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 (<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.

Key words: metamizole, N-methyl-4-aminoantipyrine, agranulocytosis, HL60 cells, hemin, iron

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 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 $2x10^5$ HL60 cells or $1x10^6$ 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 hours 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 hours 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 hours 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 hours, the cells were centrifuged at 500 g for 5 minutes and washed with PBS before PI staining (Molecular probes, Oregon, USA) at a final concentration of 10 ug/mL. After 15 minutes 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 hours, the cells were centrifuged at 500 g for 5 minutes and washed with PBS before Alexa Fluor 488® Annexin V staining (Molecular probes, Oregon, USA). After 15 minutes 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 x 10^5 HL60 cell suspensions or 1 x 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 x 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 2x10^5 HL60 cells in 2 mL Eppendorf tubes and added 100 µM MAA as described above. Before adding 10 to 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 hours 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 hours 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 hours.
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 x 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 hours and assessed membrane toxicity and ATP content as outlined previously.

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

We prepared 2 x 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 x 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 minutes 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 x 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 hours 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, 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 2B), nor for freshly isolated human granulocytes after 24 hours incubation (Fig. 2C and 2D) or 48 hours 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 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.

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 4E, 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 to 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 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 hours 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 6C). 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 7B, 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 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 supplementary 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, 39)(37, 38).

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 hours. 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 hours under our experimental conditions (Fig. 8B). The product did not undergo further transformations when incubated under these conditions for another four days.

Figures 8C and 8D 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).

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 to 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 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.

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

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Legends to Figures

Figure 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 the 4-aminoantipyrine (AA) or formylated to N-formyl-4-aminoantipyrine (FAA). The majority of AA is acetylated to N-acetyl-4-aminoantipyrine (AAA).

Figure 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 hours. 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.

Figure 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 hours. (**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.

Figure 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 hours. 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 hours. (**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.

Figure 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 hours. (**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.

Figure 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 hours. 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 hours. (**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.

Figure 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 hours. (**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 hours. (**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.

Figure 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.

Figure 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 (^{1}H, 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 (^{1}H, 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.

Fig. 1

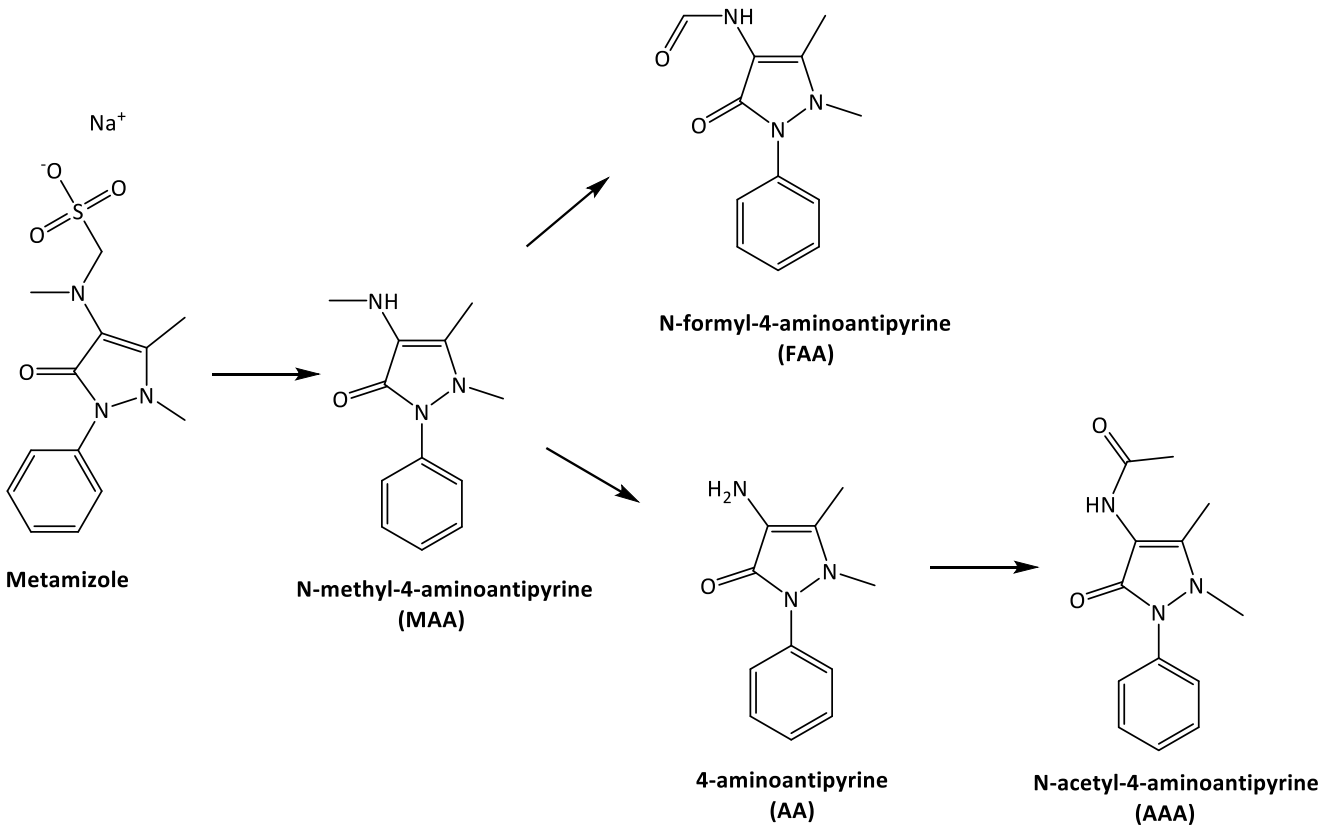

Na+
Metamizole
N-methyl-4-aminoantipyrine
(MAA)
N-formyl-4-aminoantipyrine
(FAA)
4-aminoantipyrine
(AA)
N-acetyl-4-aminoantipyrine
(AAA)

