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Effect of metamizol on promyelocytic and terminally differentiated granulocytic cells
Comparative analysis with acetylsalicylic acid and diclofenac

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Abstract

Metamizol is an analgesic and antipyretic agent that can induce agranulocytosis in certain patients. However, its effects on granulocyte viability and differentiation have been poorly evaluated. Here we analysed the effects of metamizol and its active metabolite, 4-methylaminoantipyrine (MAA), on the viability of HL60 promyelocytes and their dimethyl sulphoxide-induced differentiated granulocytes. Metamizol and MAA at 75 μM (above the peak of plasmatic concentration after 2 g intake) did not alter granulocytic differentiation of HL60 cells. Only at concentrations above 100 μM, well over the pharmacological range, metamizol-induced apoptosis in about 30% of the HL60 promyelocytes, while HL60-granulocytic terminally differentiated cells were more resistant to this apoptotic action. When the effects of metamizol were compared with those of acetylsalicylic acid (ASA) and diclofenac on cell viability, at equivalent concentrations used in analgesic and antipyretic therapy (75 μM for metamizol, and ASA and 3 μM for diclofenac) their apoptotic effects were similar. Again, the HL60 promyelocytes were more sensitive to apoptosis than granulocytic differentiated cells, as measured by the percentage of sub-G_1 cells detected by flow cytometry and by determination of caspase activity as a function of poly(ADP-ribose) polymerase cleavage. Furthermore, when human blood-derived granulocytes were treated with metamizol, MAA, and ASA at 75 μM or diclofenac at 3 μM, less than 10% of apoptotic granulocytes were detected, whereas at toxicological/suprapharmacological concentrations (10 mM), about 90% of granulocytes were apoptotic. These results demonstrate that metamizol, MAA, ASA, and diclofenac, at pharmacological concentrations, neither affect the granulocytic differentiation process nor induce relevant apoptosis on terminally differentiated granulocytes.

Keywords: Metamizol; Acetylsalicylic acid (ASA); Dimethyl sulphoxide (DMSO); 4-Methylaminoantipyrine (MAA); Non-steroidal anti-inflammatory drugs (NSAIDs); Granulocytes

1. Introduction

Metamizol is a non-opioid analgesic and antipyretic prodrug commonly used in some countries, which becomes active when converted to 4-methylaminoantipyrine (MAA) and 4-aminoantipyrine in the body [1]. Exposure to metamizol has been related with the vast induction of agranulocytosis [2], and the risk estimation for patients exposed to the drug varies broadly from country to country (from 1:2,000 to 1:1,000,000) [3]. Although the mechanism of this secondary effect is accepted to be of immunoallergic origin, a direct toxic effect of metamizol on peripheral blood granulocytes has been reported [4]. However, there is no published data about a possible direct interaction between this compound and granulocytes, therefore, we decided to study the direct toxic effect of metamizol on various differentiation stages of granulocyte maturation.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid (ASA) and diclofenac are used to

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Abbreviations: ASA, acetylsalicylic acid; COX, cyclooxygenase; DMSO, dimethyl sulphoxide; FCS, fetal calf serum; MAA, 4-methylaminoantipyrine; NSAIDs, non-steroidal anti-inflammatory drugs; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PBS, phosphate buffered saline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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treat similar pain conditions as metamizol, and they have not been related with agranulocytosis induction in epidemiological studies. Toxicological/suprapharmacological concentrations of different NSAIDs can induce apoptosis in several cell lines, including chronic lymphocyte leukaemia cells, transformed chicken embryo fibroblasts and human colon cancer cells [5–8]. All these three compounds are able to inhibit cyclooxygenase activity [9,10] and consequently to reduce prostaglandin production, although there is not a straight correlation between their potencies as prostaglandin inhibitors and their pharmacological actions [11].

Agranulocytosis induced by drugs such as metamizol, antitumorals, thyroid inhibitors or ticlopidine can be produced by a toxic effect or by an immunologic mechanism [2,4,12–14]. The massive reduction in granulocyte count could be explained either by interference with the granulocyte differentiation process and/or induction of apoptosis in mature blood granulocyte cells.

