerland). Purity and quantity of the obtained mRNA were assessed with a NanoDrop 2000 (Thermo Scientific, Wohlen, Switzerland) and cDNA synthesized from 1 µg mRNA using the Qiagen omniscript system. Real-time PCR was performed in triplicate using SYBR green (Roche Diagnostics, Rotkreuz, Basel) and specific primers (Microsynth AG, Balgach, Switzerland) for HO-1 (forward: 5′-CTTCCCCAACGAAAAGCACA-3′, reverse: 5′-ACTCAGGGCTTTTGGA GGTT-3′), SOD1 (forward: 5′-TGAAGGTGTGGGGAAGCATT-3′, reverse: 5′-GTCACATTGCCCAAGTCTCC-3′), SOD2 (forward: 5′-GGTTGTTCAC GTAGGCCG-3′, reverse: 5′-CAGCAGGCAGCTGGCT-3′), CAT (forward: 5′-ACTGAGGTCCACCCTGACTAC-3′, reverse: 5′-TCGCATTCTTAGGCT TCTCA-3′), GPX1 (forward: 5′−CCAAGCTCAATCACCTGGTCT3′, reverse: 5′-TCGATGTCAATGGTCTGGAA-3′), GPX4 (forward: 5′-GCCAG GGAGTAACGAAGAGA-3′, reverse: 5′-TCACGCAGATCTTGCTGAAC-3′),

POR (forward: 5′- TCTACGACATCGTGGCTGAG-3′ reverse: 5′−CCAAACACACCCAGGAGACT-3′), and BLVR (forward: 5′-ATCTGCAGT GAGAGCTCCAG-3′, reverse: 5′-GCTCCTCGTGCAAGACTTTT-3′). We used GAPDH as an endogenous reference (forward: 5′-CATGGCCTTCC GTGTTCCTA-3′, reverse: 5′−CCTGCTTCACCACCTTCTTGA-3′). To perform the real-time PCR, the ViiA7 system (Thermo Fisher Scientific, Ecublens, Switzerland) was used. Relative quantities of specifically amplified cDNA were calculated with the comparative threshold cycle method.

2.12. Western blot analysis

After allowing HL60 cells to differentiate for different time periods or isolating neutrophil granulocytes from whole blood, the cells were lysed in RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH adjusted to 8.0). Next, the samples were centrifuged at 500 × *g* for 10 min at 4 °C and the supernatant was collected. Protein content of each sample was determined using the BCA Protein Assay Kit (Pierce, Thermo Scientific, Rockford, USA). For protein separation, the supernatant was applied on 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Basel, Switzerland) and the samples were run under reducing conditions. After separation, the proteins were transferred to 0.2 µM nitrocellulose membranes (Bio-Rad Laboratories AG, California, USA).

Subsequently, the membranes were blocked with 5% nonfat dry milk in phosphate buffered saline (PBS) (Gibco, Paisley, UK) containing 0.1% Tween-20 (Sigma-Aldrich, MO, USA) (PBS-Tween) for 1 h at room temperature. Afterwards, the membranes were incubated overnight with a primary antibody (Abcam, Cambridge, United Kingdom) for SOD1, GPX1, GPX4, and GAPDH diluted 1:5000, SOD2 and BLVR diluted 1:1000, and HO-1, CAT, and POR diluted 1:500, 1:2000 and 1:10′000, respectively, in blocking buffer. The next day, the membranes were incubated for 1 h with the corresponding secondary antibody (Santa Cruz Biotechnology, USA) diluted 1:2000 in 5% nonfat milk in PBS-Tween. Afterwards, the membranes were washed and the immunoreactive bands were developed using enhanced chemiluminescence solution (GE Healthcare, Buckinghamshire, United Kingdom). Chemiluminescent images were scanned and band intensities analyzed using a Witec Fusion pulse imaging system (Witec, Sursee, Switzerland). To correct for loading differences, the scanning units obtained for the test proteins were divided by the scanning units obtained for the housekeeping protein GAPDH.

2.13. Mitochondrial DNA (mtDNA) determination

HL60 cells were differentiated over 5 days and neutrophil granulocytes isolated freshly from whole blood as mentioned before. Total DNA was extracted from differentiating HL60 cells and freshly isolated neutrophils and purified using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hombrechtikon, Switzerland). Purity and quantity of the obtained DNA was assessed with a NanoDrop 2000 photometer (Thermo Scientific, Wohlen, Switzerland). Real-time PCR was performed in triplicate using SYBR green (Roche Diagnostics, Rotkreuz, Basel) and specific primers (Microsynth AG, Balgach, Switzerland) for mitochondrial DNA, COX-1 (forward: 5′-TGCTTCACCAGGCAACTG-3′, reverse: 5′-GCCAATGATCTCCAAGTTCC-3′), ND1 (forward: 5′-ATGGCCAACCT CCTACTCCT-3′, reverse: 5′-CTACAACGTTGGGGCCTTT-3′), and for nuclear DNA, ASPOLG (forward: 5′-GAGCTGTTGACGGAAAGGAG-3′, reverse: 5′-CAGAAGAGAATCCCGGCTAAG-3′), 36B4 (forward: 5′-GGA ATGTGGGCTTTGTGTTC-3′, reverse: 5′−CCCAATTGTCCCCTTAC CTT-3′), were used. The mitochondrial copy number was calculated according to Quiros et al. (Quiros et al., 2017).

2.14. Determination of GSH-NEM/GSSG content by LC–MS

GSH and glutathione disulfide (GSSG) levels in differentiating HL60 cells and neutrophil granulocytes were determined by LC−MS/MS. A 10 mM stock solution of GSH and GSSG was prepared in water supplemented with 2% formic acid. GSH-d5 and GSSG-$^{13}C_4{}^{15}N^2$ were used as internal standards (1 mM in DMSO). The thiol group of GSH was derivatized with *N*-ethylmaleimide (NEM) in order to inhibit autoxidation to GSSG (Giustarini et al., 2013). GSSG was stabilized by the addition of ethylenediaminetetraacetic acid (EDTA). Thus, a derivatization solution of 20 mM NEM and 2 mM EDTA was prepared in a 3:1 mixture of acetonitrile:water. GSH and GSH-d5 were incubated for 1 h in the derivatization solution to form GSH-NEM and GSH-NEM-d5. Calibration lines of GSH-NEM (250 µM - 0.25 µM) and GSSG (25 µM–0.025 µM) were prepared in the derivatization solution. Internal standard solution of GSH-NEM-d5 and GSSG-$^{13}C_4{}^{15}N^2$ was made in 2% formic acid (1 µM).

HL60 cells were differentiated over 5 days and neutrophil granulocytes isolated from whole blood as outlined before. Approximately 10 Mio differentiating HL60 cells or freshly isolated neutrophils were harvested and the cytosolic fraction extracted. The samples were further 1:3 (v/v) diluted with IS solution and kept at − 20 °C until analysis. For LC–MS analysis, the samples were thawed; vortex mixed, and centrifuged at 15 °C and 3220*g* for 30 min. Subsequently, 2.5 µL of calibrators and cell samples were injected into the LC–MS/MS system. A Shimadzu HPLC (Kyoto, Japan) which was coupled to an API 4000 QTrap tandem mass spectrometer (ABSciex, Concord, Canada) was used to determine the GSH-NEM and GSSG levels in differentiating HL60 cells and mature neutrophils. The analytes were positively charged by electro spray ionization and analyzed with scheduled multiple reaction monitoring (MRM). The ion spray voltage was 5500 eV and the probe temperature was 700 °C. The following mass were used: 433.1 → 304.0 *m/z* for GSH-NEM, 438.1 → 304.1 *m/z* for GSH-NEM-d5, 613.2 → 355.2, 483.9, 231.0 *m/z* for GSSG, and 619.2 → 361.0, 490.0, 230.8 for GSSG-$^{13}C_4{}^{15}N_2$.

GSH-NEM and GSSG were separated on a Luna omega polar C18 analytical column (50 mm × 2.1 mm, 1.6 µm, Phenomenex, Torrance, CA, USA) at 45 °C and a flow rate of 0.4 mL/min. Water (mobile A) and acetonitrile (mobile B) both supplemented with 0.1% acetic acid were used as mobile phase. Samples were loaded onto the analytical column using 0% mobile phase B and were inline diluted via a T union with mobile phase A during the first 0.5 min of each run. After 0.5 min the gradient was linearly increased within 1.25 min, reaching 95% mobile phase B. The column was washed for 0.5 min at 95% and thereafter reconditioned for another 0.75 min at 0% mobile phase B. The retention time of GSSG and GSH-NEM was 0.56 and 1.62 min, respectively.

2.15. Statistics

Data are presented as the mean ± SEM from at least three independent experiments. All statistical analyses were performed using GraphPad Prism 7.0c (GraphPad Software, La Jolla, California, USA). Differences between many groups were tested by one-way ANOVA followed by Dunetts's multiple comparison tests to localize significant results in the ANOVA. Differences within two groups were tested by an unpaired t-test. A p-value < 0.05 was considered to be a significant difference.

3. Results

3.1. Susceptibility of HL60 cells during cellular differentiation

In a previous publication we have shown that the combination of MAA and hemin, but not MAA alone, is cytotoxic in the undifferentiated promyelocytic cell line HL60 (Rudin et al., 2019). Since the cytotoxicity of MAA in combination with hemin was observed only in promyelocytic HL60 cells but not in mature neutrophil granulocytes, the resistance against the toxicity of MAA/hemin has to develop during myeloid differentiation. The aim of the current investigation was to assess various aspects of the differentiation of the HL60 cells and to study the toxicity of MAA and/or hemin at different stages of differentiation. Promyelocytic HL60 cells can be differentiated into mature neutrophils and therefore represent a convenient model to investigate myeloid differentiation in vitro (Collins et al., 1978; Gupta et al., 2014). We differentiated promyelocytic HL60 cells to mature neutrophils over 5 days and investigated the change in susceptibility to MAA and/or hemin. We assessed the success of differentiation morphologically (Supplementary Fig. 1 A—D) and by the expression of the surface marker CD66a (Supplementary Fig. 1E). At day 0, the cells consisted mainly of promyelocytes (Supplementary Fig. 1A), at day 3 of myelocytes and metamyelocytes (Supplementary Fig. 1B) and at day 5 of band and mature neutrophils (Supplementary Fig. 1C; compare with freshly isolated human neutrophils in Supplementary Fig. 1D). The expression of the differentiation marker CD66a steadily increased during the 5 days of differentiation (Supplementary Fig. 1E). As shown in Supplementary Fig. 1F, the viable portion of the HL60 cells remained constant during differentiation.

In undifferentiated HL60 cells (day 0), both hemin and hemin/MAA significantly reduced the viable cell population and increased the portion of apoptotic cells (Fig. 1A). At this time point, MAA significantly increased the toxicity of hemin. Fig. 1B–D show the effect of MAA and hemin on apoptosis, necrosis and viability of the HL60 cells during differentiation. During differentiation, the proportion of apoptotic

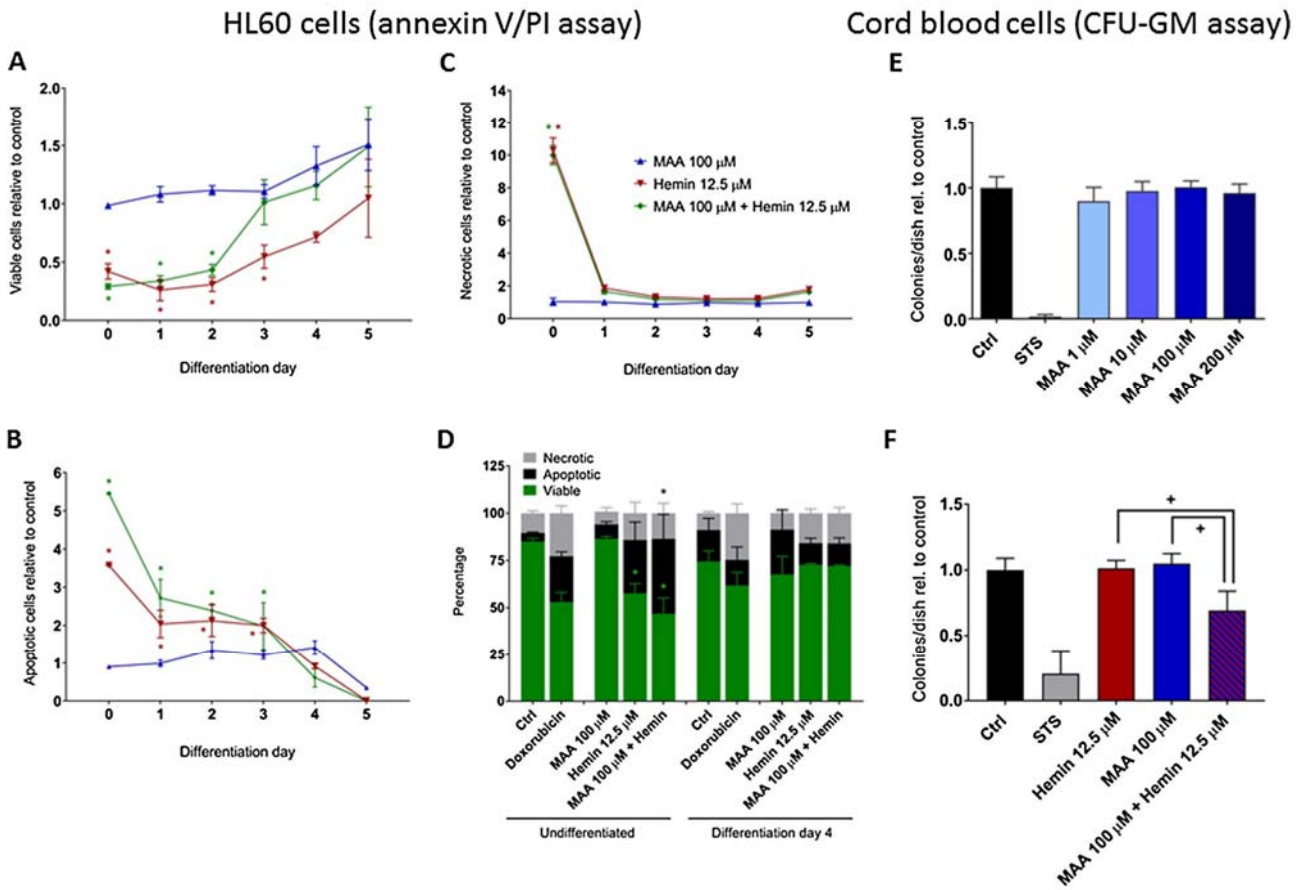

Fig. 1. *Effect of N-methyl-4-aminoantipyrine (MAA) and hemin on differentiating HL60 cells and on differentiating myeloid progenitor cells obtained from human umbilical cord blood.* Differentiating HL60 cells were treated with MAA and hemin for 24 h and (**A**) effect on viability. (**B**) effect on apoptosis, (**C**) and effect on necrosis were assessed with progressing differentiation. (**D**) Percentage of viable, apoptotic and necrotic cells at day 0 and day 5 of differentiation. Apoptosis and necrosis were assessed by staining with annexin V and propidium iodide, respectively. Myeloid progenitor cells were obtained from one donor per experiment and were treated with (**E**) MAA and (**F**) MAA and hemin for 14 days and the effect on number of colonies assessed. Data represent the mean ± SEM of three independent experiments, each value per experiment assessed in duplicate. *p < 0.05 vs. control incubations. Ctrl: control, STS: 1 µM staurosporine.

(Fig. 1B and D) and necrotic cells (Fig. 1C and D) steadily decreased in the presence of hemin or hemin and MAA, while viability increased (Fig. 1D). During differentiation, hemin alone became more toxic than the combination of hemin and MAA (Fig. 1A and D).

3.2. Susceptibility of immature myeloid cells to MAA and hemin

To confirm the findings on undifferentiated HL60 cells (day 0), we investigated the toxicity of MAA with and without hemin on immature myeloid cells from umbilical cord blood using colony forming assays. We incubated human umbilical cord blood cells for 14 days with increasing concentrations of MAA (1–200 µM) or the combination of MAA and hemin (12.5 µM). As shown in Fig. 1E, none of the tested MAA concentrations reduced the number of cell colonies. Similarly, hemin alone was not cytotoxic (Fig. 1F). However, treatment with MAA (100 µM) and hemin (12.5 µM) reduced the number of colonies significantly by approximately 30% compared to control incubations (Fig. 1F), confirming the results obtained with undifferentiated HL60 cells.

3.3. Heme metabolism

As shown in Fig. 1 and in our previous publication (Rudin et al., 2019), hemin is needed to cause cytotoxicity in MAA treated immature myeloid cells and in promyelocytic HL60 cells. However, with progressing differentiation, the previously sensitive HL60 cells become resistant to the toxicity of MAA and hemin. This may be due to increasing heme metabolism and/or the ability to neutralize the formed MAA electrophilic intermediate, which is the assumed cytotoxic reaction product of hemin and MAA (Rudin et al., 2019). The enzymes involved in heme metabolism are depicted in Supplementary Fig. 2. Heme oxygenase-1 (HO-1) is essential for degrading the toxic heme/hemin to biliverdin, which is further reduced to bilirubin and finally excreted in urine and feces (Chowdhury and Chowdhury, 1983). HO-1 and biliverdin reductase (BVR) both need electrons for their function, transferred from NADPH by cytochrome P450 reductase (CYPOR) (Sugishima et al., 2014). We therefore investigated the possibility that impairment of HO-1, CYPOR or BVR could be involved in MAA/hemin cytotoxicity by inhibition experiments.

3.4. Expression of heme oxygenase-1 (HO-1) during HL60 differentiation and effect of the inhibition of HO-1 on the cytotoxicity of MAA and hemin

As shown in Fig. 2A, HO-1 mRNA expression increased over twofold during HL60 differentiation and was more than tenfold higher in mature neutrophil granulocytes compared to undifferentiated HL60 cells. Similarly, HO-1 protein expression increased almost sixfold during differentiation and was more than tenfold higher in neutrophil granulocytes compared to undifferentiated HL60 cells (Fig. 3B, Supplementary Fig. 4A). These findings suggested that heme metabolism is important in HL60 cells and neutrophils.