Fig. 2

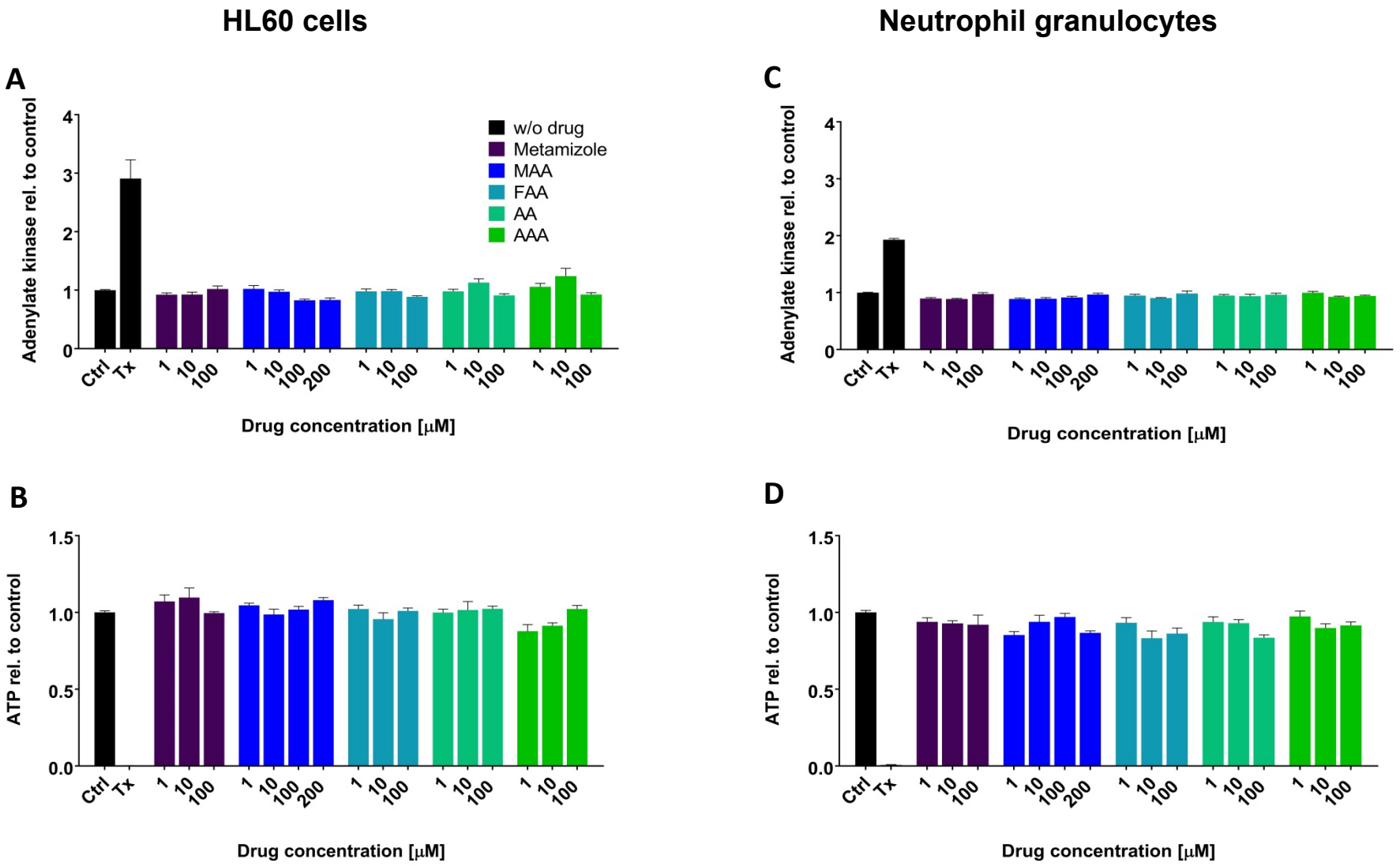

HL60 cells
Neutrophil granulocytes
A
B
C
D
Adenylate kinase rel. to control
ATP rel. to control
w/o drug
Metamizole
MAA
FAA
AA
AAA
Ctrl
Tx
1
10
100
200
Drug concentration [µM]