The HL60 cell line is derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukaemia, consists predominantly of promyelocytes, and proliferates continuously in suspension culture [15]. This cell line can be induced to terminally differentiated granulocytes by incubation with several compounds, including dimethyl sulphoxide (DMSO) [15]. Thus, DMSO-differentiated HL60 cells have many of the functional characteristics of normal peripheral blood granulocytes, including the expression of complement receptors (CD11b and CD11c), the tyrosine phosphatase CD45 [16], and the proto-oncogene c-Src [17], as well as chemotactic and phagocytic activities, or the ability to undergo respiratory burst, with production of superoxide anion radical [15,18,19], providing an excellent system to study human myeloid differentiation *in vitro*.

Here, we have used the HL60 promyelocytes as a model to study the effects of metamizol and MAA during their DMSO-induced differentiation to granulocytes. In addition, the effects of ASA and diclofenac were also analysed for comparative purposes. Herein, we report that metamizol and MAA at 75 μM did not affect the DMSO-induced expression of granulocytic differentiation markers. Also, ASA and diclofenac at equivalent concentrations, were not cytotoxic and did not induce a relevant apoptotic response both in promyelocytic and granulocytic cells. These studies were further substantiated with experiments carried out with granulocytes obtained from human buffy coats treated with these NSAIDs, where no signs of agranulocytosis were detected at pharmacological doses.

2. Materials and methods

2.1. Reagents

Tissue culture media and sera were purchased from Life Technologies. Tissue culture plastic was purchased from Becton Dickinson. Mouse mAb CD11b (clone BEAR1) [20], CD11c (clone HC1/1) [21]. Anti-[poly(ADP-ribose) polymerase] (PARP) mouse mAb was from Biomol Research Laboratories and the anti-α-tubulin mouse mAb was from Sigma Chemicals Co. FITC-conjugated $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulins were purchased from DAKO. Secondary antibodies horseradish peroxidase-conjugated were purchased from Biosource International. Metamizol and MAA were used at 75 μM (unless otherwise indicated), a high pharmacological concentration detected after standard administration of metamizol used for analgesia or antipyresis [22]. ASA and diclofenac (Sigma Chemicals Co) were used at 75 and 3 μM (unless otherwise indicated), respectively, which are within the plasma concentration range reached with analgesic and antipyretic doses of ASA (15–100 μM) and diclofenac (1–3 μM) [23–25].

2.2. Cell lines and culture methods

The HL60 cell line was cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°, in atmosphere with 5% CO_2 and 95% humidity. Differentiation of HL60 cells was induced by incubation in the presence of 1.3% (v/v) of DMSO (Carlo Erba) for 3–5 days as described [15,18,19].

2.3. Buffy coats treatments and granulocytes isolation

Buffy coats from peripheral blood were obtained from venous blood of volunteer donors and diluted 5-fold with PBS. Blood cells were then subjected overnight to corresponding treatments at 37°, 5% CO_2 and 95% humidity. After treatments granulocytes were isolated basically as described [26] with minor modifications. Briefly, after elimination of most erythrocytes by gravity sedimentation in the presence of 6% dextran/0.15 M NaCl, monocytes, and lymphocytes were separated from granulocytes by Histopaque-1077 (Sigma Chemicals Co) density gradient centrifugation at 800 *g* for 30 min (20°). Finally, contaminant erythrocytes were removed by twice-controlled hypotonic lysis with distilled water for 30 s. The isolated granulocytes were washed twice with PBS at 4° and subjected to labeling for flow cytometry analysis (see following sections). In some experiments, granulocytes were previously isolated and then incubated with the NSAIDs as described before.