In order to study a possible involvement of HO-1 in the toxicity of hemin and/or MAA, we used the competitive HO-1 inhibitor zinc protoporphyrin IX (ZnPP) (Rodgers et al., 1990; Vreman et al., 1991). HO-1

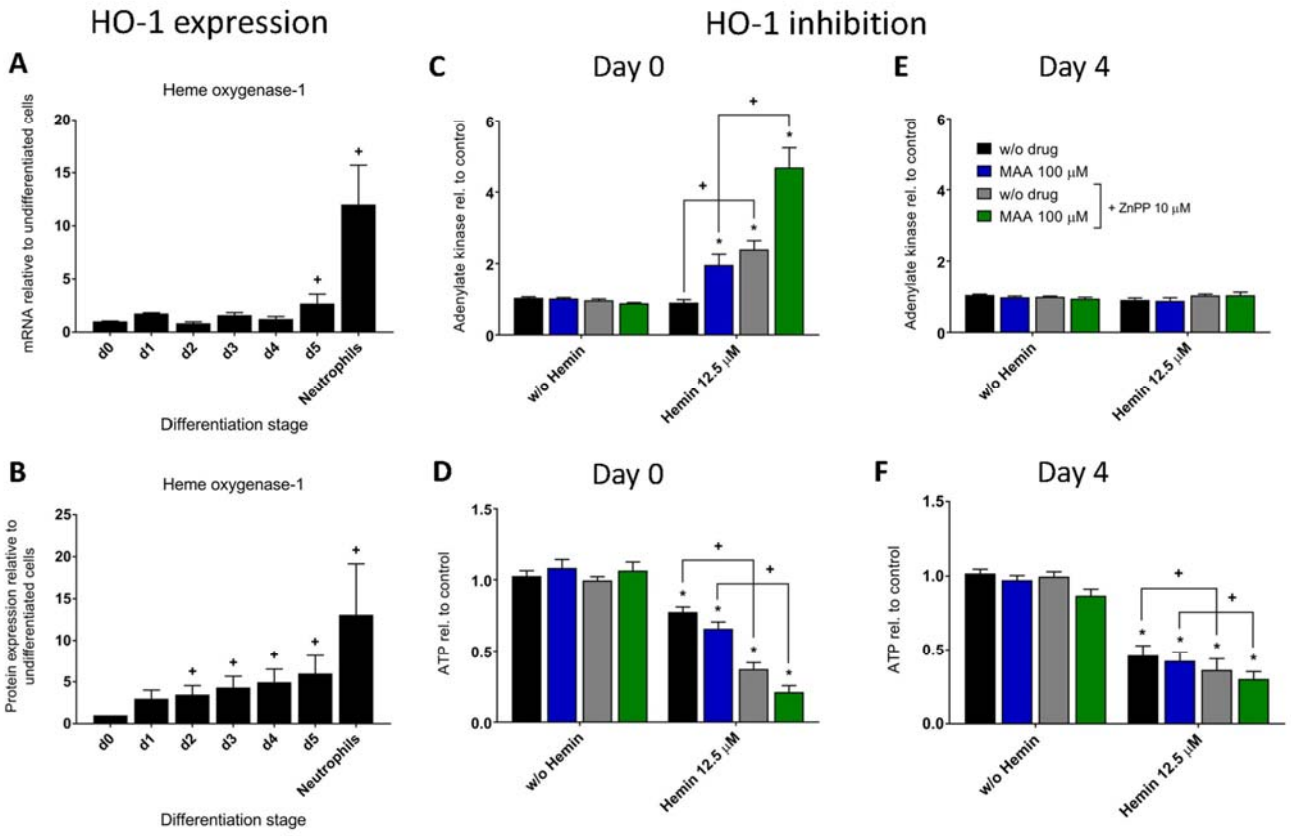

Fig. 2. *Expression of heme oxygenase-1 (HO-1) during HL60 cell differentiation and effect of the inhibition of HO-1 by zinc protoporphyrin (ZnPP) on the toxicity of MAA and hemin on undifferentiated and differentiated HL60 cells.* (**A**) Expression of HO-1 mRNA in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. $^{+}p < 0.05$ vs. undifferentiated HL60 cells. (**B**) Expression of HO-1 protein in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. $^{+}p < 0.05$ vs. undifferentiated HL60 cells. For the inhibition experiments, cells were treated with MAA and hemin for 24 h with or without pretreatment with 10 µM ZnPP. (**C**) Effect on adenylate kinase release (marker of membrane integrity) in undifferentiated HL60 cells. (**D**) effect on intracellular ATP concentration in undifferentiated HL60 cells. (**E**) effect on adenylate kinase release in HL60 cells differentiated for 4 days. (**F**) effect on intracellular ATP concentration in HL60 cells differentiated for 4 days. Data represent the mean ± SEM of three independent experiments, each value per experiment assessed in triplicate. $^{*}p < 0.05$ vs. control incubations w/o drug, $^{+}p < 0.05$ vs. incubations w/o ZnPP pretreatment of the same treatment group. Neutrophils were obtained from different healthy donors; cells from one donor were used per experiment. MAA: N-methyl-4-aminoantipyrine.

inhibition should lead to higher hemin availability and should thereby increase the toxicity of hemin and of the electrophilic intermediates of MAA after reaction with hemin. The susceptibility to hemin/MAA was assessed in undifferentiated HL60 cells and in HL60 cells after 4 days of differentiation. As shown in Fig. 2C–F, ZnPP pretreatment did not affect membrane integrity or decrease the ATP content. In incubations containing hemin and MAA, ZnPP decreased the membrane integrity significantly in undifferentiated HL60 cells (Fig. 2C), but not after 4 days of differentiation (Fig. 2E). Similarly, as shown in Fig. 2D, ZnPP significantly intensified the ATP depletion associated with hemin and MAA in undifferentiated HL60 cells and to a smaller extent in differentiated HL60 cells (Fig. 2F). These results were confirmed by staining for apoptotic and necrotic cells, where the percentage of viable cells was decreased and the necrotic cells significantly increased by ZnPP pretreatment in undifferentiated HL60 cells (Supplementary Fig. 3A). The increase in cytotoxicity of hemin and MAA by ZnPP was still observable in differentiated HL60 cells, but this increase was only numerical and did not reach statistical significance compared to cells without ZnPP pretreatment (Supplementary Fig. 3B). These findings suggested a role of heme metabolism in the differentiation of HL60 cells and in the development of resistance against the toxicity of hemin and MAA.

3.5. Expression of cytochrome P450 reductase (CYPOR) during HL60 differentiation and effect of the inhibition of cytochrome P450 reductase on the cytotoxicity of MAA and hemin

As shown in Fig. 3A, the CYPOR mRNA content increased to a maximum at day 3 followed by a decrease until day 5 of differentiation. In mature neutrophil granulocytes, the mRNA content was half as much as in undifferentiated HL60 cells. The corresponding protein expression was delayed compared to the RNA expression, reaching a maximum at differentiation day 5. In comparison, the CYPOR protein expression in mature neutrophils was lower compared to differentiated HL60 cells (Fig. 3B, Supplementary Fig. 4A).

The flavoprotein inhibitor diphenyleneiodonium (DPI) was used to inhibit cytochrome P450 reductase. Fig. 3C shows that pretreatment with DPI increased the membrane toxicity of MAA/hemin significantly compared to hemin/MAA without DPI in undifferentiated HL60 cells (day 0). Further, DPI significantly decreased the cellular ATP content of cells treated with hemin/MAA and hemin alone compared to the respective incubations without DPI (Fig. 3D). These results were confirmed by the determination of the viable, apoptotic, and necrotic portions of HL60 cells treated with these compounds in undifferentiated HL60 cells. The viable part of treated cells was decreased after DPI pretreatment with predominantly increased apoptotic cell parts (Supplementary Fig. 3C). In comparison, after 4 days of differentiation, DPI pretreatment did not significantly affect the membrane toxicity of hemin or the combination of hemin and MAA compared to control

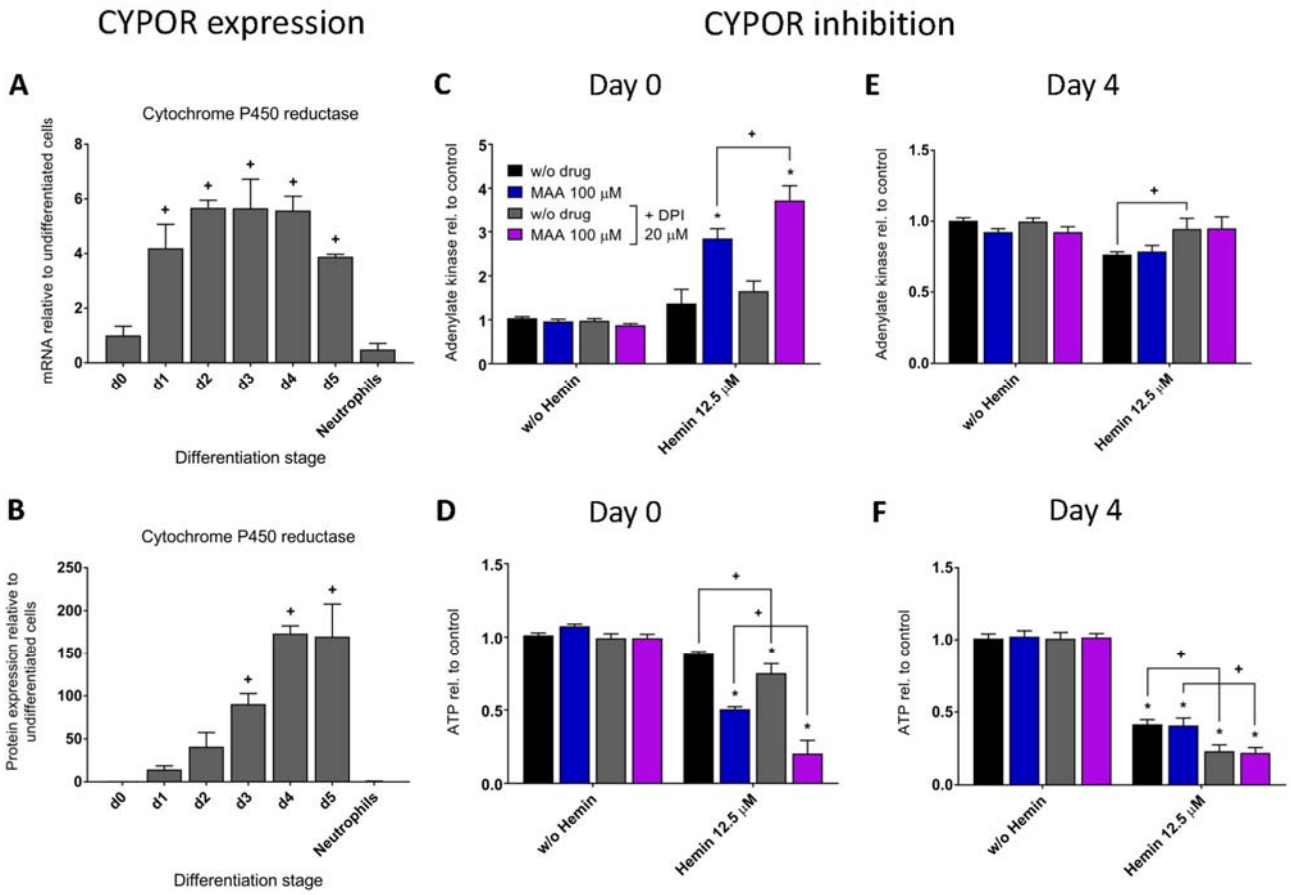

Fig. 3. *Expression of cytochrome P450 reductase (CYPOR) during HL60 cell differentiation and effect of POR inhibition by diphenyleneiodonium (DPI) on the toxicity of MAA and hemin on undifferentiated and differentiated HL60 cells.* (**A**) CYPOR mRNA expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells (day 0). $^{+}p < 0.05$ vs. undifferentiated HL60 cells. (**B**) CYPOR protein expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells (day 0). $^{+}p < 0.05$ vs. undifferentiated HL60 cells. For the inhibition experiments, cells were treated with MAA and hemin for 24 h with or without pretreatment with 20 µM DPI. (**C**) Effect on adenylate kinase release (marker of membrane integrity) in undifferentiated HL60 cells. (**D**) effect on intracellular ATP concentration in undifferentiated HL60 cells. (**E**) effect on adenylate kinase release in HL60 cells differentiated for 4 days. (**F**) effect on intracellular ATP concentration in HL60 cells differentiated for 4 days. Data represent the mean ± SEM of three independent experiments, each value per experiment assessed in triplicate. $^{*}p < 0.05$ vs. respective control incubations w/o drug, $^{+}p < 0.05$ vs. incubations w/o DPI pretreatment of the same treatment group. Neutrophils were obtained from different healthy donors; cells from one donor were used per experiment. MAA: N-methyl-4-aminoantipyrine.

incubations (Fig. 3E). However, hemin was associated with a significant drop in the cellular ATP content, which was not increased by MAA but by the addition of DPI (Fig. 3F).

Overall, DPI pretreatment amplified the toxicity of hemin and of the combination hemin/MAA in undifferentiated and of hemin in differentiated HL60 cells, suggesting an involvement of heme metabolizing enzymes in hemin/MAA cytotoxicity in undifferentiated HL60 cells.

3.6. Expression of biliverdin reductase (BVR) during HL60 cell differentiation and effect of the inhibition of biliverdin reductase on the cytotoxicity of MAA and hemin

The second degrading step of the heme metabolism consists of the conversion of biliverdin to bilirubin by BVR. Bilirubin is an antioxidant (Baranano et al., 2002; Florczyk et al., 2008) and malfunction or inhibition of biliverdin reductase, leading to decreased bilirubin levels, may increase the susceptibility of cells towards electrophilic molecules, which are produced when MAA reacts with hemin (Rudin et al., 2019). Therefore, we assessed the mRNA and protein expression of BVR during HL60 differentiation as well as in freshly isolated neutrophil granulocytes. In contrast to HO-1 and CYPOR, the mRNA expression of BVR dropped rapidly during HL60 differentiation to less than 10% after 5 days, which was comparable to the value observed in mature neutrophil granulocytes (Fig. 4A). However, the BVR protein content first increased with progressing differentiation until day 4 and then decreased at day 5 (Fig. 4B). In mature neutrophil granulocytes, the BVR protein expression was fivefold higher than in undifferentiated HL60 cells, but clearly lower than in the differentiated HL60 cells.

In undifferentiated HL60 cells, the addition of the BVR inhibitor montelukast was associated with an increase in the fraction of necrotic HL60 cells, whereas the apoptotic cell fraction decreased and viability remained unchanged compared to the corresponding incubations without montelukast (Fig. 4C). In the presence of montelukast, the addition of MAA to hemin had no significant effect on viability. After 4 days of differentiation, neither hemin nor MAA or the combination hemin and MAA decreased the viability of HL60 cells significantly, irrespectively of the presence of montelukast (Fig. 4D).

These results suggested an involvement of BVR in the emerging resistance towards the toxicity of hemin/MAA during HL60 differentiation.

3.7. Change of superoxide dismutases (SOD) 1 and 2 during myeloid differentiation

Beside increased heme metabolism capacity, the observed resistance of differentiated HL60 cells and neutrophil granulocytes to hemin and MAA may originate from a higher antioxidative capacity of these cells. The cellular antioxidative capacity, consisting of radical degrading

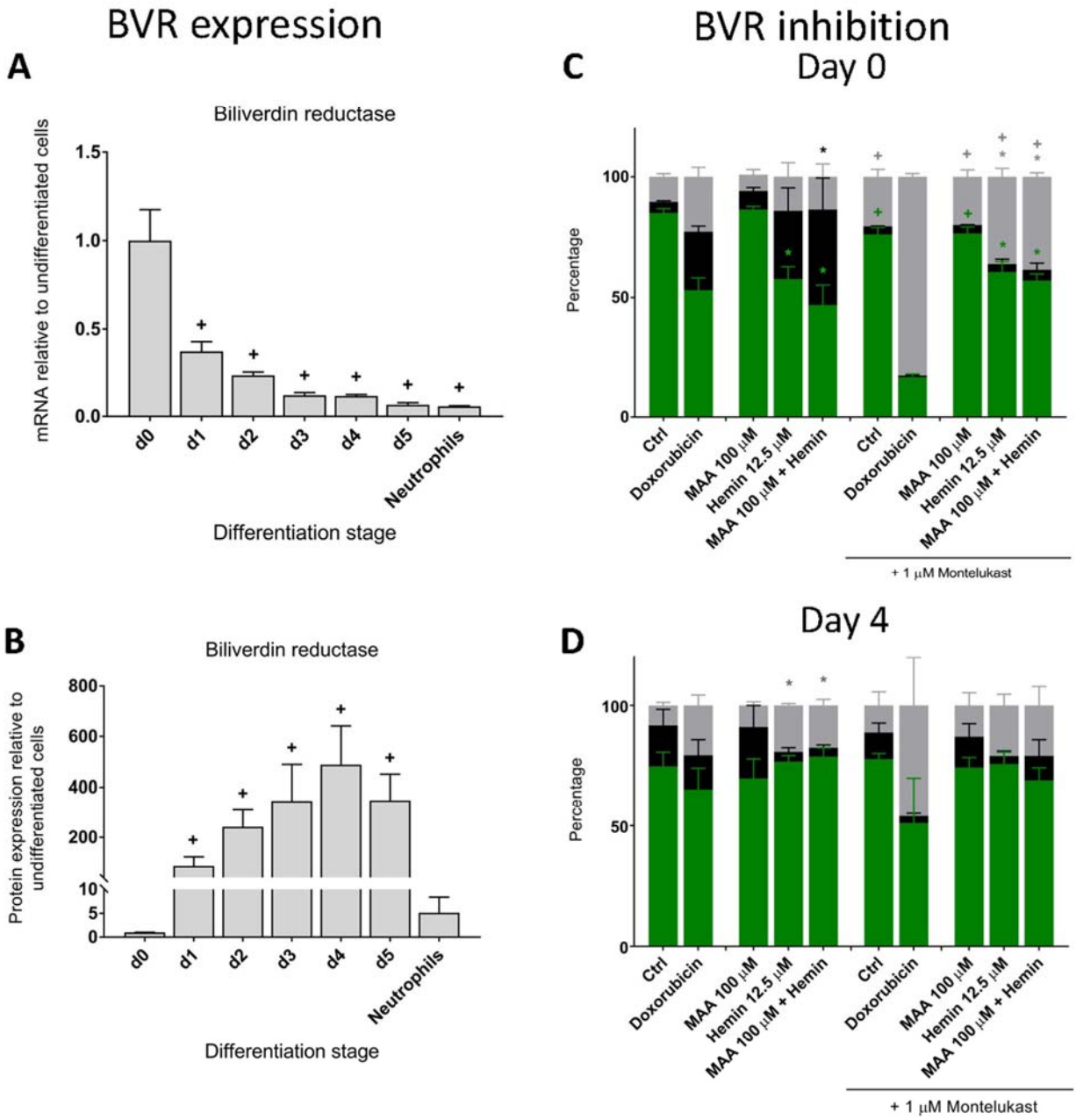

Fig. 4. *Expression of biliverdin reductase (BVR) in differentiating HL60 cells and neutrophil granulocytes and effect of the inhibition of BVR by montelukast on the toxicity of MAA and hemin on undifferentiated and differentiated HL60 cells.* HL60 cells were differentiated over 5 days and neutrophil granulocytes isolated from whole blood. (**A**) BVR mRNA expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. (**B**) BVR protein expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. $^{+}p < 0.05$ vs. undifferentiated HL60 cells. For the inhibition experiments, cells were treated with MAA and hemin for 24 h with or without pretreatment with 1 µM montelukast. (**C**) Effect of montelukast on viability of undifferentiated HL60 cells (day 0). (**D**) Effect of montelukast on viability of HL60 cells differentiated for 4 days. Data represent the mean ± SEM of three independent experiments, each value per experiment assessed in triplicate. $^{*}p < 0.05$ vs. control incubations w/o drug, $^{+}p < 0.05$ vs. incubations w/o montelukast pretreatment of the same treatment group. Neutrophils were obtained from different healthy donors; cells from one donor were used per experiment. MAA: *N*-methyl-4-aminoantipyrine.

enzymes and cellular GSH (Hayes and McLellan, 1999), may change during cell maturation and may therefore play an important role in hemin and MAA cytotoxicity. SOD1 and SOD2 are the major cellular antioxidant defense system against superoxide radicals (Fukai and Ushio-Fukai, 2011). SOD1 is mainly localized in the cytoplasm and to a smaller extent in the mitochondrial intermembrane space (Crapo et al., 1992; Okado-Matsumoto and Fridovich, 2001). In contrast, SOD2 is localized solely in the mitochondrial matrix (Hsu et al., 1996). Hence, the expression of both antioxidative enzymes was investigated in undifferentiated and differentiating HL60 cells as well as in mature neutrophil granulocytes.