Fig. 3

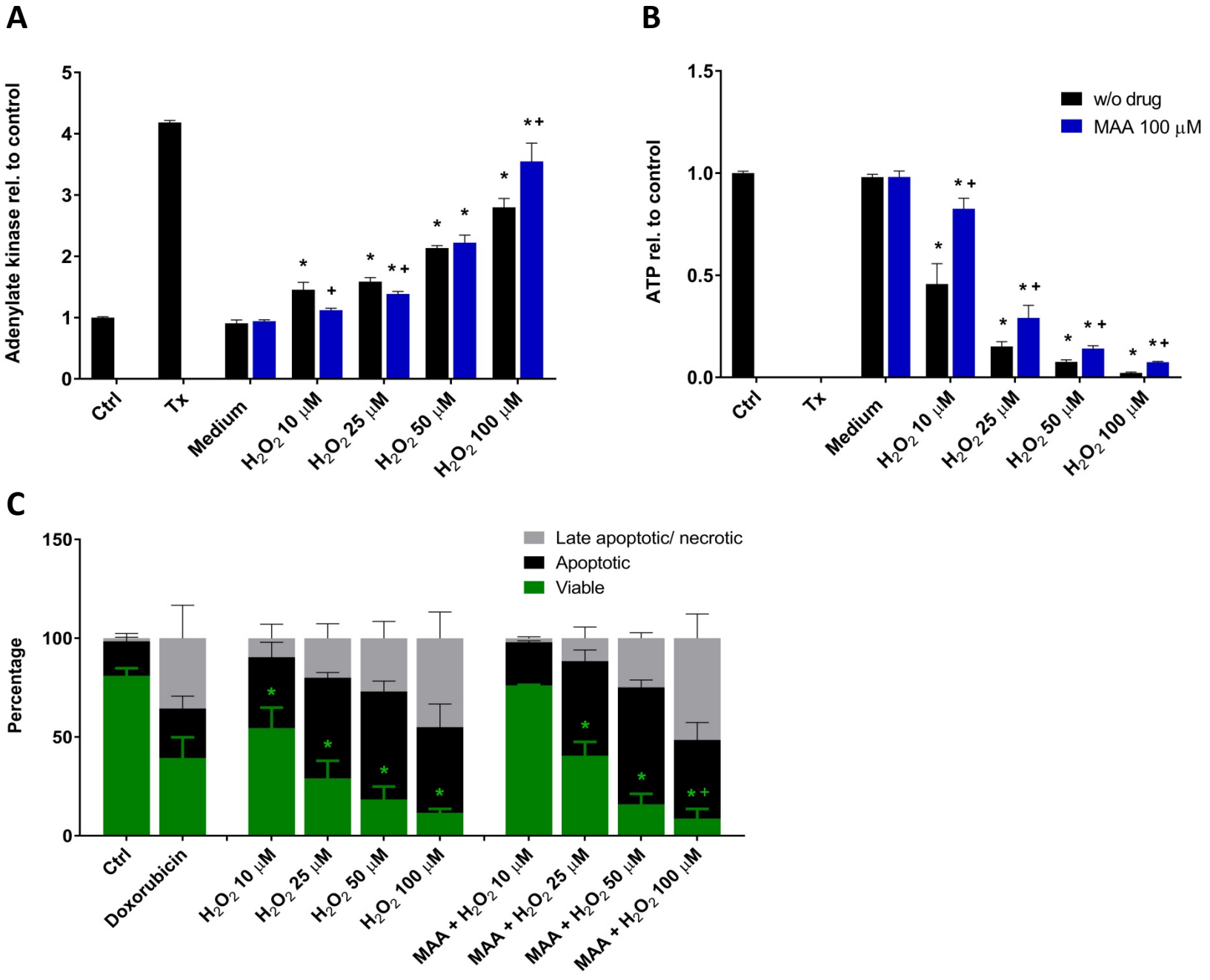
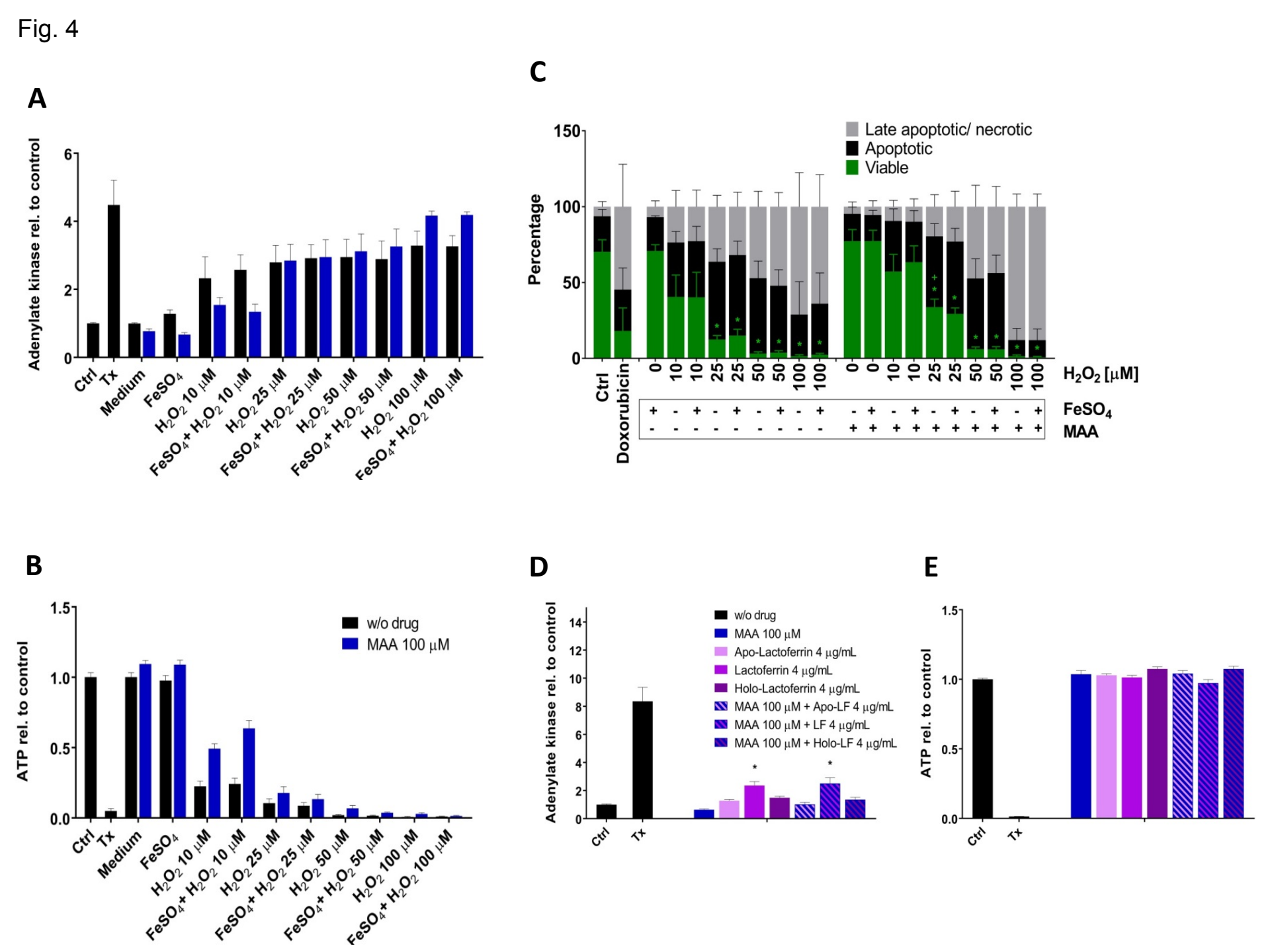