2.4. Flow cytometry analysis of differentiation markers

Cells were harvested from cultures, washed, and resuspended in staining solution (PBS, 2% FCS) at a density of 3×10^6 cells/mL. For cell surface staining, cells were incubated in optimal concentrations of each mAb on ice for 20 min. After washing, cells were incubated with

FITC-conjugated F(ab′)$_2$ fragment rabbit anti-mouse Igs. Cell debris and dead cells were excluded by light scatter parameters and propidium iodide exclusion (PI; Sigma Chemicals Co). Incubating cells with isotype-matched irrelevant mAb set background signals. The minimum number of acquired cells/sample was always 10^4 cells. Flow cytometry analyses were performed with an EPICS-XL flow cytometer (Coulter Corp.) equipped with an argon laser tuned to 488 nm. A 525 nm band pass filter collected specific fluorescence signals from FITC. The results were analysed using Expo 2.0 software (Coulter Corp.).

2.5. Analysis of apoptosis by propidium iodide DNA staining

Flow cytometry was employed to assay apoptosis by PI DNA staining as previously described [27]. Briefly, cells were collected by centrifugation at 400 *g* for 5 min at room temperature and fixed in 1 mL of PBS–70% ethanol for at least 1 hr at 4°. Fixed cells were washed in PBS and then incubated in 0.5 mL of PBS containing 50 μg/mL PI, 100 μg/mL de RNase (Sigma Chemicals Co) for 30 min at room temperature in the dark before flow cytometry analysis to determine the percentage of cells in the sub-G_0/G_1 cell cycle compartment, corresponding to apoptotic cells. The minimum number of acquired cells/sample was always 10^4 cells. Flow cytometry analyses were performed on an EPICS-XL flow cytometer (Coulter Corp.) collecting PI fluorescence signals through a 620 nm pass filter.

2.6. Analysis of cellular viability

Flow cytometry was employed to assay cellular viability. Two criteria were used, cell morphology (forward scatter vs. side scatter) and PI permeability. Briefly, cells (10^4 cells/mL) were collected by centrifugation at 400 *g* for 5 min at room temperature, washed with 1 mL of PBS, and resuspended in 0.5 mL of PBS containing 10 μg/mL PI and immediately analysed on the EPICS-XL flow cytometer. The minimum number of acquired cells/sample was always 10^4 cells. Cells permeable to PI were considered non-viable.

2.7. Western blot analysis

Cells were collected by centrifugation at 400 *g* for 5 min (4°), washed once in ice-cold PBS and subsequently lysed with 1 mL per 2×10^7 cells of lysis buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride, 1 mM phenantroline, 1 mM benzamidine hydrochloride, 1 mM iodoacetamide). Total cell lysates, supernatants from a 15,000 *g* for 30 min (4°) centrifugation, were compensated with lysis buffer for the same protein concentration after being determined by the BCA protein assay (Pierce). They were finally boiled in 1 × SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol) and stored at −80° until further use. For Western blotting analysis, samples were subjected to SDS-PAGE and transferred to Immobilon-P membranes. Filters were blocked with 5% fat-free dried milk (Fluka Chemie AG) in TTBS (10 mM Tris–HCl, pH 7.4, 0.1% Tween 20). Membranes were incubated with the primary antibody in blocking buffer, washed three times with TTBS, and further incubated with the suitable horseradish peroxidase-conjugated anti-species-specific antibody. Proteins were detected by chemiluminiscence assay (ECL; Amersham Biosciences).

3. Results

Granulocytic differentiation of promyelocytic HL60 cells by retinoic acid or by polar compounds like DMSO is characterised by settled changes in expression of cell surface markers. Among them, the up-regulated expression of the α subunit of the β2 integrin CR3, CD11b [28], the α subunit of the β2 integrin CR4, CD11c, the tyrosine phosphatase CD45 [16], and the protoncogene c-Src which also increases in activity during granulocytic differentiation [17].