As shown in Fig. 5A, SOD1 mRNA expression in HL60 cells decreased with progressing differentiation and was lowest in mature neutrophils. The corresponding protein expression increased to a maximum at day 4 of differentiation followed by a decrease at day 5 and likewise lower SOD1 levels in mature neutrophils (Fig. 5B, Supplementary Fig. 4A). Based on these observations, we considered it as unlikely that SOD1 contributed to the increasing resistance against hemin/MAA toxicity of differentiating HL60 cells. This may be due to the fact that the main function of mature neutrophils is to destroy invading pathogens by superoxide production, which could be impaired by the cytoplasmic SOD1. In comparison, the highest SOD2 mRNA

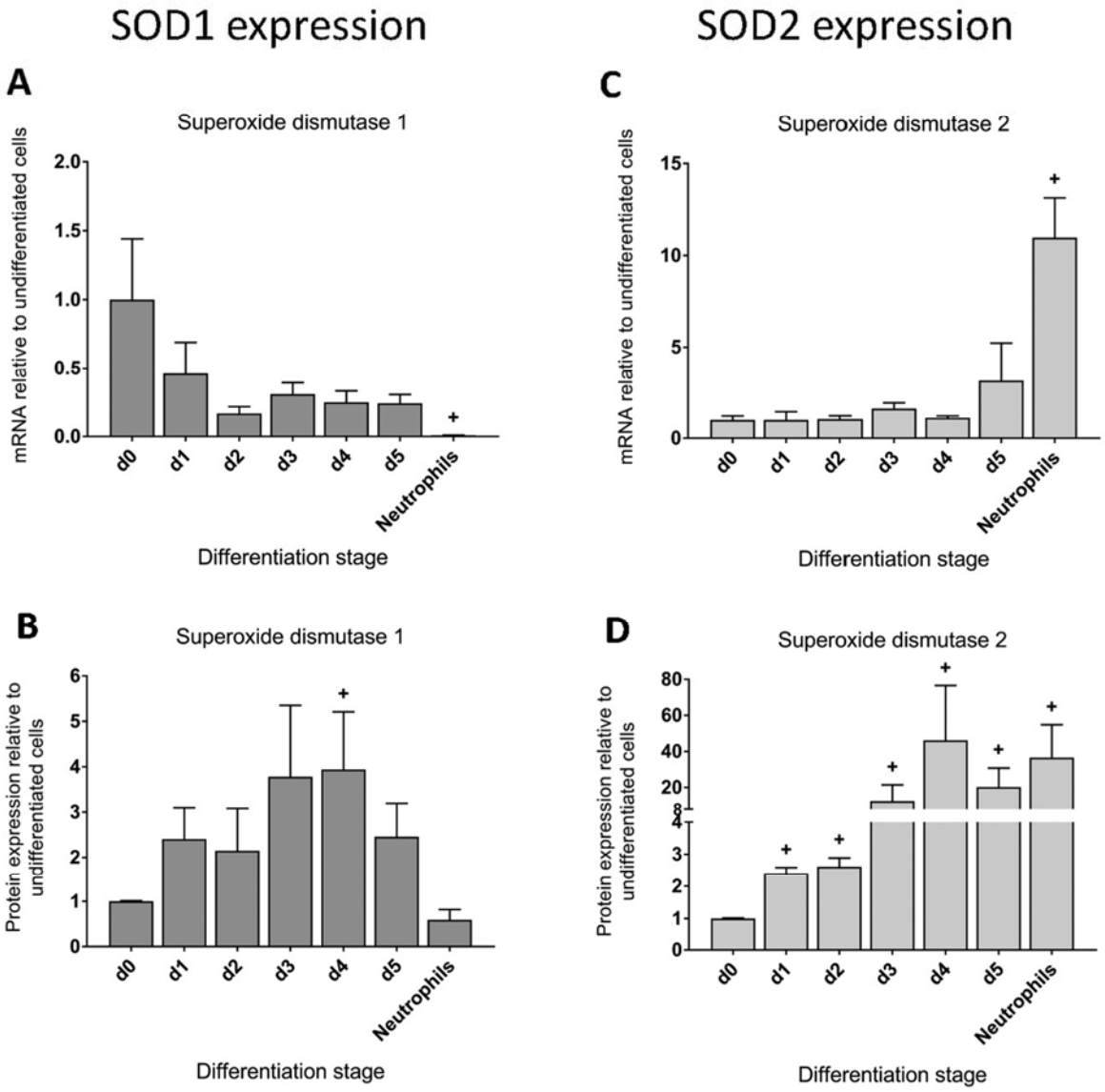

Fig. 5. *Expression of superoxide dismutase in differentiating HL60 cells and neutrophil granulocytes.* HL60 cells were differentiated over 5 days and neutrophil granulocytes isolated from whole blood. (**A**) Superoxide dismutase 1 (SOD1) mRNA expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. (**B**) SOD1 protein expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. (**C**) Superoxide dismutase 2 (SOD2) mRNA expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. (**D**) SOD2 protein expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. Data represent the mean ± SEM of three independent experiments, each value per experiment assessed in triplicate. $^{+}p < 0.05$ vs. undifferentiated HL60 cells. Neutrophils were obtained from different healthy donors; cells from one donor were used per experiment.

expression was measured at day 5 of differentiation in HL60 cells and was even higher in mature neutrophil granulocytes (Fig. 5C). Accordingly, the protein expression increased about 40-fold after 4 days of differentiation and was similar in mature neutrophils (Fig. 5D, Supplementary Fig. 4A). Since SOD2 is localized in the mitochondrial matrix, high enzyme levels will not interfere with the cellular superoxide production during host defense. To distinguish whether the increasing SOD2 content was due to an increasing amount of mitochondria or if the SOD2 concentration increased within the mitochondria, we assessed the mitochondrial DNA copy number during HL60 cell differentiation and in mature neutrophil granulocytes. As shown in Supplementary Fig. 5A, the mitochondrial copy number decreased slightly to 90% after one day of HL60 cell differentiation and remained at this level until day 5. In mature neutrophil granulocytes the mitochondrial copy number was significantly lower than in undifferentiated HL60 cells. As shown in suppl. Fig. 5B, after 3 days of differentiation, the SOD2 expression significantly increased when expressed relative to the mitochondrial copy number, reaching a maximum at day 4 of differentiation. In mature neutrophils, the SOD2 content relative to the mitochondrial copy number was even higher than the observed maximum in differentiated HL60 cells. Thus, the increasing SOD2 expression during HL60 differentiation as well as the high values in mature neutrophils underscore the importance of this enzyme for neutrophil granulocytes and suggests that it is involved in the resistance of differentiated granulocytes towards hemin and MAA.

3.8. Change of hydrogen peroxide degrading enzymes during myeloid differentiation

After degradation of superoxide radicals by superoxide dismutase, the formed hydrogen peroxide is further metabolized to water and oxygen by catalase and glutathione peroxidases (GPX) (Ighodaro and Akinloye, 2018). Hence, we investigated the expression of catalase, GPX1, and GPX4 in undifferentiated and differentiating HL60 cells as

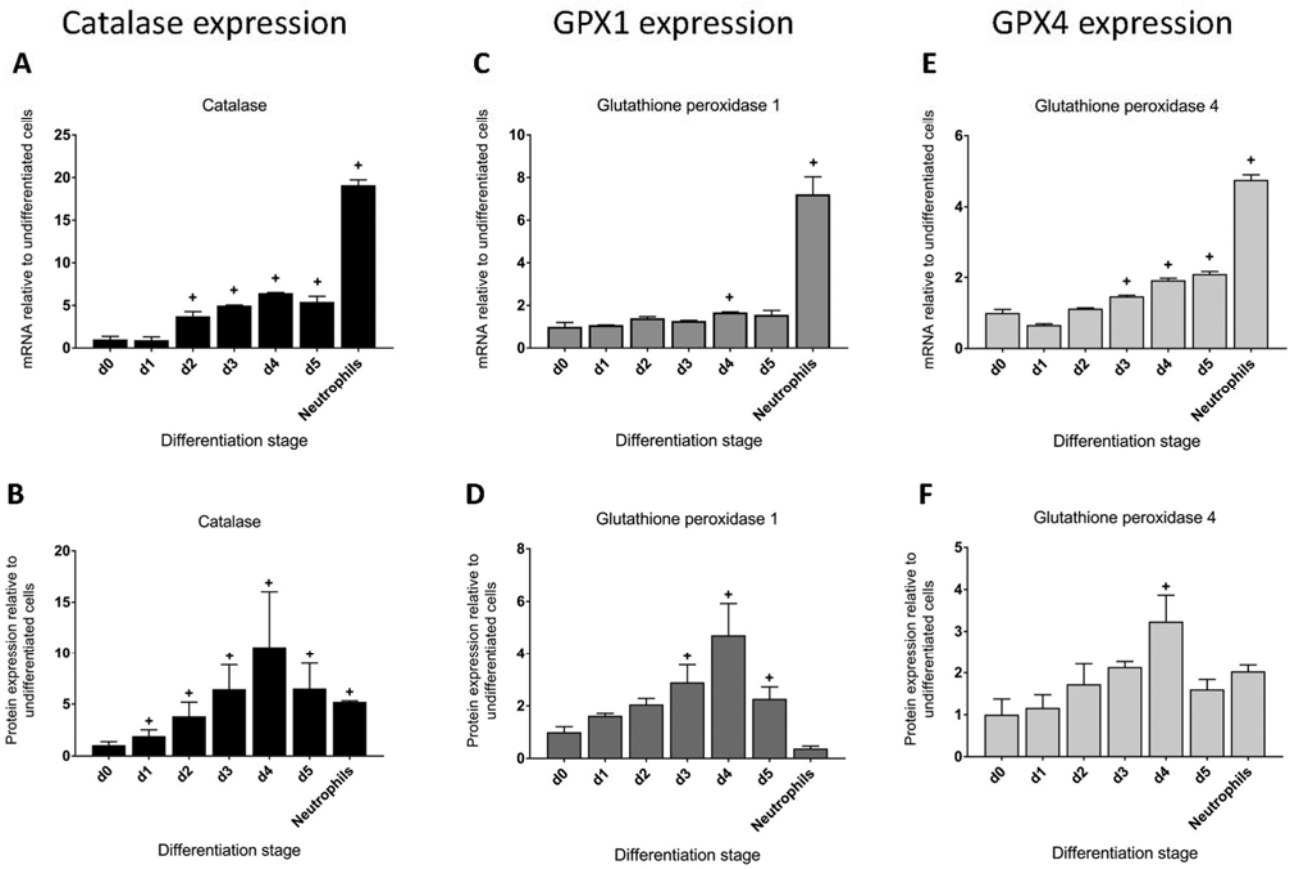

Fig. 6. *Expression of catalase and glutathione peroxidases (GPX) in differentiating HL60 cells and neutrophil granulocytes.* HL60 cells were differentiated over 5 days and neutrophil granulocytes isolated from whole blood. (**A**) Catalase mRNA expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. (**B**) Catalase protein expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. (**C**) GPX1 mRNA expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. (**D**) GPX1 protein expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. (**E**) GPX4 mRNA expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. (**F**) GPX4 protein expression in differentiating HL60 cells and neutrophil granulocytes relative to undifferentiated HL60 cells. Data represent the mean ± SEM of three independent experiments. Neutrophils were obtained from different healthy donors; cells from one donor were used per experiment.

well as mature neutrophil granulocytes.

Catalase is predominantly located at the major site of hydrogen peroxide production, the peroxisomes, but can also be found in the cytoplasm (Chelikani et al., 2004; Djordjević, 2004; Zhou and Kang, 2000). Since neutrophils produce the myeloperoxidase substrate hydrogen peroxide for host defense purposes, catalase might be necessary to protect the cell against the self-made oxidant. As shown in Fig. 6A, catalase mRNA content increased over 5 fold until day 5 in differentiating HL60 cells and was almost 20 fold higher in neutrophil granulocytes compared to undifferentiated HL60 cells. Accordingly, the catalase protein expression increased significantly starting at day 1 reaching a maximum at day 4 of differentiation (Fig. 6B, Supplementary Fig. 4B). In neutrophil granulocytes, the catalase protein expression was over 5 times higher than in undifferentiated HL60 cells, but lower than in HL60 cells after 4 days of differentiation. Hence, the cellular capacity to degrade hydrogen peroxide strongly increased with progressing maturation of the cells. Therefore, catalase may be involved in the cellular resistance of differentiated HL60 cells and neutrophil granulocytes against reactive MAA intermediates.

GPX1 and GPX4 are abundant in all mammalian cells, being responsible for breakdown of hydrogen peroxide in mitochondria (GPX1) and phospholipid hydroperoxides (GPX4) (Chelikani et al., 2004; Ursini et al., 1985). Fig. 6C and E show that the mRNA content of GPX1 and GPX4 increased with proceeding HL60 differentiation 1.5 fold and 2 fold, respectively, compared to undifferentiated HL60 cells. The increase in GPX1 and GPX4 mRNA was even more pronounced in mature neutrophil granulocytes (Fig. 6C and E). As shown in Fig. 6D and Supplementary Fig. 4B, the GPX1 protein expression increased during HL60 differentiation to almost 5 times higher expression than in undifferentiated cells, but decreased with further differentiation and was lower in neutrophil granulocytes than in undifferentiated HL60 cells. Similarly, GPX4 protein expression was the highest after 4 days of differentiation (Fig. 6F, Supplementary Fig. 4B), but, in contrast to GPX1, was still two fold higher in neutrophil granulocytes than in undifferentiated HL60 cells. These results suggested that GPX1 is not involved in the resistance of neutrophil granulocytes towards hemin/MAA toxicity, but seems to play a role during myeloid differentiation. However, GPX4 may support the resistance of differentiated HL60 cells and neutrophil granulocytes against reactive MAA intermediates.

3.9. Change of cellular GSH concentration during myeloid differentiation

Beside enzymatic antioxidative defense mechanisms, GSH, the most abundant cytosolic thiol, is important for the cellular non-enzymatic antioxidant defense system by scavenging various free radicals (Galano and Alvarez-Idaboy, 2011). Cellular GSH and GSSG concentrations were measured in undifferentiated and differentiating HL60 cells. As shown in Fig. 7A, the GSH concentration increased during HL60 cell differentiation while the corresponding GSSG levels remained almost constant. This led to a rapidly decreasing GSSG/GSH ratio during progressing HL60 cell differentiation (Fig. 7B). The observed increase in the cellular GSH pool may support the emerging resistance of

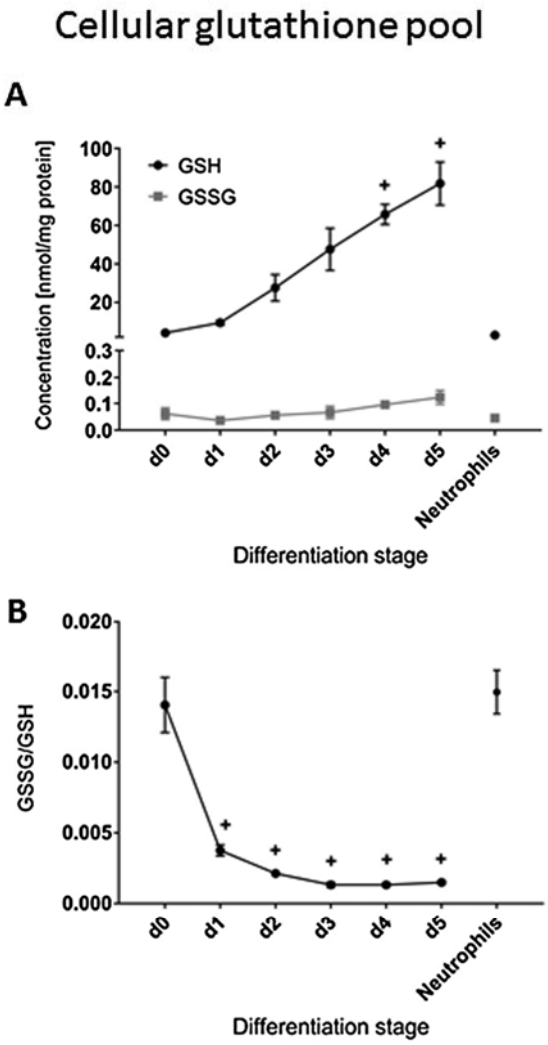

Fig. 7. *Glutathione pool in differentiating HL60 cells and neutrophil granulocytes.* HL60 cells were differentiated over 5 days and neutrophil granulocytes isolated from whole blood. (**A**) Content of reduced (GSH) and oxidized glutathione (GSSG) in HL60 cells and neutrophil granulocytes. (**B**) GSSG/GSH ratio in HL60 cells and neutrophil granulocytes. Data represent the mean ± SEM of three independent experiments, each value per experiment assessed in triplicate. $^{+}p < 0.05$ vs. undifferentiated HL60 cells. Neutrophils were obtained from different healthy donors; cells from one donor were used per experiment.

differentiating HL60 cells against the toxicity of hemin/MAA.

4. Discussion

The current study shows that MAA increases the toxicity of hemin in immature myeloid cells but less so in HL60 cells after 3 days of differentiation and that hemin/MAA is not toxic in mature neutrophil granulocytes. These results demonstrate that the cells become resistant towards the toxicity of hemin/MAA during differentiation.

We could show that the susceptibility of HL60 cells in the metamyelocytic differentiation stage (day 4 of differentiation) to MAA and hemin was decreased compared to undifferentiated HL60 cells (day 0 of differentiation). This is in agreement with bone marrow biopsies of affected patients, which revealed a stop at the promyelocyte/myelocyte stage in granulocyte maturation (Kummer et al., 2006). Taking into account our previous results showing that MAA interacts with hemin in a reaction that produces electrophilic intermediates (Rudin et al., 2019), potential mechanisms leading to resistance to the toxicity of hemin/MAA of differentiating HL60 cells include increased metabolism of heme/hemin and/or increased oxidative defense capacity.

The cellular metabolism of heme (see Supplementary Fig. 2) represents an important detoxification mechanism of the highly reactive heme (Alam, 2002). Heme or its oxidized form hemin consist of a ferrous or ferric iron ion, respectively, in a porphyrin ring (Robinson et al., 2009). Heme (or hemin) is contained in the prosthetic group of various enzymes and hemoproteins (Ponka, 1999; Severance and Hamza, 2009). It is synthesized through an enzymatic cascade that is located in the cytosol and in the mitochondria (Lange et al., 1999; Schultz et al., 2010). Additionally, free heme can be released from hemoglobin (or hemin from methemoglobin) after hemoglobin degradation (Hargrove et al., 1997; Kassa et al., 2016; Robinson et al., 2009). Extracellular hemin can also be taken up by cells, where it can react with MAA to produce cytotoxic reactive intermediates (Pierre et al., 2007; Rudin et al., 2019). Heme and hemin are highly reactive molecules, damaging various cell types by oxidizing cellular proteins and lipids (Fitch et al., 1983; Goldstein et al., 2003; Owen et al., 2016).