A
Adenylate kinase rel. to control
0
1
2
3
4
5
Ctrl
Tx
Medium
H2O2 10 µM
H2O2 25 µM
H2O2 50 µM
H2O2 100 µM
B
ATP rel. to control
0.0
0.5
1.0
1.5
w/o drug
MAA 100 µM
C
Percentage
0
50
100
150
Late apoptotic/ necrotic
Apoptotic
Viable
Ctrl
Doxorubicin
H2O2 10 µM
H2O2 25 µM
H2O2 50 µM
H2O2 100 µM
MAA + H2O2 10 µM
MAA + H2O2 25 µM
MAA + H2O2 50 µM
MAA + H2O2 100 µM

Fig. 4


A
Adenylate kinase rel. to control
Ctrl
Tx
Medium
FeSO4
H2O2 10 µM
FeSO4 + H2O2 10 µM
H2O2 25 µM
FeSO4 + H2O2 25 µM
H2O2 50 µM
FeSO4 + H2O2 50 µM
H2O2 100 µM
FeSO4 + H2O2 100 µM
B
ATP rel. to control
w/o drug
MAA 100 µM
C
Percentage
Late apoptotic/ necrotic
Apoptotic
Viable
Doxorubicin
H2O2 [µM]
FeSO4
MAA
D
Adenylate kinase rel. to control
w/o drug
MAA 100 µM
Apo-Lactoferrin 4 µg/mL
Lactoferrin 4 µg/mL
Holo-Lactoferrin 4 µg/mL
MAA 100 µM + Apo-LF 4 µg/mL
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MAA 100 µM + Holo-LF 4 µg/mL
E
ATP rel. to control