Using this *in vitro* differentiation system, we have analysed the effects of metamizol and its active metabolite MAA, on the viability of promyelocytic and granulocytic cells. First, we assessed whether metamizol and MAA alters the granulocytic differentiation process. Untreated or DMSO-treated HL60 cells were seeded at 10^4 cells/mL, and a set of them was also incubated with metamizol or MAA at 75 μM, concentration that doubles the MAA plasmatic concentration detected after oral administration of 2 g of metamizol [22]. Untreated and metamizol- or MAA-treated cultures were tested for the expression of the above cell differentiation markers. As expected, DMSO-induced granulocytic differentiation of the promyelocytic HL60 cells. Thus, the expression of CD11b and CD11c clearly increased above control cells (Fig. 1A). The analysis of the CD45, c-Src and p-c-Src by Western blotting also showed up-regulation in granulocytic-like differentiated HL60 cells (data not shown). Consistent with a differentiation process, there is a cessation of cellular growth as observed in a cell cycle resolution by PI labeling followed by flow cytometric analysis (Fig. 2, Control). Since HL60 are growing cells, they are distributed into the three PI-defined cell cycle compartments (G_1, S, and $G_2 + M$). In contrast, most of the HL60-DMSO treated cells (differentiated to granulocytes; HL60-granulocytes) are arrested into the G_1 phase of the cell cycle. The results obtained when HL60 cells were treated with DMSO in the presence of metamizol or MAA (75 μM) were identical to those obtained in their absence (Figs. 1 and 2), indicating that metamizol and MAA did not alter the granulocytic differentiation of HL60-DMSO treated cells. In addition,