HO-1 is the first enzyme involved in heme degradation, producing biliverdin and the important signaling molecule CO (Dennery, 2014; Tenhunen et al., 1968). HO-1 also exerts anti-inflammatory and antioxidative effects by regulation of gene expression and protein translation, and by inducing DNA repair (Cai et al., 2012; Lin et al., 2007, 2008). In the absence of the HO-1 inhibitor ZnPP, the addition of MAA to hemin increased membrane toxicity, the drop in the cellular ATP content and the fraction of apoptotic/necrotic cells in undifferentiated HL60 cells. In contrast, at 4 days of differentiation, MAA did not increase the toxicity of hemin. The addition of the HO-1 inhibitor ZnPP increased the toxicity of hemin and hemin/MAA at day 0. At day 4, the addition of ZnPP only increased the drop in the cellular ATP, but did not impair membrane integrity or cell viability. At the same time, the protein expression of HO-1 and of the antioxidative enzymes as well as the cellular GSH pool increased dramatically over the 4 days of differentiation. At day 0, HL60 cells were extremely sensitive to the toxicity of hemin, most probably due to the fact that hemin could almost not be degraded. As expected, when the degradation was inhibited by the addition of ZnPP, the toxicity on HL60 cells by hemin increased. Also the addition of MAA increased the toxicity of hemin, most probably due the production of electrophilic intermediates upon the reaction of MAA with hemin (Pierre et al., 2007; Rudin et al., 2019). The antioxidative defense system was almost not existing in undifferentiated HL60 cells, explaining the increase in hemin toxicity by MAA. After 4 days of differentiation, the hemin degradation and antioxidative defense systems had been developed. Accordingly, the toxicity of hemin was only detectable regarding the cellular ATP content, but not for membrane integrity or cell viability. The toxicity of hemin could be increased by inhibition of HO-1, demonstrating the importance of the heme degrading system in the presence of hemin. Importantly, MAA did not increase the toxicity of hemin at this stage, most probably due to the antioxidative defense system, which had been developed. These findings are in agreement with our previous report showing that MAA reacts with hemin and that an electrophilic intermediate is formed, which increases the toxicity of hemin (Rudin et al., 2019). In this report we have shown that GSH could prevent the toxicity of MAA in combination with hemin, suggesting that the increase in the cellular GSH content was critical for limiting the toxicity of MAA. As shown in Fig. 6, the cellular content of GSH increased from day 0 to day 5 of the differentiation by almost a factor of ten, suggesting a role of GSH in the development of resistance against the toxicity of MAA.

The findings regarding CYPOR were similar to HO-1. MAA increased the toxicity of hemin in undifferentiated but not in differentiated HL60 cells and the inhibitor DPI increased the toxicity of hemin and hemin/MAA in undifferentiated and partially also in differentiated HL60 cells. These findings are not surprising, since HO-1 (and BVR) needs electrons transferred from NADPH by CYPOR for its function (Florczyk et al., 2008; Sugishima et al., 2014). Similar to HO-1, the protein expression of CYPOR increased dramatically during HL60 differentiation.

However, in contrast to HO-1, the protein expression of CYPOR was low in neutrophils. CYPOR is an electron donor for all microsomal CYP enzymes and therefore important during cell maturation (Gocek et al., 2014). In mature neutrophils, which have a limited lifetime, CYPOR appears to be less important than HO-1. Due to the generation of CO, which is an important signaling molecule (Dennery, 2014), the function of HO-1 is not restricted to heme or hemin degradation.

After degradation of heme to biliverdin by HO-1, biliverdin is reduced to bilirubin by BVR. Since bilirubin is an important antioxidative substance, BVR has a role in the antioxidative capacity of a cell (Maines, 2005; Tudor et al., 2008). In undifferentiated HL60 cells, MAA increased the toxicity of hemin on cell viability. This effect was absent at day 4 of cell differentiation, where hemin or the combination hemin and MAA were not toxic. In the presence of montelukast, an inhibitor of BVR (van Dijk et al., 2017), hemin decreased the viability of undifferentiated HL60 cells. This effect was not increased by MAA and was not observed in HL60 after 4 days of differentiation. The effects of BVR inhibition on hemin/MAA toxicity were qualitatively comparable, but quantitatively less accentuated than those observed for the inhibition of HO-1 or CYPOR, suggesting that biliverdin reductase is important for neutrophil maturation, but less so for resistance against the toxicity of heme/MAA. The importance of biliverdin reductase for HL60 cell differentiation is underscored by a high expression during the differentiation phase but a relatively low expression in mature neutrophils.

As mentioned above, the observed resistance of differentiated HL60 cells to the toxicity of hemin and MAA may also be due to the increased antioxidative defense. The antioxidative defense becomes particularly important in differentiating myeloid cells as the respiratory burst enzyme system matures, reaching the capacity of generating high intracellular ROS concentrations (Babior et al., 2002). Superoxide can dismutate spontaneously to hydrogen peroxide or enzymatically by SOD1 and SOD2 (Fukai and Ushio-Fukai, 2011; Ighodaro and Akinloye, 2018; Pietarinen-Runtti et al., 2000). While SOD1 protein expression reached a peak at day 4 of HL60 cell differentiation and was low in mature neutrophils, SOD2 protein levels increased constantly during HL60 cell differentiation and were high in mature neutrophils. Regarding SOD1, the results of the current study are in agreement with the study of Auwerx et al., who described a decrease in the SOD1 mRNA expression during HL60 cell differentiation (Auwerx et al., 1989). These results suggest that SOD1 is needed during cellular differentiation, but not in mature neutrophils. Since SOD1 is mainly localized in the cytoplasm (Crapo et al., 1992; Okado-Matsumoto and Fridovich, 2001), it could interfere with the superoxide production of neutrophils during host defense. In contrary, SOD2 is located to the mitochondrial matrix, where it supports the degradation of superoxide formed by mitochondrial respiration (Fukai and Ushio-Fukai, 2011). Importantly, Maianski et al. observed high mitochondrial levels of SOD2 in mature neutrophil granulocytes (Maianski et al., 2004). They explained it by the low cytochrome c expression in mature neutrophils, which impairs the flow of electrons between complex III and IV of the electron transport chain, which could be associated with excessive ROS production.

The hydrogen peroxide produced by SOD1, SOD2, and further oxidases may react with Fe^{2+} to form the highly reactive hydroxyl radicals (Ighodaro and Akinloye, 2018). To prevent this reaction, hydrogen peroxide is enzymatically degraded by catalase and glutathione peroxidases (Chelikani et al., 2004; Djordjević, 2004; Ursini et al., 1985; Zhou and Kang, 2000). Similar to SOD 1 and 2, the protein expression of catalase and GPX1 and 4 showed a peak at 4 days of differentiation, demonstrating their importance for the differentiation of the HL60 cells. While catalase and GPX4 had also a high expression in mature neutrophils, the expression of GPX1 was low in neutrophils. GPX4 is located in mitochondria and in the cytosol, but also in the nucleus, where it regulates the content of phospholipid hydroperoxides and fatty acid hydroperoxides (Imai and Nakagawa, 2003; Ursini et al., 1985). Mitochondrial GPX4 suppresses cytochrome c release, whereas non-mitochondrial GPX4 inhibits the activation of lipoxygenase and cyclooxygenase in the nucleus and the endoplasmic reticulum (Nomura et al., 1999; Sakamoto et al., 2000). Similar to HO-1, the function of GPX4 appears to be too important for mature neutrophils to be reduced or eliminated during the differentiation.

Regarding the GSH content in HL60 cells, the increase in GSH during the development is dramatic (from 3.9 nmol/mg protein at day 0–80 nmol/mg protein at day 5, Fig. 7A), suggesting that antioxidative defense is important during HL60 cell maturation. In comparison to mature HL60 cells, the GSH content of human neutrophils was lower (3 nmol/mg protein, similar to undifferentiated HL60 cells). An almost identical GSH content in human neutrophil granulocytes has been reported in a previous study (Kose and Naziroglu, 2015). While we cannot exclude that some GSH was lost during the granulocyte isolation, it appears also to be possible that a high GSH content is needed for granulocyte maturation in the bone marrow, but not for granulocytes in circulation. In contrast to undifferentiated HL60 cells, neutrophil granulocytes were not sensitive to hemin/MAA despite similar cellular GSH contents. A possible explanation for this apparent discrepancy is the finding that neutrophils had a higher expression of enzymes degrading hemin (HO-1), superoxide (SOD2) and hydrogen peroxide (catalase) than undifferentiated HL60 cells.

Although we could show that undifferentiated HL60 cells are susceptible to hemin/MAA and we could explain the resistance to hemin/MAA during HL60 cell maturation by the expression of heme degrading and antioxidative metabolic pathways, it remains unclear whether HL60 cells reflect granulocyte maturation in the human bone marrow. In this context, it is important to take into account that we demonstrated the toxicity of hemin/MAA in a second, independent human cell system, which supports the results obtained in HL60 cells. Nevertheless, HL60 cells are a tumor cell line, which may have different properties compared to neutrophil granulocytes and granulocyte precursors. The results of the current study should therefore be confirmed by investigations in humans focusing on susceptibility factors for this idiosyncratic adverse reaction.

In conclusion, the current studies show that hemin alone is toxic for HL60 cells, in particular when the antioxidative systems are not fully developed and when the hemin degrading system is not fully developed or inhibited. MAA increases the toxicity of hemin, but only in undifferentiated HL60 cells, where the antioxidative system is almost absent. This suggests that the toxicity of MAA is mainly due to the formation of a reactive metabolite, which cannot be degraded when the antioxidative capacity is lacking. Risk factors for MAA toxicity may therefore be a high intracellular hemin concentration and a lack of antioxidative capacity in developing neutrophil granulocytes, e.g. impaired expression of key enzymes in the heme degrading and/or antioxidative metabolic pathways.

Author contributions

DR and SK designed the experiments and DR performed all the experiments. NR assisted with LC–MS measurements. DR, UD, and SK wrote the manuscript.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.tox.2019.152254.

References

Abraham, N.G., Kappas, A., 2008. Pharmacological and clinical aspects of heme oxygenase. Pharmacological reviews 60, 79–127.
Alam, J., 2002. Heme oxygenase-1: past, present, and future. Antioxid. Redox Signal. 4, 559–562.
Andres, E., Maloisel, F., 2008. Idiosyncratic drug-induced agranulocytosis or acute neutropenia. Curr. Opin. Hematol. 15, 15–21.
Auwerx, J.H., Chait, A., Wolfbauer, G., Deeb, S.S., 1989. Loss of copper-zinc superoxide dismutase gene expression in differentiated cells of myelo-monocytic origin. Blood 74, 1807–1810.
Babior, B.M., Lambeth, J.D., Nauseef, W., 2002. The neutrophil NADPH oxidase. Arch. Biochem. Biophys. 397, 342–344.
Ballinger, C.A., Mendis-Handagama, C., Kalmar, J.R., Arnold, R.R., Kinkade Jr., J.M., 1994. Changes in the localization of catalase during differentiation of neutrophilic granulocytes. Blood 83, 2654–2668.
Baranano, D.E., Rao, M., Ferris, C.D., Snyder, S.H., 2002. Biliverdin reductase: a major physiologic cytoprotectant. PNAS 99, 16093–16098.
Bardoel, B.W., Kenny, E.F., Sollberger, G., Zychlinsky, A., 2014. The balancing act of neutrophils. Cell host & microbe 15, 526–536.
Blaser, L., Hassna, H., Hofmann, S., Holbro, A., Haschke, M., Ratz Bravo, A., Zeller, A., Krahenbuhl, S., Taegtmeyer, A., 2017. Leucopenia associated with metamizole: a case-control study. Swiss medical weekly 147 w14438.
Blaser, L.S., Tramonti, A., Egger, P., Haschke, M., Krahenbuhl, S., Ratz Bravo, A.E., 2015. Hematological safety of metamizole: retrospective analysis of WHO and swiss spontaneous safety reports. Eur. J. Clin. Pharmacol. 71, 209–217.
Breitman, T.R., Selonick, S.E., Collins, S.J., 1980. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. PNAS 77, 2936–2940.
Cai, C., Teng, L., Vu, D., He, J.Q., Guo, Y., Li, Q., Tang, X.L., Rokosh, G., Bhatnagar, A., Bolli, R., 2012. The heme oxygenase 1 inducer (CoPP) protects human cardiac stem cells against apoptosis through activation of the extracellular signal-regulated kinase (ERK)/NRF2 signaling pathway and cytokine release. The Journal of biological chemistry 287, 33720–33732.
Chelikani, P., Fita, I., Loewen, P.C., 2004. Diversity of structures and properties among catalases. Cellular and Molecular Life Sciences CMLS 61, 192–208.
Chowdhury, J.R., Chowdhury, N.R., 1983. Conjugation and excretion of bilirubin. Semin Liver Dis 3, 11–23.
Collins, S.J., Ruscetti, F.W., Gallagher, R.E., Gallo, R.C., 1978. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. PNAS 75, 2458–2462.
Crapo, J.D., Oury, T., Rabouille, C., Slot, J.W., Chang, L.Y., 1992. Copper,zinc superoxide dismutase is primarily a cytosolic protein in human cells. PNAS 89, 10405–10409.
Dennery, P.A., 2014. Signaling function of heme oxygenase proteins. Antioxid. Redox Signal. 20, 1743–1753.
Djordjević, V.B., 2004. Free Radicals in Cell Biology. International Review of Cytology. Academic Press, pp. 57–89.
Emerson, M.R., LeVine, S.M., 2000. Heme oxygenase-1 and NADPH cytochrome P450 reductase expression in experimental allergic encephalomyelitis: an expanded view of the stress response. J. Neurochem. 75, 2555–2562.
Fitch, C.D., Chevli, R., Kanjananggulpan, P., Dutta, P., Chevli, K., Chou, A.C., 1983. Intracellular ferriprotoporphyrin IX is a lytic agent. Blood 62, 1165–1168.
Florczyk, U.M., Jozkowicz, A., Dulak, J., 2008. Biliverdin reductase: new features of an old enzyme and its potential therapeutic significance. Pharmacological reports : PR 60, 38–48.
Fukai, T., Ushio-Fukai, M., 2011. Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxidants & redox signaling 15, 1583–1606.
Galano, A., Alvarez-Idaboy, J.R., 2011. Glutathione: mechanism and kinetics of its non-enzymatic defense action against free radicals. RSC Adv. 1, 1763–1771.
Garbe, E., 2007. Non-chemotherapy drug-induced agranulocytosis. Expert Opin Drug Saf 6, 323–335.
Giustarini, D., Dalle-Donne, I., Milzani, A., Fanti, P., Rossi, R., 2013. Analysis of GSH and GSSG after derivatization with N-ethylmaleimide. Nat. Protoc. 8, 1660.
Gocek, E., Marchwicka, A., Bujko, K., Marcinkowska, E., 2014. NADPH-cytochrome P450 reductase is regulated by all-trans retinoic acid and by 1,25-dihydroxyvitamin D3 in human acute myeloid leukemia cells. PloS one 9, e91752.
Goldstein, L., Teng, Z.P., Zeserson, E., Patel, M., Regan, R.F., 2003. Hemin induces an iron-dependent, oxidative injury to human neuron-like cells. J. Neurosci. Res. 73, 113–121.
Gupta, D., Shah, H.P., Malu, K., Berliner, N., Gaines, P., 2014. Differentiation and Characterization of Myeloid Cells. Current Protocols in Immunology 104, Unit 22F.25.
Hargrove, M.S., Whitaker, T., Olson, J.S., Vali, R.J., Mathews, A.J., 1997. Quaternary structure regulates hemin dissociation from human hemoglobin. The Journal of biological chemistry 272, 17385–17389.
Hayes, J.D., McLellan, L.I., 1999. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radical Res. 31, 273–300.
Hsu, J.L., Hsieh, Y., Tu, C., O'Connor, D., Nick, H.S., Silverman, D.N., 1996. Catalytic properties of human manganese superoxide dismutase. The Journal of biological chemistry 271, 17687–17691.
Ighodaro, O.M., Akinloye, O.A., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. Alexandria Journal of Medicine 54, 287–293.
Imai, H., Nakagawa, Y., 2003. Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. Free radical biology & medicine 34, 145–169.
Kassa, T., Jana, S., Meng, F., Alayash, A.I., 2016. Differential heme release from various hemoglobin redox states and the upregulation of cellular heme oxygenase-1. FEBS open bio 6, 876–884.
Klebanoff, S.J., Olszowski, S., Van Voorhis, W.C., Ledbetter, J.A., Waltersdorph, A.M., Schlechte, K.G., 1992. Effects of gamma-interferon on human neutrophils: protection from deterioration on storage. Blood 80, 225–234.
Kose, S.A., Naziroglu, M., 2015. N-acetyl cysteine reduces oxidative toxicity, apoptosis, and calcium entry through TRPV1 channels in the neutrophils of patients with polycystic ovary syndrome. Free Radical Res. 49, 338–346.
Kummer, O., Haschke, M., Tuchscherer, D., Lampert, M., Martius, F., Krahenbuhl, S., 2006. Agranulocytosis in a patient treated with metamizole and clopidogrel. Praxis (Bern 1994) 95, 1743–1745 quiz 1746-1747.
Lange, H., Kispal, G., Lill, R., 1999. Mechanism of iron transport to the site of heme synthesis inside yeast mitochondria. The Journal of biological chemistry 274, 18989–18996.
Levy, M., Flusser, D., Zylber-Katz, E., Granit, L., 1984. Plasma kinetics of dipyrone metabolites in rapid and slow acetylators. Eur. J. Clin. Pharmacol. 27, 453–458.
Lin, Q., Weis, S., Yang, G., Weng, Y.H., Helston, R., Rish, K., Smith, A., Bordner, J., Polte, T., Gaunitz, F., Dennery, P.A., 2007. Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. The Journal of biological chemistry 282, 20621–20633.
Lin, Q.S., Weis, S., Yang, G., Zhuang, T., Abate, A., Dennery, P.A., 2008. Catalytic inactive heme oxygenase-1 protein regulates its own expression in oxidative stress. Free radical biology & medicine 44, 847–855.
Maianski, N.A., Geissler, J., Srinivasula, S.M., Alnemri, E.S., Roos, D., Kuijpers, T.W., 2004. Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. Cell Death Differ. 11, 143–153.
Maines, M.D., 2005. New insights into biliverdin reductase functions: linking heme metabolism to cell signaling. Physiology (Bethesda, Md.) 20, 382–389.
McGuire, J.J., Anderson, D.J., McDonald, B.J., Narayanasami, R., Bennett, B.M., 1998. Inhibition of NADPH-cytochrome P450 reductase and glyceryl trinitrate biotransformation by diphenyleneiodonium sulfate. Biochem. Pharmacol. 56, 881–893.
Nomura, K., Imai, H., Koumura, T., Arai, M., Nakagawa, Y., 1999. Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. The Journal of biological chemistry 274, 29294–29302.
Okado-Matsumoto, A., Fridovich, I., 2001. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. The Journal of biological chemistry 276, 38388–38393.
Owen, J.E., Bishop, G.M., Robinson, S.R., 2016. Uptake and toxicity of hemin and iron in cultured mouse astrocytes. Neurochem. Res. 41, 298–306.
Pessina, A., Albella, B., Bueren, J., Brantom, P., Casati, S., Gribaldo, L., Croera, C., Gagliardi, G., Foti, P., Parchment, R., Parent-Massin, D., Sibiril, Y., Van Den Heuvel, R., 2001. Prevalidation of a model for predicting acute neutropenia by colony forming unit granulocyte/macrophage (CFU-GM) assay. Toxicology in vitro: an international journal published in association with BIBRA 15, 729–740.
Pierre, S.C., Schmidt, R., Brenneis, C., Michaelis, M., Geisslinger, G., Scholich, K., 2007. Inhibition of cyclooxygenases by dipyrone. Br. J. Pharmacol. 151, 494–503.
Pietarinen-Runtti, P., Lakari, E., Raivio, K.O., Kinnula, V.L., 2000. Expression of antioxidant enzymes in human inflammatory cells. American journal of physiology. Cell physiology 278, C118–125.
Pimstone, N.R., Tenhunen, R., Seitz, P.T., Marver, H.S., Schmid, R., 1971. The enzymatic degradation of hemoglobin to bile pigments by macrophages. The Journal of experimental medicine 133, 1264–1281.
Ponka, P., 1999. Cell biology of heme. The American journal of the medical sciences 318, 241–256.
Quiros, P.M., Goyal, A., Jha, P., Auwerx, J., 2017. Analysis of mtDNA/nDNA ratio in mice. Current protocols in mouse biology 7, 47–54.
Robinson, S.R., Dang, T.N., Dringen, R., Bishop, G.M., 2009. Hemin toxicity: a preventable source of brain damage following hemorrhagic stroke. Redox report: communications in free radical research 14, 228–235.
Rodgers, P.A., Vreman, H.J., Stevenson, D.K., 1990. Heme catabolism in rhesus neonates inhibited by zinc protoporphyrin. Dev. Pharmacol. Ther. 14, 216–222.
Rudin, D., Lanzilotto, A., Bachmann, F., Housecroft, C.E., Constable, E.C., Drewe, J., Haschke, M., Krahenbuhl, S., 2019. Non-immunological toxicological mechanisms of metamizole-associated neutropenia in HL60 cells. Biochem. Pharmacol. 163 (May), 345–356.
Sakamoto, H., Imai, H., Nakagawa, Y., 2000. Involvement of phospholipid hydroperoxide glutathione peroxidase in the modulation of prostaglandin D2 synthesis. The Journal of biological chemistry 275, 40028–40035.
Sánchez, S., De La Lastra, C., Ortiz, P., Motilva, V., Martín, M.J., 2002. Gastrointestinal tolerability of metamizol, acetaminophen, and diclofenac in subchronic treatment in rats. Dig. Dis. Sci. 47, 2791–2798.
Schultz, I.J., Chen, C., Paw, B.H., Hamza, I., 2010. Iron and porphyrin trafficking in heme biogenesis. The Journal of biological chemistry 285, 26753–26759.
Severance, S., Hamza, I., 2009. Trafficking of heme and porphyrins in metazoa. Chem. Rev. 109, 4596–4616.
Sugishima, M., Sato, H., Higashimoto, Y., Harada, J., Wada, K., Fukuyama, K., Noguchi, M., 2014. Structural basis for the electron transfer from an open form of NADPH-cytochrome P450 oxidoreductase to heme oxygenase. PNAS 111, 2524–2529.
Takao, H., Hirabayashi, K., Nishigaya, Y., Kouriki, H., Nakaniwa, T., Hagiwara, Y., Harada, J., Sato, H., Yamazaki, T., Sakakibara, Y., Suiko, M., Asada, Y., Takahashi, Y., Yamamoto, K., Fukuyama, K., Sugishima, M., Wada, K., 2017. A substrate-bound