Fig. 5

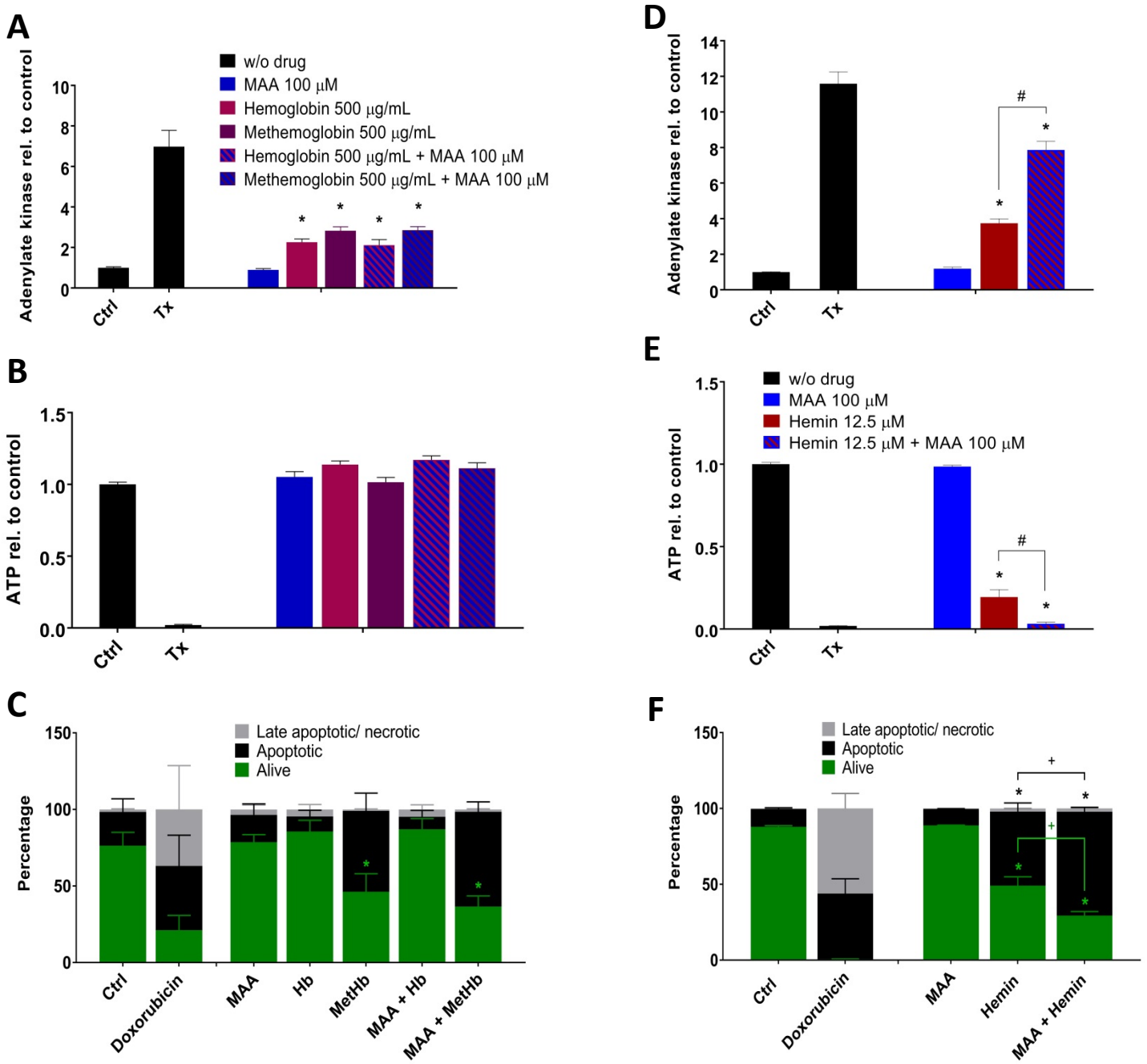

A
Adenylate kinase rel. to control
w/o drug
MAA 100 μM
Hemoglobin 500 μg/mL
Methemoglobin 500 μg/mL
Hemoglobin 500 μg/mL + MAA 100 μM
Methemoglobin 500 μg/mL + MAA 100 μM
Ctrl
Tx
B
ATP rel. to control
C
Percentage
Late apoptotic/ necrotic
Apoptotic
Alive
Ctrl
Doxorubicin
MAA
Hb
MetHb
MAA + Hb
MAA + MetHb
D
Adenylate kinase rel. to control
E
ATP rel. to control
Hemin 12.5 μM
Hemin 12.5 μM + MAA 100 μM
F
Hemin
MAA + Hemin

Fig. 6

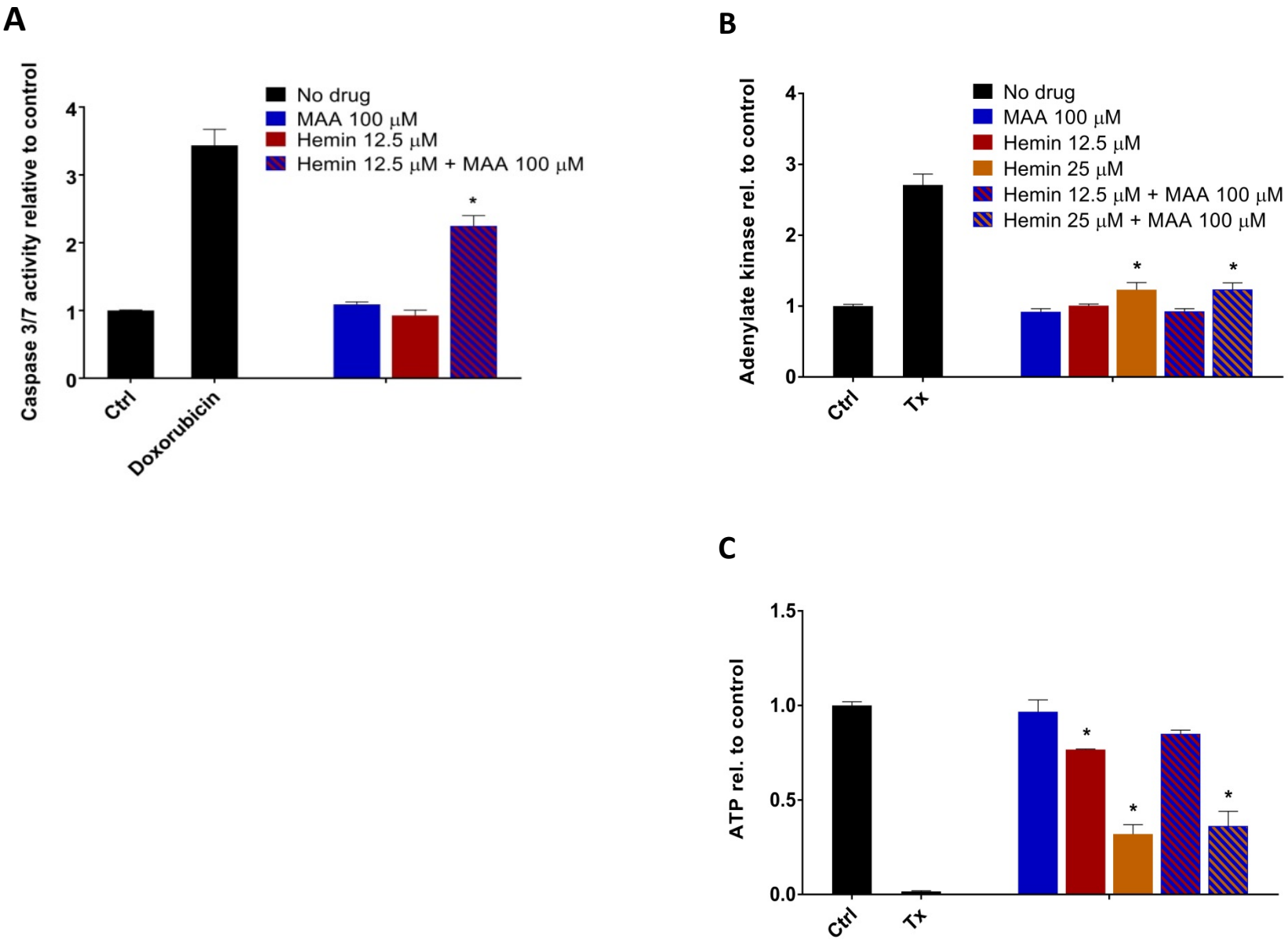

A
Caspase 3/7 activity relative to control
No drug
MAA 100 μM
Hemin 12.5 μM
Hemin 12.5 μM + MAA 100 μM
Ctrl
Doxorubicin
B
Adenylate kinase rel. to control
No drug
MAA 100 μM
Hemin 12.5 μM
Hemin 25 μM
Hemin 12.5 μM + MAA 100 μM
Hemin 25 μM + MAA 100 μM
Ctrl
Tx
C
ATP rel. to control
Ctrl
Tx

Fig. 7

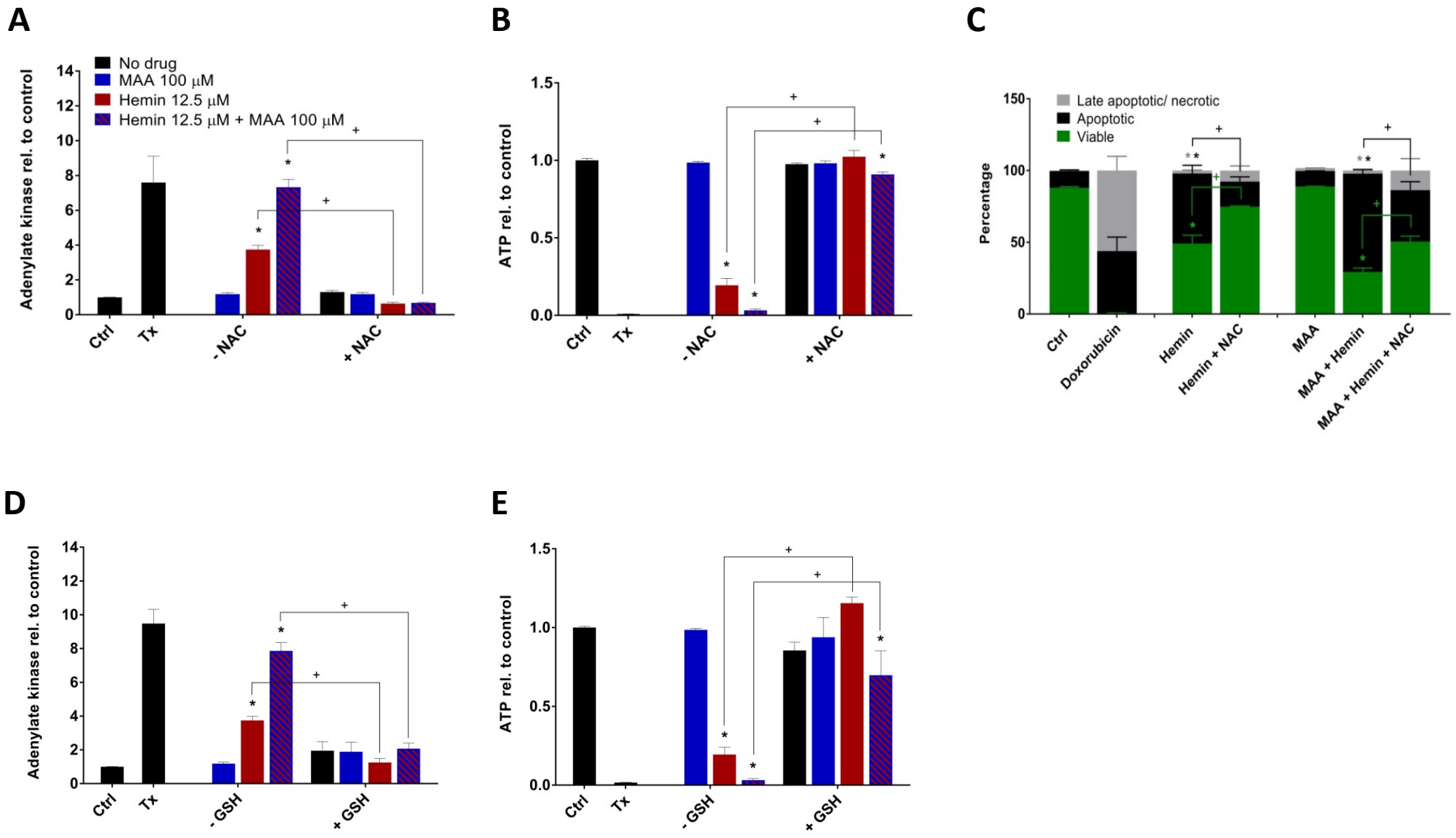

A
No drug
MAA 100 μM
Hemin 12.5 μM
Hemin 12.5 μM + MAA 100 μM
Adenylate kinase rel. to control
Ctrl
Tx
- NAC
+ NAC
B
ATP rel. to control
Ctrl
Tx
- NAC
+ NAC
C
Late apoptotic/ necrotic
Apoptotic
Viable
Percentage
Ctrl
Doxorubicin
Hemin
Hemin + NAC
MAA
MAA + Hemin
MAA + Hemin + NAC
D
Adenylate kinase rel. to control
Ctrl
Tx
- GSH
+ GSH
E
ATP rel. to control
Ctrl
Tx
- GSH
+ GSH