structure of cyanobacterial biliverdin reductase identifies stacked substrates as critical for activity. Nat. Commun. 8, 14397.
Tenhunen, R., Marver, H.S., Schmid, R., 1968. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. PNAS 61, 748–755.
Tudor, C., Lerner-Marmarosh, N., Engelborghs, Y., Gibbs, P.E.M., Maines, M.D., 2008. Biliverdin reductase is a transporter of haem into the nucleus and is essential for regulation of HO-1 gene expression by haematin. The Biochemical journal 413, 405–416.
Ursini, F., Maiorino, M., Gregolin, C., 1985. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochim. Biophys. Acta 839, 62–70.
van Dijk, R., Aronson, S.J., de Waart, D.R., van de Graaf, S.F., Duijst, S., Seppen, J., Elferink, R.O., Beuers, U., Bosma, P.J., 2017. Biliverdin reductase inhibitors did not improve severe unconjugated hyperbilirubinemia in vivo. Sci. Rep. 7, 1646.
Vreman, H.J., Lee, O.K., Stevenson, D.K., 1991. In vitro and in vivo characteristics of a heme oxygenase inhibitor: ZnBG. The American journal of the medical sciences 302, 335–341.
Waldhauser, K.M., Torok, M., Ha, H.R., Thomet, U., Konrad, D., Brecht, K., Follath, F., Krahenbuhl, S., 2006. Hepatocellular toxicity and pharmacological effect of amiodarone and amiodarone derivatives. J. Pharmacol. Exp. Ther. 319, 1413–1423.
Zhou, Z., Kang, Y.J., 2000. Cellular and subcellular localization of catalase in the heart of transgenic mice. The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society 48, 585–594.

Supplement

Toxicity of metamizole on differentiating HL60 cells and human neutrophil granulocytes

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Keywords: metamizole, HL60 cells, neutrophils, antioxidative defense, heme metabolism

Legends to supplementary figures

Fig. S1

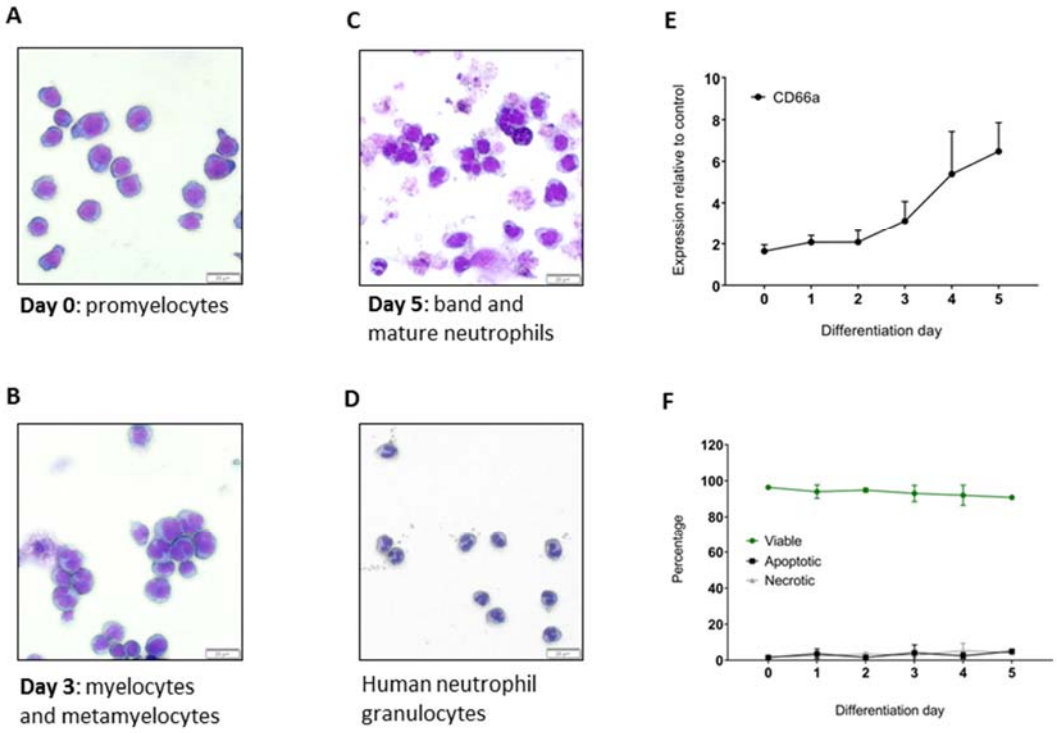

Figure S1

Differentiation of HL60 cells. Promyelocytic HL60 cells were differentiated towards neutrophil granulocytes over 5 days and the success of differentiation was assessed morphologically and by expression of the surface marker CD66a. (**A-D**) Light microscopy. Cell preparations of HL60 cells at (**A**) day 0, (**B**) day 3, and (**C**) day 5 of differentiation. (**D**) freshly isolated neutrophil granulocytes. Cells were stained with Hemacolor® Rapid staining set (Merck, Darmstadt, Germany) for evaluation of cell morphology. Bars, 20 µm. (**E**) Expression of the surface marker CD66a on differentiating HL60 cells. (**F**) Assessment of cell viability during the differentiation process. Apoptosis and necrosis were assessed by staining with annexin V and propidium iodide, respectively.

Fig. S2

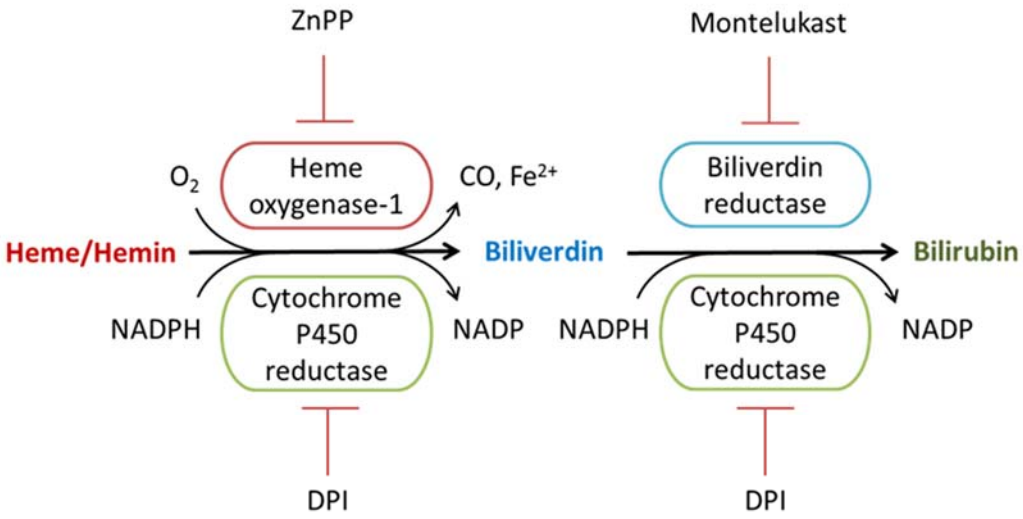

Figure S2

Metabolism of heme/hemin by heme oxygenase-1 (HO-1) and biliverdin reductase (BVR). HO-1 forms biliverdin by breaking the protoporphyrin ring of heme or hemin. The reaction consumes oxygen (O_2) and releases an iron ion (Fe^{2+}or Fe^{3+}) and carbon monoxide (CO). HO-1 can be inhibited by zinc protoporphyrine (ZnPP). Biliverdin is reduced to bilirubin by BVR. BVR can be inhibited by montelukast. HO-1 and BVR both use electrons for their reactions, which are transferred from NADPH by cytochrome P450 reductase (CYPOR). CYPOR can be inhibited by the flavoprotein inhibitor diphenylene iodonium (DPI).

Fig. S3

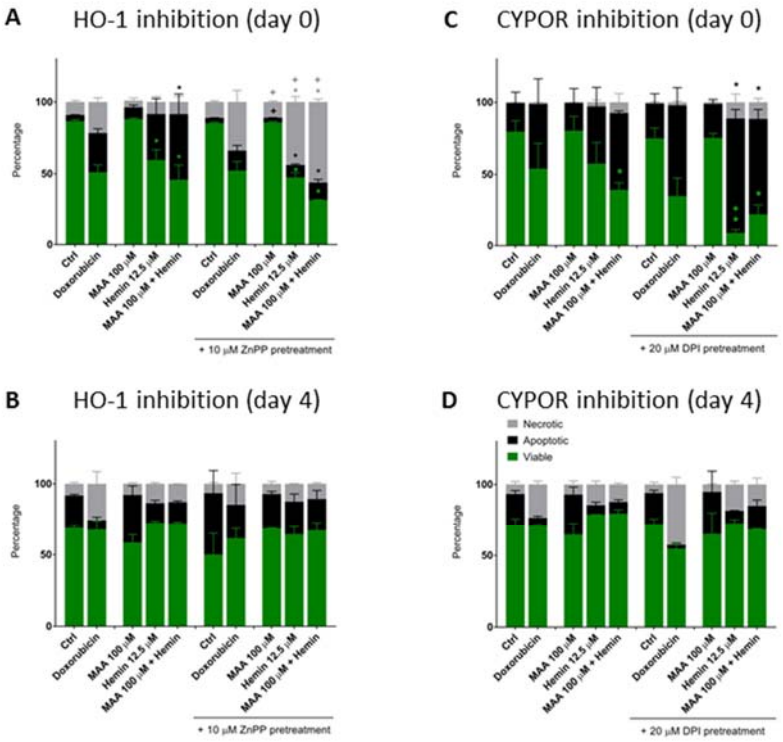

Figure S3

Effect of heme metabolism enzyme inhibitors on the effect of MAA and hemin on undifferentiated and differentiated HL60 cells. Cells were treated with MAA and hemin for 24 hours with or without inhibitors of heme metabolism enzymes and the effect on cell death by apoptosis or necrosis assessed. (**A**) Effect of heme oxygenase-1 inhibition by pretreatment with 10 µM zinc protoporphyrin (ZnPP) on undifferentiated HL60 cells. (**B**) Effect of HO-1 inhibition by pretreatment with 10 µM ZnPP on HL60 cells differentiated for 4 days. (**C**) Effect of CYPOR inhibition by pretreatment with 20 µM diphenylene iodonium (DPI) on undifferentiated HL60 cells. (**D**) Effect of CYPOR inhibition by pretreatment with 20 µM DPI on HL60 cells differentiated for 4 days. Apoptosis and necrosis were assessed by staining with annexin V and propidium iodide, respectively. Doxorubicin (0.5 µM) was used as positive control. *p<0.05 vs. control incubations (Ctrl), +p<0.05 vs. incubations w/o enzyme inhibitor of the same treatment group. Ctrl: control.

Fig. S4

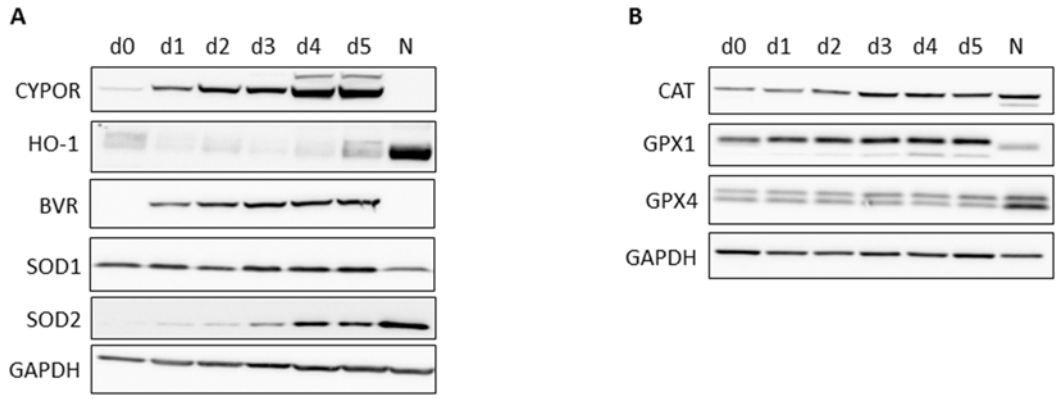

Figure S4

Western blot of heme degrading enzymes and antioxidative defense enzymes in differentiating HL60 cells and neutrophil granulocytes. HL60 cells were differentiated over 5 days and neutrophil granulocytes isolated from whole blood. (**A**) Protein expression of cytochrome P450 reductase (CYPOR), heme oxygenase-1 (HO-1), biliverdin reductase (BVR), superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2). GAPDH was used as loading control. (**B**) Protein expression of catalase (CAT), glutathione peroxidase 1 (GPX1), and glutathione peroxidase 4 (GPX4). GAPDH was used as loading control, d0-d5: days of HL60 differentiation, N: neutrophil granulocytes.

Fig. S5

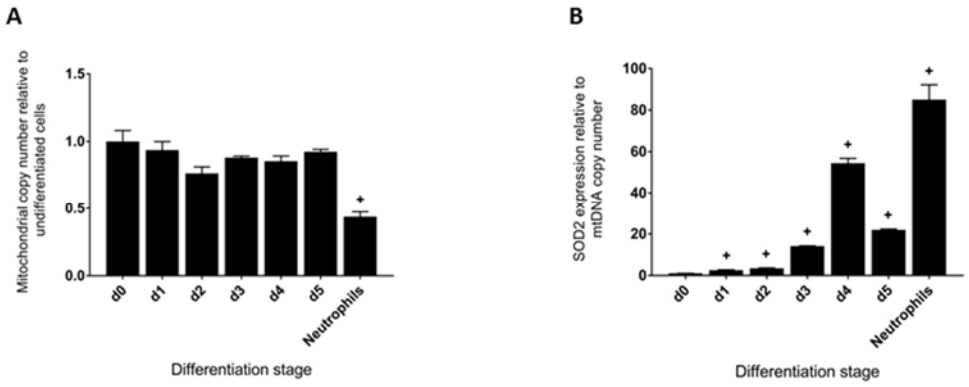

Figure S5

Mitochondrial copy number and protein expression of superoxide dismutase 2 (SOD2) relative to the mitochondrial copy number. SOD2 protein expression and the mitochondrial copy number were determined in differentiating HL60 cells and neutrophil granulocytes. (**A**) Mitochondrial copy number relative to undifferentiated HL60 cells. (**B**) SOD2 protein expression relative to mitochondrial copy number. $^{+}p<0.05$ vs. undifferentiated HL60 cells, mtDNA: mitochondrial DNA.

4. OBSERVATIONAL COMPARATIVE STUDY

4.1. Metamizole-associated neutropenia: Comparison of patients with neutropenia and metamizole-tolerant patients

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Original Article

Metamizole-associated neutropenia: Comparison of patients with neutropenia and metamizole-tolerant patients

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ABSTRACT

Reports of metamizole-induced neutropenia have increased in Switzerland and Germany over the last decades, most likely reflecting increased use of metamizole. To date, there are no effective strategies to identify patients at increased risk of metamizole-induced neutropenia. In this observational, multi-center comparative study, characteristics of patients with metamizole-associated neutropenia were compared with patients treated with metamizole without developing adverse hematological reactions. Patients with metamizole-induced neutropenia treated at the University Hospitals Basel and Bern between 2005 and 2017 were included. Tolerant comparison patients with continuous metamizole treatment (≥500 mg/day for at least 28 days) were recruited from GP offices and community pharmacies. Forty-eight patients with metamizole-induced neutropenia, consisting of 23 and 25 cases with inpatient-acquired and outpatient-acquired neutropenia, respectively, were compared to 39 metamizole tolerant comparison patients. Median latency until first diagnosis of neutropenia was 6 days (1–61 days) in inpatient cases and 19 days (2–204 days) in outpatient cases. There was no association between non-myelotoxic and non-immunosuppressive co-medication (p = .6627), history of drug allergy (p = .1304), and preexisting auto-immune diseases (p = .2313) and the development of metamizole-induced neutropenia. Our results suggest that autoimmune diseases, history of drug allergy, and concomitant treatment with non-myelotoxic and non-immunosuppressive drugs are likely not individual risk factors for metamizole-associated neutropenia.