Fig. 8

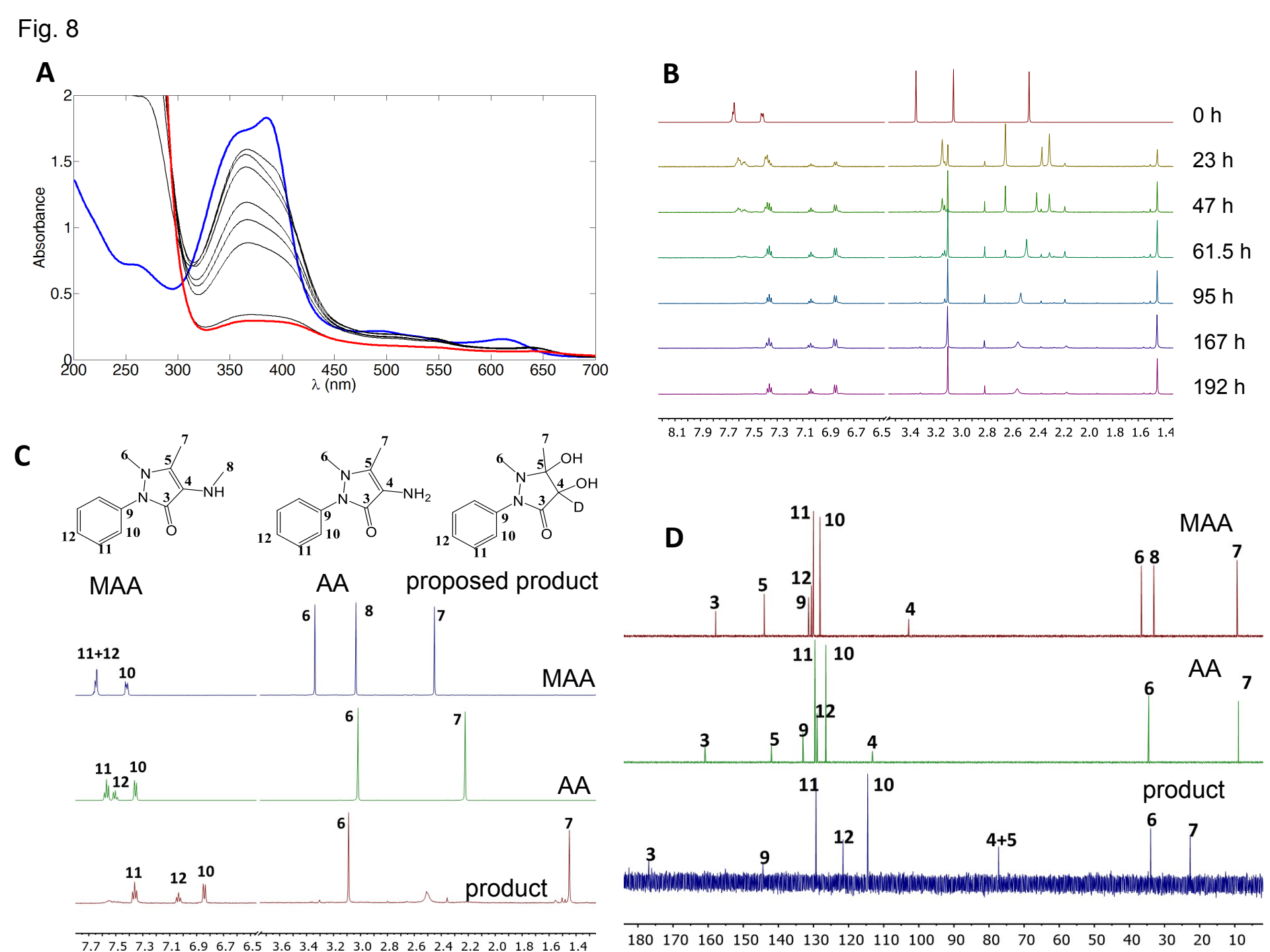
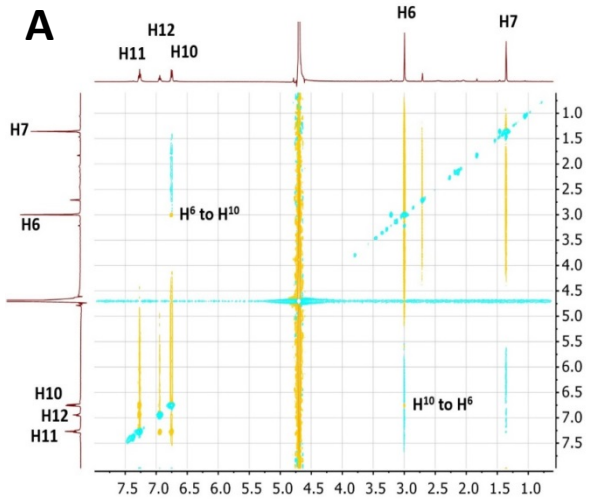
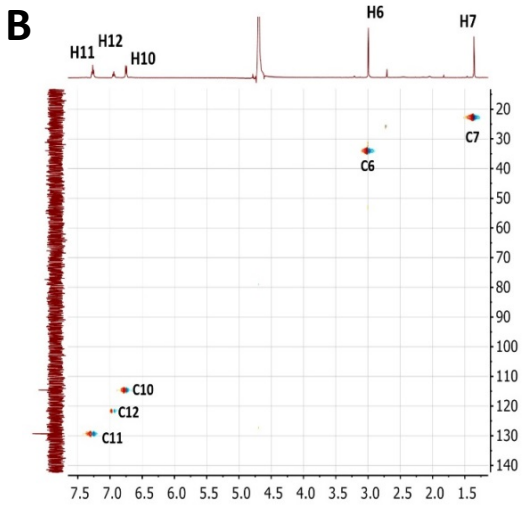
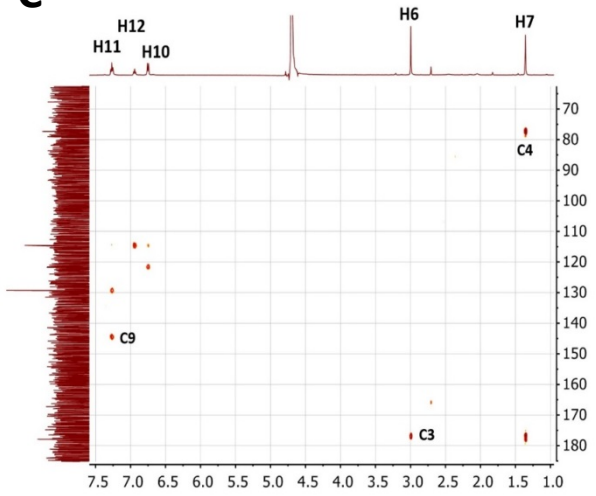
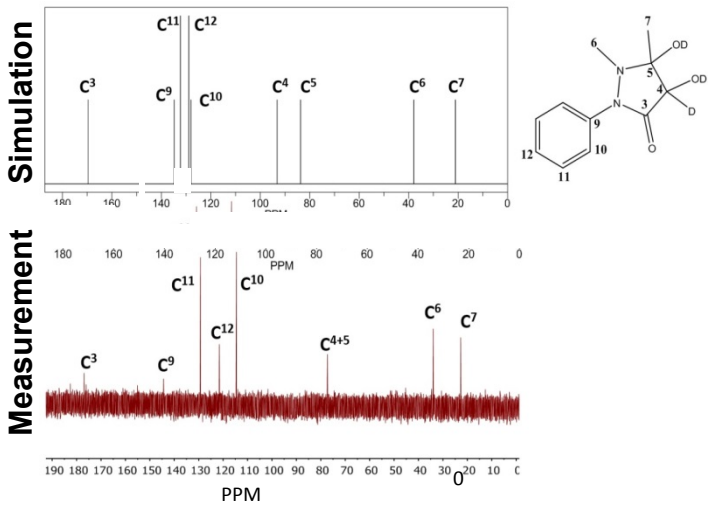