1. Introduction

Metamizole (dipyrone) is a non-opioid analgesic and antipyretic drug, widely prescribed in many countries due to its good efficacy and low gastrointestinal toxicity [1–3]. Despite the favorable safety profile of metamizole, susceptible patients may experience neutropenia or agranulocytosis, a severe and potentially fatal decrease in circulating neutrophil granulocytes [1,4]. Previous studies reported incidence rates of approximately 1:1500 prescriptions in Sweden and 1 case per one million inhabitants and year in Spain [5,6]. In Switzerland, the incidence rate of metamizole-associated agranulocytosis was estimated to be 0.46–1.63 cases per million person-days of use between 2006 and 2012, whereas the incidence of metamizole-associated neutropenia is likely higher [1]. Based on reports of metamizole-induced neutropenia (MIN), metamizole has been withdrawn from the market in several countries including Sweden and the USA, but it is still available and increasingly prescribed in other countries such as Switzerland, Germany, and France [5,7]. The mechanism by which metamizole induces

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neutropenia is currently not known and there are no effective strategies to predict or prevent occurrence of MIN. Immunologic mechanisms, including T-cell mediated elimination of neutrophils, have been proposed due to clinical observations such as increased symptom-severity upon re-exposure in some patients [8–10]. Conversely, certain features such as the rapid onset within a few hours after the first few doses in some patients without known previous exposure and the observation that metamizole can also induce bi- or pancytopenia [5] are more compatible with direct toxicity on precursors of neutrophil granulocytes and other hematopoietic cells in the bone marrow. This hypothesis is supported by a recent study revealing cytotoxicity in neutrophil progenitor cells involving electrophilic intermediates of the main metamizole metabolite [11]. Additionally, genetic predisposition has been shown for various neutropenia-inducing drugs such as sulfasalazine and clozapine, which might also be present in MIN [12,13]. It has been shown that neither metamizole dose, nor treatment duration increase the risk of MIN [1]. However, various factors have been suggested to influence the development of MIN, such as co-medication with immunosuppressants, e.g. low dose methotrexate or with non-myelotoxic and non-immunosuppressive drugs, and comorbidities such as infections with hepatitis B or C or HIV [1,4,6,14–16]. However, to prevent MIN, a better understanding of factors associated with increased susceptibility is needed. By excluding at-risk patients from metamizole exposure instead of banning its use overall, metamizole could remain a valuable treatment option e.g. for patients with contra-indications for nonsteroidal anti-inflammatory drugs such as many elderly patients. Hence, the current study aimed to identify differences between patients who had experienced MIN and patients tolerating metamizole treatment without developing any adverse hematological events, which could represent potential susceptibility factors for MIN.

This study is part of an interdisciplinary collaboration investigating direct toxic and immunological mechanisms of MIN as well as genetic factors potentially associated with MIN. The current work describes the findings of the characteristics evaluated during clinical assessment of cases and comparison patients and aims to evaluate the association between potential risk factors and the development of MIN. Results of the toxicological, immunological, and genetic investigations will be published separately.

2. Methods

2.1. Setting and study design

An observational, multi-center comparative study was performed including patients with new-onset neutropenia during metamizole therapy and metamizole-tolerant comparison patients between 2005 and 2017.

The study was performed following the principles of good clinical practice according to the Declaration of Helsinki. The study was approved by the local ethics committee “Ethikkommission Nordwest- und Zentralschweiz” (protocol number EKNZ BASEC 2015–00231). Written informed consent was obtained from all study participants.

2.2. Selection and assessment of MIN cases

In order to identify patients with metamizole-associated neutropenia, two different approaches were used. In a first approach, we screened the available electronic medical records of all wards of the University Hospitals Basel and Bern for the keywords “metamizole”, “Novalgin”, or “Minalgin” in conjunction with “neutropenia”, “agranulocytosis”, or “leucopenia” for the time period between 2005 and 2017. In a second approach, neutropenia cases who were hospitalized during the recruitment period of the study (2016–2018) were reported by the treating physician per email to the study authors or by report to the pharmacovigilance center by the processing physician or pharmacist (Fig. 1).

Included patients were at least 18 years old and developed neutropenia at the earliest one day after the first metamizole intake and at the latest 2 weeks after stopping metamizole intake. Only patients were included where a causal association between neutropenia and metamizole intake was classified at least as “possible” according to the Naranjo score [17]. Patients with idiopathic neutropenia or concomitant use of cytotoxic drugs or immunosuppressants (1 case low dose methotrexate, 2 cases tacrolimus, and 1 case sirolimus) were excluded. A previous analysis of Swiss pharmacovigilance data showed that myelotoxic drugs such as methotrexate increase the risk of MIN [1], which is why we excluded cases with myelotoxic co-medication to focus on yet unknown potential risk factors. Additionally, although non-myelotoxic, immunosuppressants are known to reduce the neutrophil count as ADR [18]. Since only 3 cases were under immunosuppressive co-medication, it would have been not possible to differentiate, whether MIN was caused by metamizole alone or by the combination with the immunosuppressant. Therefore, these cases were excluded from the analysis.

Eligible patients were contacted by mail and by phone. Patients who agreed to participate were invited for a study visit, where they were again informed about the study and provided written informed consent. Neutropenia patients were subdivided into inpatient cases who developed neutropenia in an inpatient setting, or outpatient cases who developed neutropenia in an outpatient setting with subsequent hospitalization.

2.3. Selection and assessment of metamizole tolerant comparison patients

Metamizole-tolerant comparison patients were recruited by contacting local family practitioners and community pharmacies. After asking patients under metamizole treatment with a daily metamizole dose of at least 500 mg for at least 28 consecutive days for consent of potential study participation, the practitioners and pharmacists forwarded the patients' contact information to the study authors. Additionally, some control patients contacted the authors after receiving a study information letter from their practitioner or pharmacist. Tolerant comparison patients, who agreed to participate, were invited for a study visit identically to case patients, where they were informed about the study and provided written informed consent. Good clinical drug tolerability was confirmed by the absence of fever, sore throat, or mucositis during metamizole treatment. Additionally, tolerant comparison patients were required to have a medical history without any drug-related hematological complications.

2.4. Covariates

During the study visit, demographic data (age, body mass index (BMI), ethnicity), hypersensitivity reactions (“allergy”) to drugs, concomitant diseases, co-medication, dose, duration, route, and frequency of metamizole intake of cases and comparison patients were recorded identically for cases and comparison patients by interview. Based on dosage information and medical records if available, daily dose, cumulative dose, and treatment duration were calculated. For neutropenia cases, latency until diagnosis of neutropenia, as well as duration of neutropenia were calculated. In case metamizole dosage regimen or latency could not be assessed with certainty, a minimal daily dose of 500 mg or a minimal latency of one day of treatment were assumed (n = 10 of 48 cases). Laboratory values and treatment of neutropenia were obtained from the patients' inpatient medical records. Co-medication of drugs with known association with neutropenia (level 1 and 2 evidence according to Andersohn et al.) was taken into consideration when assessing adverse drug reaction (ADR) causality [16]. Blood counts consisting of neutrophil and platelet counts and hemoglobin values were recorded at the nadir of neutropenia. Neutropenia was defined as a neutrophil count below 1.5×10^9/L. Thrombocytopenia was defined as a thrombocyte count below

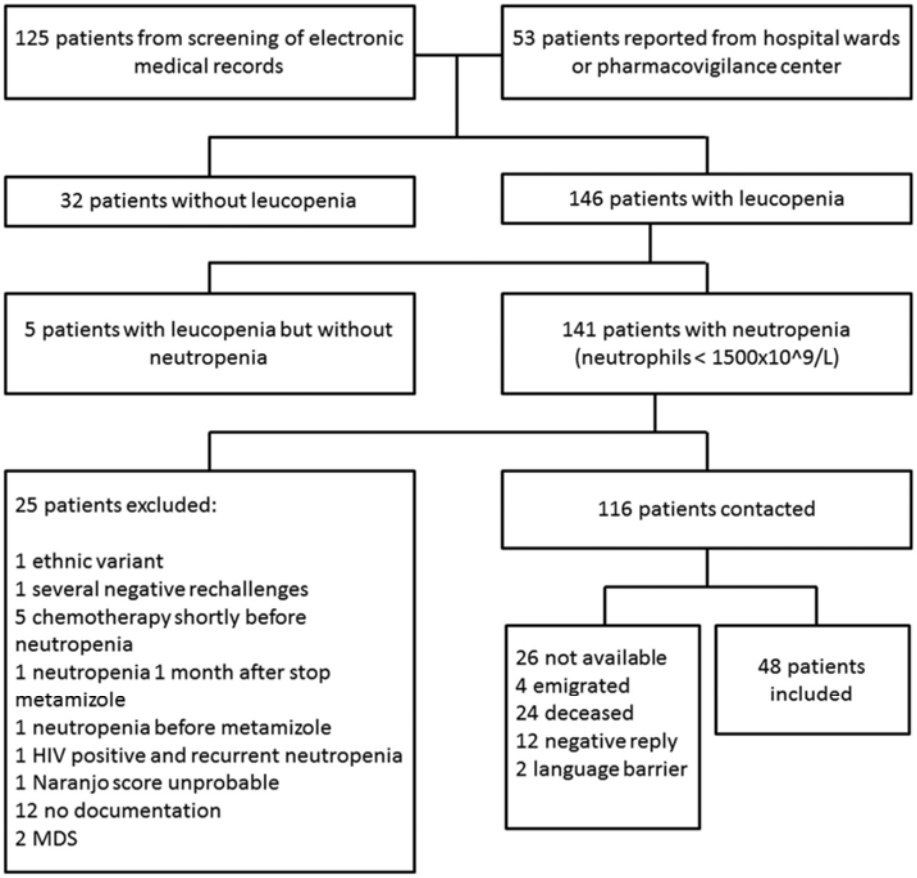

Fig. 1. Selection process of case patient recruitment.

150×10^9/L, and anemia as a hemoglobin concentration below 120 g/L and 140 g/L for women and men, respectively. For tolerant comparison patients, hematological laboratory values during metamizole treatment were evaluated if available. Additionally, a full blood count was obtained on the day of the study visit to confirm normal blood values.

Since immunologic reactions may be involved in metamizole-associated neutropenia, the allergy history of cases and comparison patients was obtained and grouped into probably T-cell mediated (delayed), anaphylactic, or non-specific allergic reactions to any drug. Details about possible T-cell mediated allergies or anaphylactic/anaphylactoid reactions were extracted from patient charts and patient history. Comorbidities were grouped according to their potential to affect neutrophil counts into chronic infections, autoimmune diseases, and other comorbidities (i.e. cardiovascular and metabolic diseases, renal insufficiency, psychiatric disorders and others).

2.5. Statistical analysis

We summarized the frequency distribution of continuous covariates and present normally distributed parameters as arithmetic mean (and standard deviation) and not normally distributed parameters as median (and range). Between-group comparisons were done using *t*-tests (for $n = 25$) and Fisher exact tests (when $n < 25$) for normally distributed variables and Wilcoxon signed rank tests for not normally distributed variables. Normality was assessed graphically. Microsoft Office Excel (Version 2010, Redmond, Washington, USA) and GraphPad PRISM (Version 7, La Jolla, California, USA) were used for analyses. Differences were considered as statistically significant when $p < .05$.

We conducted an explorative (hypothesis generating) comparative analysis using multivariable logistic regression analysis to compute odds ratios (OR) with 95% confidence intervals (CI). To increase homogeneity of study groups, we excluded all patients who had developed neutropenia in an inpatient setting, because control patients were recruited from an outpatient setting only. We compared the prevalence of potential risk factors for neutropenia between cases and comparison patients. Evaluated risk factors were considered based on their potential to influence development of neutropenia reported in previous studies. These included daily metamizole dose (< 1500 mg or ≥ 1500 mg), co-medication with level 1 evidence and level 2 evidence according to Andersohn et al., and two composite covariates including 1) autoimmune diseases and drug allergies and 2) cardiovascular and metabolic diseases (including hypertension, hypertensive cardiopathy, diabetes, and hypercholesterinemia). Covariates were dichotomized due to the limited number of included neutropenia cases ($n = 25$). We were not able to analyze acute infections and age as potential risk factors of MIN due to potential bias by the underlying indication for metamizole treatment. Several cases received short-term metamizole treatment for fever or pain during an acute infection, whereas tolerant comparison patients were required a minimum treatment duration with metamizole of 28 days, which inherently excludes patients with short-term therapy and over-samples controls with chronic diseases and long-term metamizole therapy. Multivariable logistic regression analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC).

3. Results

3.1. Patients and metamizole treatment

Forty-eight patients with MIN managed at the University Hospital Basel and the University Hospital Bern between 2005 and 2017 and 39

Table 1
Patient and comparison subject characteristics.

	All cases (n = 48)	Inpatient cases (n = 23)	Outpatient cases (n = 25)	Tolerant comparison patients (n = 39)	p-value
Age (years) at neutropenia diagnosis (cases), or at last metamizole intake (comparison patients); median (range)	44 (17–77)	50 (22–77)	35 (17–76)	60 (19–91)	0.0007[a]
Women; N (%)	24 (50.0)	11 (48.0)	13 (52.0)	21 (53.8)	> 0.9999[b]
Body mass index in kg/m2; median (range)	24 (18–47)	23 (18–37)	24 (19–47)	28 (16–39)	0.0320[a]
Ethnicity; N (%)					
Caucasian	47 (97.9)	22 (95.7)	25 (100)	37 (94.9)	0.5164[b]
Other	1 (2.1)	1 (4.3)	0 (0)	2 (5)	

[a] Wilcoxon signed rank test between outpatient cases and tolerant comparison patients
[b] Fishers exact test between outpatient cases and tolerant comparison patients.

Table 2
Metamizole treatment.

	All cases (n = 48)	Inpatient cases (n = 23)	Outpatient cases (n = 25)	Tolerant comparison patients (n = 39)	p-value
Dosage (g/day); mean (SD)	1.78 (1.26)	1.91 (1.23)	1.67 (1.31)	1.64 (1.06)	0.9384[d]
Cumulative dose (g); median (range)	21 (0.5–859)	26 (0.5–196)	18 (0.5–859)	214 (23.5–13'149)	< 0.0001[e]
Latency time[a]/ treatment duration[b](days); median (range)	12 (1–204)	6 (1–61)	19 (2–204)	177 (30–5480)	< 0.0001[e]
Latency time[a]/ treatment duration[b]categorized: Patients N (%)					
1–7 days	19 (40)	12 (52)	7 (28)	n/a	
8–30 days[a]/ 28–30 days[b]	18 (38)	9 (39)	9 (36)	2 (5)	
31–60 days	8 (17)	1 (4)	7 (28)	9 (23)	
61–120 days	2 (4)	1 (4)	1 (8)	5 (13)	
121–180 days	0 (0)	0 (0)	0 (0)	4 (10)	
181–365 days	1 (2)	0 (0)	1 (4)	4 (10)	
> 365 days	0 (0)	0 (0)	1 (4)	15 (38)	
Route of application; N (%)					0.0594[f]
Oral	82	62	100	95	
Intravenous[c]	18	38	0	5	

[a] Cases.
[b] Comparison patients.
[c] Initial intravenous administration followed by per oral administration.
[d] t-test between outpatient cases and tolerant comparison patients.
[e] Wilcoxon signed rank test between outpatient cases and tolerant comparison patients.
[f] Fishers exact test between outpatient cases and tolerant comparison patients.

metamizole tolerant control patients were included. Characteristics of neutropenia patients and control subjects are summarized in Table 1. The median age of neutropenia cases was 44 years (17–77 years) and of tolerant comparison patients was 60 years (19–91 years). The majority of cases and comparison patients (> 90%) were Caucasian, reducing genetic variability of the results. The application route for metamizole was predominantly oral among tolerant comparison patients (95%) and cases (100% in outpatient and 62% in inpatient cases, Table. 2). Mean daily metamizole dose did not differ significantly between cases and tolerant comparison patients and was within the recommended maximum dose of 4 g per day (Table. 2).

The median latency between first intake of metamizole and neutropenia diagnosis among outpatient and inpatient cases was 19 and 6 days, respectively. Among all cases, 40% received metamizole for < 7 days and 38% for 8 to 30 days. Latency between 31 and 60 days and longer than 60 days was observed in 17% and 6% of cases, respectively. By definition of the study inclusion criteria, tolerant control patients took metamizole for at least 28 days. The median treatment duration in the tolerant comparison group was almost six months, with more than one third taking metamizole for more than one year.

3.2. Metamizole-associated neutropenia

According to the Naranjo scale, none of the cases met the criteria for a "definite" causality for metamizole and neutropenia (Table. 3). In half of the cases, causality was formally assessed as "probable" and the other half as "possible". Over two thirds of all included cases experienced agranulocytosis, i.e. a drop in neutrophils below 0.5×10^9 cells per liter (90% among outpatient cases and 40% among inpatient cases).

Ten patients (all inpatient cases) had a known rechallenge with metamizole, of whom 2 experienced a new episode of neutropenia whereas 5 cases had a negative rechallenge. For 3 patients, the outcome of the rechallenge was unknown. Table 4 shows that anemia was present in 19 (49%) tolerant comparison patients and in over half of these comparison patients anemia was either present before the start of metamizole treatment or could be attributed to other causes than metamizole. In contrast, 39 cases (81%) had anemia at neutropenia diagnosis and thereof almost half was of unknown cause. There was no difference between inpatient and outpatient cases.

Thrombocytopenia was less frequent among tolerant comparison patients (13%) than among cases (33%). In 80% of the tolerant comparison patients, thrombocytopenia was either already present before the start of metamizole treatment or had a known cause, whereas the cause for thrombocytopenia was only known for two of the inpatient cases and for none of the outpatient cases.

None of the cases had new onset pancytopenia, but in 33% of cases, cell counts were reduced in all three cell lines at the time of neutropenia diagnosis.

3.3. Preexisting drug-related allergies

As shown in Table 5, evidence for T-cell mediated allergies was only

Table 3
Characteristics of neutropenia.

	All cases (n = 48)	Inpatient cases (n = 23)	Outpatient cases (n = 25)
Naranjo Causality assessment; N (%)			
Definite (> 9)	0 (0)	0 (0)	0 (0)
Probable (5–8)	25 (52)	8 (35)	17 (68)
Possible (1–4)	23 (48)	15 (65)	8 (32)
Rechallenge; N (%)			
Known positive rechallenge	2 (4)	2 (9)	0 (0)
Known negative rechallenge	5 (10)	5 (22)	0 (0)
Rechallenge with unkown outcome	3 (6)	3 (13)	0 (0)
No known rechallenge	38 (79)	13 (57)	25 (100)
ADR duration in days; median (range)	5 (1–15)	3 (1–8)	7 (1–15)
Severity of neutropenia (n = 48 + 1)[a]; N (%)			
Neutrophils 1–1.5 × 10^9/l	4 (8)	4 (17)	0 (0)
Neutrophils 0.5–1 × 10^9/l	13 (27)	11 (46)	2 (8)
Neutrophils < 0.5 × 10^9/l	32 (65)	10 (42)	23 (92)

[a] One case with positive rechallenge and recorded blood values.

found in one neutropenia case and in no tolerant control. One inpatient case and one tolerant control each had a history of anaphylactic/anaphylactoid reaction to drugs (penicillin, diclofenac). In addition, one third of the tolerant comparison patients had experienced unspecific drug rashes compared to approximately 20% and 10% in the inpatient and outpatient cases, respectively.

3.4. Comorbidities

Half of the cases had experienced an infection shortly before or at diagnosis of neutropenia whereas only one quarter of the tolerant comparison patients reported a possible infection during metamizole treatment (Table 5). Eight cases with infection received metamizole to treat fever and/or pain related to the infection. There was a higher proportion of chronic comorbidities among inpatient than among outpatient cases. Self-reported autoimmune diseases were present in 8% and 15% of cases and tolerant comparison patients, respectively. Three quarters of case patients with an autoimmune disease were inpatient cases. Other diseases without any expected effect on the neutrophil count were most prevalent in the tolerant comparison group.

3.5. Co-medication

The median number of co-medications per patient was higher among the tolerant comparison group compared with all neutropenia cases (Table 5). Inpatient cases received twice as many co-medications during metamizole treatment compared with outpatient cases.

Besides metamizole, various drugs have been associated with neutropenia. According to the inclusion criteria, none of the cases had received myelotoxic or immunosuppressive co-medications. Among the tolerant comparison patients, one patient was under once weekly methotrexate treatment (10 mg/ week) and his blood values were within the normal range.

Certain non-myelotoxic and non-immunosuppressive medications have also been associated with neutropenia. Over 40% of cases and tolerant comparison patients had at least one co-medication with evidence 1 according to the classification after Andersohn et al. [16]. However, 13% of tolerant comparison patients had 2 or more evidence 1 drugs compared to 2% among cases. Inpatient cases received more evidence 1 co-medications than outpatient cases. Prevalence of drugs with evidence 2 was slightly higher among cases compared to tolerant comparison patients. Evidence 2 co-medication was twice as frequent among inpatient cases compared with outpatient cases (Table 5).

3.6. Multivariable comparison of cases and tolerant comparison patients

One third of outpatient cases and almost half of tolerant comparison patients received a median daily metamizole dose of 1.5 g or higher. The corresponding adjusted OR revealed no association of metamizole dose and the risk of neutropenia (OR 1.24, 95% CI 0.35–4.33). After multivariable adjustment, the adjusted OR for neutropenia in association with autoimmune diseases and/or any previous allergic reaction to drugs was 0.22 (95% CI 0.06–0.81). Cardiovascular and/or metabolic diseases were twice as frequent among tolerant comparison patients compared with outpatient cases. Thus, the corresponding adjusted OR revealed no association of cardiovascular and/or metabolic diseases and the risk of neutropenia (OR 0.58, 95% CI 0.18–1.84). Half of the tolerant control patients and 40% of outpatient cases had at least one co-medication with drugs associated with neutropenia of evidence level 1. Similarly, co-treatment with drugs associated with neutropenia of evidence level 2 was reported by half of the tolerant control patients and 36% of outpatient cases. The corresponding adjusted ORs showed no association between evidence level 1 (OR 0.43, 95% CI 0.13–1.41) or evidence level 2 (OR 0.54, 95% CI 0.18–1.64) co-medication and MIN (Table 6).

Table 4
Additional blood values.

	All cases (n = 48)	Inpatient cases (n = 23)	Outpatient cases (n = 25)	Tolerant comparison patients (n = 39)
Other affected cell lines; N (%)				
Anemia (w: < 120 g/L; m: < 140 g/L)	39 (81)	19 (83)	20 (80)	19 (49)
Anemia with known cause or preexisting	17 (35)	8 (35)	9 (36)	10 (26)
Thrombocytopenia (< 150 × 10^9/L)	16 (33)	9 (39)	7 (28)	5 (13)
Thrombocytopenia with known cause or preexisting	2 (4)	2 (9)	0 (0)	4 (10)
New onset pancytopenia[a]	0 (0)	0 (0)	0 (0)	0 (0)
Pancytopenia at presentation[b]	16 (33)	10 (43)	6 (24)	0 (0)

[a] New onset after metamizole treatment.
[b] With known cause or preexisting anemia or thrombocytopenia.

Table 5
Health characteristics.

	All cases (n = 48)	Inpatient cases (n = 23)	Outpatient cases (n = 25)	Tolerant comparison patients (n = 39)	p-value
Allergies to drugs; N (%)					
T-cell mediated[a]	1 (2)	1 (4)	0 (0)	0 (0)	> 0.9999[e]
Anaphylactic reactions to drugs	1 (2)	1 (4)	0 (0)	1 (3)	> 0.9999[e]
Drug rash, non-specified	8 (17)	5 (22)	3 (12)	12 (31)	0.1304[e]
Comorbidities; N (%)					
Infections[b]	23 (48)	11 (48)	12 (48)	10 (26)	0.1048[e]
Acute infections	22 (46)	10 (43)	12 (48)	9 (23)	0.0563[e]
Chronic infections	4 (8)	3 (13)	1 (4)	1 (3)	1.0000[e]
Preexisting auto-immune diseases	4 (8)	3 (13)	1 (4)	6 (15)	0.2313[e]
Cardiovascular diseases	12 (25)	5 (22)	7 (28)	14 (36)	0.5919[e]
Metabolic diseases	4 (8)	3 (13)	1 (4)	13 (33)	0.0055[e]
Psychiatric disorders	3 (6)	1 (4)	2 (8)	4 (10)	1.0000[e]
Renal insufficiency	4 (8)	1 (4)	3 (12)	0 (0)	0.0552[e]
Other	10 (21)	3 (13)	7 (28)	14 (36)	0.5919[e]
Co-medication					
Median number of medications per subject (range)	5 (0 – 20)	7 (0–14)	3 (0–20)	8 (0–20)	< 0.0001[h]
N (%) of patients with evidence 1 co-medication[c]					0.6627[e]
1 evidence 1 drug	20 (42)	11 (48)	9 (36)	16 (41)	
2 evidence 1 drugs	1 (2)	0 (0)	1 (4)	4 (10)	
> 2 evidence 1 drugs	0 (0)	0 (0)	0 (0)	1 (3)	
N (%) of patients with evidence 2 co-medication[d]					0.5732[e]
1 evidence 2 drug	21 (44)	13 (57)	8 (32)	15 (38)	
2 evidence 2 drugs	9 (19)	8 (35)	1 (4)	5 (13)	
> 2 evidence 2 drugs	1 (2)	1 (4)	0	1 (3)	

[a] According to patient chart/detailed patient history.
[b] Several infections per patient counted as one infection.
[c] Evidence 1: At least 1 ADR report with a definite relationship.
[d] Evidence 2: At least 1 ADR report with a probable relationship but no report with a definite relationship.
[e] Fishers exact test between outpatient cases and tolerant comparison patients
[h] Wilcoxon signed rank test between outpatient cases and tolerant comparison patients.

4. Discussion

In the current retrospective observational comparative study, patients with autoimmune diseases or previous allergic reactions to drugs were not at increased risk of MIN. Additionally, co-medication with non-myelotoxic and non-immunosuppressive drugs, which have previously been associated with neutropenia [16], were not associated with an increased risk of MIN.

A possible involvement of the immune system in the development of MIN has previously been suggested [19,20]. Patients who had previously experienced an immune-mediated reaction to drugs might be more susceptible to also react to other drugs. Accordingly, patients with autoimmune diseases might have a more reactive immune system, which could also react to xenobiotics. However, in the current study, autoimmune diseases and previous immune-mediated reactions to drugs were more frequent among tolerant comparison patients than in neutropenia cases, which may be due to the older age of the tolerant comparison patients. Our results therefore suggest that autoimmune diseases and prior immune mediated reactions are not a key risk factor for developing MIN. In a previous study investigating risk factors for metamizole induced leucopenia [4], history of medication allergies has been associated with an increased risk for leucopenia after metamizole treatment. This divergence to the current study may be attributable due to the different data collection methods. In the current study, patients' drug allergy history was assessed by personal interviews, whereas in the previous retrospective study, data were collected from medical records only, introducing a risk of reporting bias.

Regarding co-medication, also non-myelotoxic and non-immunosuppressive drugs have been associated with the development of neutropenia [16] and may facilitate the development of MIN or even be the main cause for neutropenia in patients treated with metamizole. However, in the current study, administration of drugs associated with neutropenia was more frequent among tolerant comparison patients compared with neutropenia cases and was not associated with increased risk for MIN after multivariable adjusting. This is in agreement with a previous study, which did not find evidence that co-medication with non-myelotoxic and non-immunosuppressive drugs is a potential risk factor for MIN [4].

Table 6
Multivariable comparative analysis of neutropenia risk factors.

	Outpatient cases (n = 25)	Tolerant comparison patients (n = 39)	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Risk factor; N (%)				
Metamizole dose > 1.5 gramm	8 (32)	18 (46)	1.19 (0.42–3.35)	1.24 (0.35–4.33)
Autoimmune disease and/or any drug related allergic reaction	4 (16)	19 (49)	0.25 (0.07–0.85)	0.22 (0.06–0.81)
Cardiovascular and/or metabolic disease	8 (32)	27 (69)	0.50 (0.17–1.41)	0.58 (0.18–1.84)
Evidence level 1 co-medication	10 (40)	21 (54)	0.57 (0.21–1.58)	0.43 (0.13–1.41)
Evidence level 2 co-medication	9 (36)	21 (54)	0.59 (0.21–1.66)	0.54 (0.18–1.64)

Previous retrospective studies investigating MIN identified concomitant use of myelotoxic and immunosuppressive drugs as potential risk factor [1,4]. Since myelotoxic and immunosuppressive drugs themselves can cause neutropenia or lead to a decrease in circulating neutrophils, a high number of patients and comparison patients treated with such drugs would be needed in order to differentiate whether neutropenia was caused by metamizole, the myelotoxic or immunosuppressive co-medication, or the combination of both. Since this was not the case in the current study, we excluded patients with any myelotoxic or immunosuppressive treatment, which allowed searching for other potentially involved factors.

A previous analysis of Swiss pharmacovigilance data showed that only few patients developed neutropenia after > 28 days of metamizole treatment [1]. Therefore, tolerant comparison patients included in the current study were required to be on metamizole treatment for at least 28 days. Previous studies reported contradictory findings regarding the latency time of MIN. The median latency time of 12 days among all cases is within the latency time period of 7 to 14 days reported by Swiss pharmacovigilance data [1]. However, it is longer than the 2 days reported in a review of Andersohn et al., describing latency times of various non-chemotherapy drugs [16]. Interestingly, outpatient cases in our study had a longer median latency compared to inpatient cases. This difference may be due to the predominantly intermittent metamizole use among outpatient cases compared to the regular daily treatment in-hospital. Alternatively, the shorter latency time among inpatient cases might reflect the closer surveillance of hospital patients where a drop in the neutrophil count is detected earlier than in outpatient cases with less frequent blood counts. This could also explain the higher degree of neutropenia severity among outpatient cases, reflecting the later detection of the neutropenia and thus later metamizole discontinuation. Similar observations of lower neutrophil counts in outpatient cases compared to inpatient cases had been reported in a German case-control surveillance study [21]. In accordance with the more pronounced severity of neutropenia, the ADR duration of 7 days was longer among outpatient cases compared to 3 days in inpatient cases in our study, reflecting the time needed to restore a sufficient amount of circulating mature neutrophils from the bone marrow [22]. This is in agreement with reports of Andersohn et al., who investigated agranulocytosis and reported a median ADR duration of 8 days. In the current study, the median ADR duration of all cases was 5 days, which is similar to previously reported findings of Swiss, German and Swedish studies investigating MIN [4,5,23].

4.1. Limitations

The observations in the current study are mainly limited by the small sample size, a consequence of the rarity of MIN, as well as the difficulty of recruiting a larger number of tolerant comparison patients, who had metamizole treatment for at least 28 consecutive days. Several potential risk factors could therefore not be evaluated. The required minimum treatment duration of 28 days for metamizole tolerant comparison patients, but not for cases, resulted in different indications for metamizole treatment in cases and comparison patients. While acute diseases such as infections, pain, menstrual cramps, and migraine were predominant indications in cases, chronic diseases such as chronic lumbar spine syndrome, gout, and fibromyalgia were the most frequent indications in comparison patients. This difference between cases and comparison patients prevented the analysis of acute infections and age as potential risk factors of MIN, because they are associated with the underlying indication and would thus have resulted in biased effect estimates. Prior studies reported an increased ADR risk for other drugs in the presence of viral infections [24]. Two of the neutropenia cases had an HIV infection and one neutropenia patient had an ongoing chronic hepatitis C infection, but all with normal neutrophil counts before and after metamizole treatment. Taking into account these observations, it would be interesting to investigate the impact of viral, bacterial, and fungal infections on the risk to develop MIN. WHO and Swiss spontaneous safety reports mentioned more metamizole-associated hematological disorders in elderly people (median age close to 60 years), but without data on metamizole exposure of the specific age groups [1]. Since the median age of neutropenia cases in the current study was distinctly lower, further studies on the influence of age on the risk of MIN would be interesting. Further, the included patients represent those, who were willing to participate in the study and in case of the neutropenia patients, who survived the adverse drug reaction. The inclusion of cases with not only a “probable” but also a “possible” association according to the Naranjo scale could have led to the inclusion of a number of cases where metamizole was not the main cause of neutropenia. The recruitment of inpatient cases and tolerant comparison patients in different settings limited a direct comparison, which is why we only compared cases who developed neutropenia in an outpatient setting with tolerant comparison patients in the multivariable regression analysis. However, we cannot rule out residual selection bias due to different recruitment channels for cases and comparison patients.

5. Conclusion

Our results suggest that neither autoimmune diseases, history of drug allergies, nor concomitant treatment with drugs associated with neutropenia of evidence level 1 or evidence level 2 are associated with an increased individual risk of metamizole-associated neutropenia.

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Declaration of Competing Interest

None of the authors has a conflict of interest regarding this study.

References

[1] Blaser LS, Tramonti A, Egger P, Haschke M, Krahenbuhl S, Ratz Bravo AE. Hematological safety of metamizole: retrospective analysis of WHO and Swiss spontaneous safety reports. Eur J Clin Pharmacol 2015;71(2):209–17.
[2] Sanchez S, Alarcon de la Lastra C, Ortiz P, Motilva V, Martin MJ. Gastrointestinal tolerability of metamizol, acetaminophen, and diclofenac in subchronic treatment in rats. Dig Dis Sci 2002;47(12):2791–8.
[3] Andrade SE, Martinez C, Walker AM. Comparative safety evaluation of non-narcotic analgesics. J Clin Epidemiol 1998;51(12):1357–65.
[4] Blaser L, Hassna H, Hofmann S, Holbro A, Haschke M, Ratz Bravo A, et al. Leucopenia associated with metamizole: a case-control study. Swiss Med Wkly 2017;147:w14438.
[5] Hedenmalm K, Spigset O. Agranulocytosis and other blood dyscrasias associated with dipyrone (metamizole). Eur J Clin Pharmacol 2002;58(4):265–74.
[6] Ibanez L, Vidal X, Ballarin E, Laporte JR. Agranulocytosis associated with dipyrone (metamizol). Eur J Clin Pharmacol 2005;60(11):821–9.
[7] Andrade S, Bartels DB, Lange R, Sandford L, Gurwitz J. Safety of metamizole: a systematic review of the literature. J Clin Pharm Ther 2016;41(5):459–77.
[8] Andres E, Dali-Youcef N, Serraj K, Zimmer J. Recognition and management of drug-induced cytopenias: the example of idiosyncratic drug-induced thrombocytopenia. Expert Opin Drug Saf 2009;8(2):183–90.
[9] Uetrecht JP. Reactive metabolites and agranulocytosis. Eur J Haematol Suppl 1996;60:83–8.
[10] Claas FH. Immune mechanisms leading to drug-induced blood dyscrasias. Eur J Haematol Suppl 1996;60:64–8.
[11] Rudin D, Lanzilotto A, Bachmann F, Housecroft CE, Constable EC, Drewe J, et al. Non-immunological toxicological mechanisms of metamizole-associated neutropenia in HL60 cells. Biochem Pharmacol 2019;163:345–56. https://doi.org/10.1016/j.bcp.2019.01.011.

[12] Wadelius M, Eriksson N, Kreutz R, Bondon-Guitton E, Ibanez L, Carvajal A, et al. Sulfasalazine-induced agranulocytosis is associated with the human leukocyte antigen locus. Clin Pharmacol Ther 2018;103(5):843–53.
[13] Legge SE, Hamshere ML, Ripke S, Pardinas AF, Goldstein JI, Rees E, et al. Genome-wide common and rare variant analysis provides novel insights into clozapine-associated neutropenia. Mol Psychiatry 2017;22(10):1502–8.
[14] Duval X, Journot V, Leport C, Chene G, Dupon M, Cuzin L, et al. Incidence of and risk factors for adverse drug reactions in a prospective cohort of HIV-infected adults initiating protease inhibitor-containing therapy. Clin Infect Dis 2004;39(2):248–55.
[15] Guitton E, Montastruc JL, Lapeyre-Mestre M. Influence of HCV or HBV coinfection on adverse drug reactions to antiretroviral drugs in HIV patients. Eur J Clin Pharmacol 2006;62(3):243–9.
[16] Andersohn F, Konzen C, Garbe E. Systematic review: agranulocytosis induced by nonchemotherapy drugs. Ann Intern Med 2007;146(9):657–65.
[17] Naranjo CA, Busto U, Sellers EM, Sandor P, Ruiz I, Roberts EA, et al. A method for estimating the probability of adverse drug reactions. Clin Pharmacol Ther 1981;30(2):239–45.
[18] Documed. Swiss product information online. updated 17 Jan 2018. Available from http://www.compendium.ch; 2017.
[19] Lampl C, Likar R. Metamizol: Wirkmechanismen, Interaktionen und Agranulozytoserisiko. Der Schmerz 2014;28(6):584–90.
[20] Nikolova I, Tencheva J, Voinikov J, Petkova V, Benbasat N, Danchev N. Metamizole: a review profile of a well-known "forgotten" drug. Part I: pharmaceutical and nonclinical profile. Biotechnol Biotechnol Equip 2012;26(6):3329–37.
[21] Huber M, Andersohn F, Sarganas G, Bronder E, Klimpel A, Thomae M, et al. Metamizole-induced agranulocytosis revisited: results from the prospective Berlin Case-Control Surveillance Study. Eur J Clin Pharmacol 2015;71(2):219–27.
[22] Boxer L, Dale DC. Neutropenia: causes and consequences. Semin Hematol 2002;39(2):75–81.
[23] Stammschulte T, Ludwig WD, Muhlbauer B, Bronder E, Gundert-Remy U. Metamizole (dipyrone)-associated agranulocytosis. an analysis of German spontaneous reports 1990-2012. Eur J Clin Pharmacol 2015;71(9):1129–38.
[24] Levy M. Role of viral infections in the induction of adverse drug reactions. Drug Saf 1997;16(1):1–8.