A
Absorbance
λ (nm)
B
0 h
23 h
47 h
61.5 h
95 h
167 h
192 h
C
MAA
AA
proposed product
11+12
10
product
D
MAA
AA
product
4+5

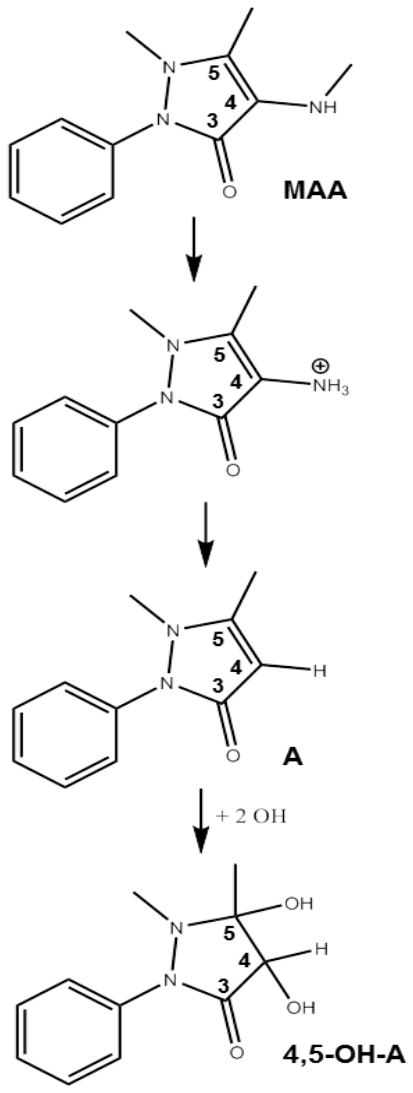
Fig. 9


A
H12
H11
H10
H6
H7
H7
H6
H10
H12
H11
H⁶ to H¹⁰
H¹⁰ to H⁶


B
H11
H12
H10
H6
H7
C7
C6
C10
C12
C11


C
H12
H11
H10
H6
H7
C4
C9
C3


D
Simulation
Measurement
C³
C⁹
C¹⁰
C¹¹
C¹²
C⁴
C⁵
C⁶
C⁷
C⁴⁺⁵
PPM


E
MAA
A
+ 2 OH
4,5-OH-A