5. DISCUSSION

In several assays elucidating the cytotoxic potential of metamizole and its metabolites, the main metabolite MAA was showing unexpected characteristics. While no cytotoxicity was detectable at pharmacological concentrations, MAA improved the viability of the treated cells compared to untreated cells. Especially under stress-inducing conditions, when cells are treated with hydrogen peroxide for example, MAA increased the cell viability by its radical scavenging characteristic. This circumstance first hampered the process to find a cytotoxic mechanism of metamizole-associated neutropenia, but then also revealed a potential mechanism, how MAA could elicit cytotoxicity. While detoxifying radicals by donating protons or adsorbing electrons, radical scavengers themselves form intermediate radicals, which normally are immediately further degraded to unreactive compounds (Fujii et al. 2011; Valko et al. 2006). But in presence of a highly oxidative compound, radical scavengers cannot regenerate their antioxidative properties and turn themselves into reactive electrophilic intermediates (Valko et al. 2006). I have shown that this is the case for MAA when combined with hemin. Heme is part of hemoglobin and myoglobin where it carries oxygen as well as part of several enzymes, which use heme as active site to perform electron-transfer, oxygen delivery, catalysis, and signaling (Kumar and Bandyopadhyay 2005; Yan et al. 2017). In my studies, heme or hemin did not react with MAA when they were bound inside of hemoglobin or methemoglobin, respectively. The globin chains seemed to shield the iron ions strong enough so that MAA was not able to get in contact with the iron to react. Heme or hemin can be released in case of hemoglobin or methemoglobin degradation. Heme and hemin normally do not occur freely since they damage lipids, proteins, and DNA through reactive oxygen species (ROS) generation (Wagener et al. 2001). Free heme is avoided by immediate further degradation by heme-oxygenase (Chung et al. 2012; Kumar and Bandyopadhyay 2005). But in case of excess erythrocyte degradation during hemolysis, the heme degrading system is overcharged and hemoglobin undergoes accelerated oxidation and oxidative changes leading to heme loss (Buehler et al. 2011). Free heme is readily oxidized to hemin and is able to enter neutrophils and their precursors, where it can react with MAA to form reactive MAA intermediates. Since heme is also often contained in enzymes, most cells, including neutrophils, produce heme to incorporate it into their hemoproteins (Hentze et al. 2010). Hence, heme is naturally present in neutrophils and neutrophil precursor cells and could in case of impaired heme metabolism inside the cells accumulate and potentially react with MAA, thereby forming reactive intermediates leading to neutropenia. The iron ion of hemin is bound to four pyrrole nitrogens resulting in an open axial coordination site that can bind oxygen (Ryu et al.

2016). Its reversible redox characteristics enable rapid electron transfer to and from these iron ions (Que Jr and Tolman 2008). Hence, one hemin molecule can oxidize several other compounds, which reflects its high oxidative capacity. In case of cytotoxicity assays described within this thesis, eight times less hemin than MAA was enough to produce sufficient MAA electrophilic intermediates to induce apoptosis in HL60 cells. Hemin itself showed a concentration dependent cytotoxicity, which was strongly amplified in combination with MAA. Then again, MAA-hemin cytotoxicity showed different characteristics than hemin cytotoxicity, especially in differentiating HL60 cells. Hemin cytotoxicity was detectable up to higher differentiation stages than MAA-hemin cytotoxicity, indicating a different mechanism leading to apoptosis. Especially the observed marginally mitochondrial toxicity could all be contributed to hemin, which was potentially due to ROS production (Kumar and Bandyopadhyay 2005). The different maturation stages of cells investigated within this thesis showed striking differences regarding their resistance not only against MAA-hemin, but also when treated with hemin alone or the oxidant hydrogen peroxide. Compared to other immune cells, neutrophils have a very short life-span in the circulation (Boxer and Dale 2002). Therefore, neutrophils do not need to maintain higher cellular functions, which enable these cells to invest everything into defense systems instead of survival mechanisms. In contrast, immature neutrophils have more days of existence ahead and are still developing their cellular properties, which need more energy and resources from outside. This renders immature cells more susceptible for external and internal damaging influences. During myeloid differentiation from myeloblasts to mature neutrophils, which takes around 10 to 16 days *in vivo*, the cells undergo drastic morphologic, metabolic and enzymatic changes (Boxer and Dale 2002; Boyunaga et al. 2010; Jacque et al. 2015). In view of the final purpose of neutrophils, the protection of the organism against invading pathogens by production of highly oxidative compounds, neutrophils need a resilient system to protect themselves from their own weapons. Hence, neutrophils possess a strong antioxidative defense system able to degrade high concentrations of hydrogen peroxide (Bardoel et al. 2014; Splettstoesser and Schuff-Werner 2002). In the second *in vitro* study of this thesis, I have shown that the antioxidative defense system is significantly less pronounced in promyelocytic HL60 cells. This difference explains the observed susceptibility of HL60 cells to MAA-hemin as well as to hydrogen peroxide, which was not detectable in mature neutrophils. Besides the antioxidative defense, also the heme-metabolizing capacity increases during neutrophil maturation. Hence, the cells are able to degrade hemin faster and thus to interrupt potential radical formation with MAA. This is of importance since, as mentioned above; one hemin molecule can react with several MAA molecules. Thus, fewer MAA radicals can be formed and damage the cells. It cannot be distinguished, whether the antioxidative defense or the heme-metabolizing capacity is more

important in the observed resistance of higher differentiated cells. It is also likely that both mechanisms synergistically are responsible for the changed susceptibility.

During infections, immune cells are recruited and activated. This leads to metabolic changes within the cells that potentially increase their susceptibility to MAA-hemin. Neutropenia patients of our cohort often also showed decreased lymphocyte counts. This has also been described for other drug-induced neutropenia (Tesfa et al. 2009), supporting the theory that metabolic changes and/or increased hemolysis due to infections could have an impact on cellular susceptibility to MAA-hemin. In contrast to mature neutrophils, mature lymphocytes predominantly use oxidative phosphorylation for their ATP generation (Kramer et al. 2014). During infections, when lymphocytes are activated, they undergo a metabolic switch from oxidative phosphorylation to glycolysis (Pearce and Pearce 2013). This might turn lymphocytes more susceptible to metamizole toxicity. Mature neutrophils only rely on glycolysis for ATP generation, but in contrast to lymphocytes, they possess a stronger antioxidative capacity due to their own ROS production (Chacko et al. 2013; Kramer et al. 2014). Immature neutrophils also predominantly generate ATP by glycolysis but have a weaker antioxidative defense and hemin-metabolism capacity (Boyunaga et al. 2010). Hence, MAA-hemin cytotoxicity might affect cellular glycolysis leading to impaired ATP generation followed by cell death. In case there are sufficient defense mechanisms to detoxify formed MAA radicals, the cellular ATP generation will not be affected, and cell viability is maintained. Moreover, infections can lead to increased hemolysis (Berkowitz 1991), making more hemin available to react with MAA. Hemin can be taken up into the neutrophil precursor cells or, as mentioned before, be produced within the cells themselves. Furthermore, acute infections stimulate granulopoiesis within the bone marrow, thereby increasing the number of neutrophils in the circulation to limit the infection and to replenish the depleted progenitor populations in the bone marrow (Glatman Zaretsky et al. 2014). Thus, bone marrow and progenitor cell metabolism are increased, which could lead to higher susceptibility to reactive MAA intermediates. Since hemin, but not hydrogen peroxide, was able to form enough reactive MAA intermediates *in vitro*, it is possible that a constant source of oxidants is needed. This could be hemin, which can react with several MAA molecules, or continuously produced oxidizing free radicals. During oxidative burst due to inflammatory processes, immune cells produce superoxide anions and nitric oxide, which may react to even more reactive peroxynitrite anions (Carr et al. 2000). Peroxinitrite can cause DNA fragmentation and lipid peroxidation and if continuously produced, peroxinitirite potentially could oxidize MAA to electrophilic intermediates. Hence, the combination of continuously produced oxidants during infection and impaired antioxidative defense in neutropenia patients could be the crucial difference compared

to tolerant control patients who also had infections, but a fully functional antioxidative defense system.

The observation that lymphocytes are also often affected by metamizole in neutropenia patients points to a toxicological mechanism *in vivo*. Additionally, the often mentioned potential risk factors for immune-mediated neutropenia, history of positive drug allergy or autoimmune diseases (Andres et al. 2011), were less frequent among neutropenia patients compared to tolerant control patients of our cohort. This does not exclude an immune-mediated mechanism of metamizole-associated neutropenia but makes an immunological predisposition less likely. A systematic analysis of the peripheral blood in neutropenia patients and tolerant controls from our cohort did not reveal any activation of the adaptive immune system (Dina et al. 2019, under review). These findings support a cytotoxic mechanism of metamizole-associated neutropenia. Furthermore, immune reactions to drugs need about 2 weeks to develop (Schonhofer et al. 2003), but many neutropenia cases elicited neutropenia after only a few days of metamizole treatment without previous contact to the drug.

5.1. References

Andres E, Zimmer J, Mecili M, et al. (2011) Clinical presentation and management of drug-induced agranulocytosis. Expert review of hematology 4(2):143-51 doi:10.1586/ehm.11.12

Bardoel BW, Kenny EF, Sollberger G, et al. (2014) The balancing act of neutrophils. Cell host & microbe 15(5):526-36 doi:10.1016/j.chom.2014.04.011

Berkowitz FE (1991) Hemolysis and Infection: Categories and Mechanisms of Their Interrelationship. Reviews of Infectious Diseases 13(6):1151-1162 doi:10.1093/clinids/13.6.1151

Boxer L, Dale DC (2002) Neutropenia: causes and consequences. Seminars in hematology 39(2):75-81

Boyunaga H, Keles H, Kenar L, et al. (2010) Anaerobic Glycolysis is the Main Pathway for Energy Generation in HL-60 Acute Promyelocytic Leukemia Cells. Journal of Clinical and Analytical Medicine 1(2):5-8 doi:10.4328

Buehler PW, Karnaukhova E, Gelderman MP, et al. (2011) Blood aging, safety, and transfusion: capturing the "radical" menace. Antioxidants & redox signaling 14(9):1713-28 doi:10.1089/ars.2010.3447

Carr AC, McCall MR, Frei B (2000) Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. Arteriosclerosis, thrombosis, and vascular biology 20(7):1716-23

Chacko BK, Kramer PA, Ravi S, et al. (2013) Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood. Laboratory investigation; a journal of technical methods and pathology 93(6):690-700 doi:10.1038/labinvest.2013.53

Chung J, Chen C, Paw BH (2012) Heme metabolism and erythropoiesis. Current opinion in hematology 19(3):156-62 doi:10.1097/MOH.0b013e328351c48b

Fujii J, Ito JI, Zhang X, et al. (2011) Unveiling the roles of the glutathione redox system in vivo by analyzing genetically modified mice. Journal of clinical biochemistry and nutrition 49(2):70-8 doi:10.3164/jcbn.10-138SR

Glatman Zaretsky A, Engiles JB, Hunter CA (2014) Infection-induced changes in hematopoiesis. Journal of immunology (Baltimore, Md : 1950) 192(1):27-33 doi:10.4049/jimmunol.1302061

Hentze MW, Muckenthaler MU, Galy B, et al. (2010) Two to tango: regulation of Mammalian iron metabolism. Cell 142(1):24-38 doi:10.1016/j.cell.2010.06.028

Jacque N, Ronchetti AM, Larrue C, et al. (2015) Targeting glutaminolysis has antileukemic activity in acute myeloid leukemia and synergizes with BCL-2 inhibition. Blood 126(11):1346-56 doi:10.1182/blood-2015-01-621870

Kramer PA, Ravi S, Chacko B, et al. (2014) A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: implications for their use as bioenergetic biomarkers. Redox biology 2:206-10 doi:10.1016/j.redox.2013.12.026

Kumar S, Bandyopadhyay U (2005) Free heme toxicity and its detoxification systems in human. Toxicology letters 157(3):175-88 doi:10.1016/j.toxlet.2005.03.004

Pearce EL, Pearce EJ (2013) Metabolic pathways in immune cell activation and quiescence. Immunity 38(4):633-43 doi:10.1016/j.immuni.2013.04.005

Que Jr L, Tolman WB (2008) Biologically inspired oxidation catalysis. Nature 455:333 doi:10.1038/nature07371

Ryu W-H, Gittleson FS, Thomsen JM, et al. (2016) Heme biomolecule as redox mediator and oxygen shuttle for efficient charging of lithium-oxygen batteries. Nature communications 7:12925 doi:10.1038/ncomms12925

https://www.nature.com/articles/ncomms12925#supplementary-information

Schonhofer P, Offerhaus L, Herxheimer A (2003) Dipyrone and agranulocytosis: what is the risk? Lancet (London, England) 361(9361):968-9 doi:10.1016/s0140-6736(03)12751-1

Splettstoesser WD, Schuff-Werner P (2002) Oxidative stress in phagocytes--"the enemy within". Microscopy research and technique 57(6):441-55 doi:10.1002/jemt.10098

Tesfa D, Keisu M, Palmblad J (2009) Idiosyncratic drug-induced agranulocytosis: Possible mechanisms and management. Am J Hematol 84(7):428-434 doi:10.1002/ajh.21433

Valko M, Rhodes CJ, Moncol J, et al. (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-biological interactions 160(1):1-40 doi:10.1016/j.cbi.2005.12.009

Wagener FA, Eggert A, Boerman OC, et al. (2001) Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. Blood 98(6):1802-11

Yan D, Lin YW, Tan X (2017) Heme-containing enzymes and inhibitors for tryptophan metabolism. Metallomics : integrated biometal science 9(9):1230-1240 doi:10.1039/c7mt00105c

6. CONCLUSION

The different susceptibility of immature and mature neutrophils to MAA-hemin cytotoxicity might point to a possible mechanism of metamizole-associated neutropenia observed *in vivo*. MAA can be transformed to an electrophilic intermediate in presence of highly oxidative compounds such as hemin. Thus, increased radical formation or decreased radical detoxification capacity might be crucial to whether a patient under metamizole treatment elicits neutropenia or not. The main result of the clinical data assessment showed that infections were twice as frequent among neutropenia patients compared to tolerant control patients. These infections potentially increased the susceptibility of neutrophil precursor cells to MAA radicals. Thus, highly oxidative compounds, which are generated continuously during infections, could form more electrophilic MAA intermediates than MAA is able to scavenge. However, the development of metamizole-associated neutropenia seems to require more than an infection during metamizole treatment, since also tolerant control patients had infections without any hematological adverse events. Cellular predisposition consisting of either low antioxidative capacity, poor heme metabolism, increased free heme generation, or increased production of other highly oxidative compounds may render cells more susceptible to MAA toxicity. Hence, addition of infections to already susceptible cells may facilitate or accelerate the development of neutropenia.

In contrast to general view, the results presented in this thesis favor a cytotoxic mechanism of metamizole-associated neutropenia. *In vitro* data illustrated a pathway, by which MAA can form a reactive electrophile intermediate that induces apoptosis in promyelocytic HL60 cells. Clinical data revealed no association between autoimmune diseases or drug-allergy history and neutropenia development, suggesting no involvement of the adaptive immune system. Additionally, high frequency of infections among neutropenia patients before neutropenia diagnosis points to an involvement of an aberrant cellular environment. This could be altered cell metabolism, increased heme concentration due to hemolysis or impaired heme metabolism, increased ROS production, or a too weak antioxidative defense system.

The aim of this thesis was to investigate the mechanism of metamizole-associated neutropenia to identify potential risk factors for patients. The clinical study data assessment showed a smaller impact of non-myelotoxic and non-immunosuppressive co-medication and the immune system than assumed. Nevertheless, acute infections seem to increase the risk to develop metamizole-associated neutropenia and may require additional surveillance in patients under metamizole treatment. Additionally, more awareness is needed concerning adequate patient information

including first signs of neutropenia and how to react in case of suspected neutropenia to prevent future fatal cases by early diagnosis and treatment.

7. OUTLOOK

Some further *in vitro* investigations regarding the mechanism by which reactive MAA intermediates are damaging neutrophil precursor cells have already been pursued. Since MAA-hemin leads to a drop in ATP in HL60 cells, the next step was to investigate a potential impairment of cellular energy generation by MAA radicals. First assays showed that when HL60 cells are forced to use only oxidative phosphorylation for ATP generation, cytotoxicity of MAA-hemin was significantly reduced. This suggests that mitochondria are not involved in the observed cytotoxicity. In a next step, the impact of MAA-hemin on cellular glycolysis was investigated. Since neutrophils and also neutrophil precursor cells rely predominantly on glycolysis for ATP generation, any damage to this system would strongly impair the cellular energy metabolism. The assessment of the cellular energy metabolism showed that MAA-hemin compromises glycolysis, leading to the observed drop in ATP, which may also be the cause of apoptosis.

The genetic study of the collaborating genetics group investigating potential predispositions for metamizole-associated neutropenia is still ongoing. So far, no significant genetic differences between neutropenia cases and controls have been detected. Since the sample size is relatively small, significant outcomes would need a larger control group. It is therefore planned to include more healthy control subjects from a genetic databank in Bern to enlarge the control group. Moreover, there is the possibility to receive genetic data from metamizole-associated neutropenia cases from other countries. This would make it possible to verify so far not significant but promising genetic variants in other neutropenia cohorts.

The main finding of the clinical data assessment of the case-control study was the higher frequency of infections among neutropenia cases compared to controls. To verify the impact of infection on the risk to develop metamizole-associated neutropenia, a higher number of study participants, which are compared to age-matched controls, is required. Therefore, control patients without inclusion criteria of a minimal metamizole intake period should be included, since long-term analgesic treatment is more common at higher age.

For subsequent *in vitro* investigations, cellular changes during infections and the involved enzymes may be assessed. These cellular changes would normally not lead to neutropenia, but in combination with possibly impaired antioxidative defense or low heme-metabolizing capacity could serve as the triggering factor for neutropenia development. Thus, potential *in vitro* findings

could be verified in an animal model with humanized immune cells. Mouse models with humanized neutrophils as well as lymphocytes have been successfully generated by several groups for investigations of the immune system (Coughlan et al. 2012; Evering and Tsuji 2018). Hence, the involvement of the previously identified enzymes *in vitro* could be investigated by knock-down of these enzymes and MAA treatment in presence of an infection.

7.1. References

Coughlan AM, Freeley SJ, Robson MG (2012) Humanised mice have functional human neutrophils. Journal of immunological methods 385(1-2):96-104 doi:10.1016/j.jim.2012.08.005

Evering TH, Tsuji M (2018) Human Immune System Mice for the Study of Human Immunodeficiency Virus-Type 1 Infection of the Central Nervous System. Frontiers in immunology 9:649 doi:10.3389/fimmu.2018.00649