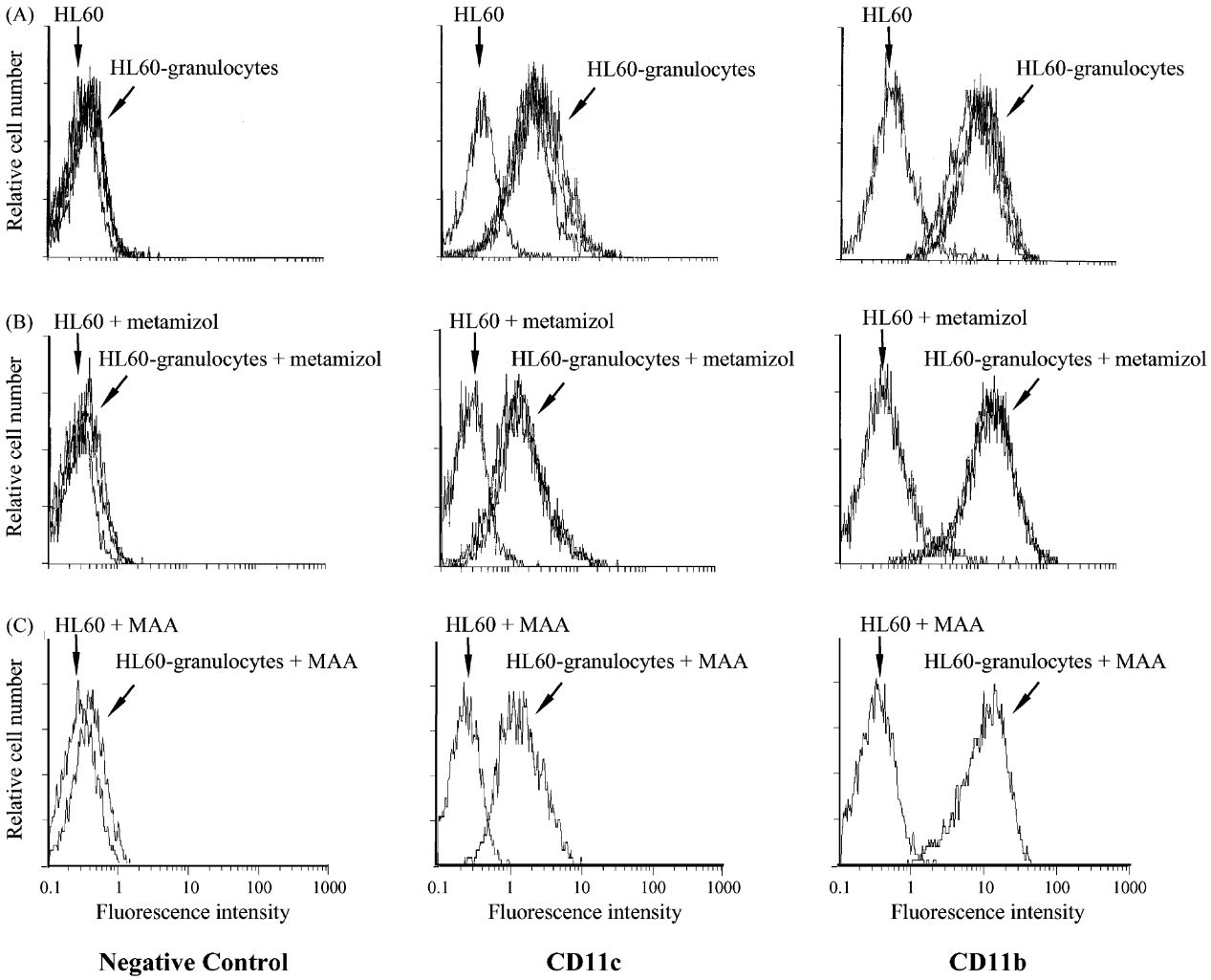

Fig. 1. Differentiation of HL60 cells to granulocytes by DMSO induces expression of CD11b and CD11c: effect of metamizol and MAA. HL60 cells were seeded at 10^4 cells/mL on day 0 and cultured in the absence (HL60) or presence of 1.3% (v/v) DMSO to induce granulocytic differentiation (HL60-granulocytes). HL60 and HL60-granulocytes were cultured in the absence (A) or in the presence of 75 μM of either metamizol (B) or MAA (C). Cells were harvested and incubated with anti-CD11b, anti-CD11c, or an irrelevant mAb (negative control), then with FITC-conjugated $F(ab')_2$ fragment rabbit anti-mouse Igs, and subsequently analysed by flow cytometry as described in Section 2. Panels A and B show overlapping fluorescence patterns of HL60-granulocytes obtained after 3, 4 or 5 days treatment with DMSO, while panel C shows a single fluorescence pattern of HL60-granulocytes after 4 days of treatment with DMSO. Histograms are representative of three independent experiments.

treatment of HL60 and HL60-granulocytes with metamizol or MAA (75 μM) did not induce apoptosis, as the percentage of cells in the sub-G1 compartment of the cell cycle is similar to that observed in control untreated cells (Fig. 2).

There is a large body of scientific reports describing apoptotic effects of the NSAIDs. We have, therefore, investigated the possible apoptotic effects of metamizol on HL60 and granulocytic cells. As shown in Fig. 3, promyelocytic HL60 cells are more sensitive than HL60-granulocytes to the apoptotic action of metamizol. Within the pharmacological range of metamizol concentrations found in the human plasma (hatched area in Fig. 3), the percentage of apoptotic HL60 cells was lower than 20% and even smaller in HL60-granulocytes (Fig. 4). Only at suprapharmacological concentrations, over 100 μM, which are toxic to HL60 cells (data not shown), metamizol provoked high level of apoptosis.

Next, we compared the capacity of metamizol with that of ASA and diclofenac to induce apoptosis on HL60, HL60 during the differentiation to granulocytes induced by DMSO (v/v) (1.3%) and on terminally differentiated HL60-granulocytes (HL60 treated for 3 days with DMSO). Cells were seeded at 10^4 cells/mL and incubated with increasing concentrations of these compounds. The promyelocytic HL60 cells were more sensitive to the apoptotic effect of these NSAIDs than the HL60-DMSO or the granulocytic cells. Nevertheless, the degree of apoptosis of HL60 cells induced by these three drugs was similar and did not exceed 10% of the cell population, while the apoptotic population in HL60-DMSO or in HL60-granulocytes was smaller than 5% of the cell population (Fig. 4A). We also analysed the apoptotic effects of these NSAIDs by determining the proteolytic cleavage of PARP, a substrate of caspases, cysteine proteases specifically activated in

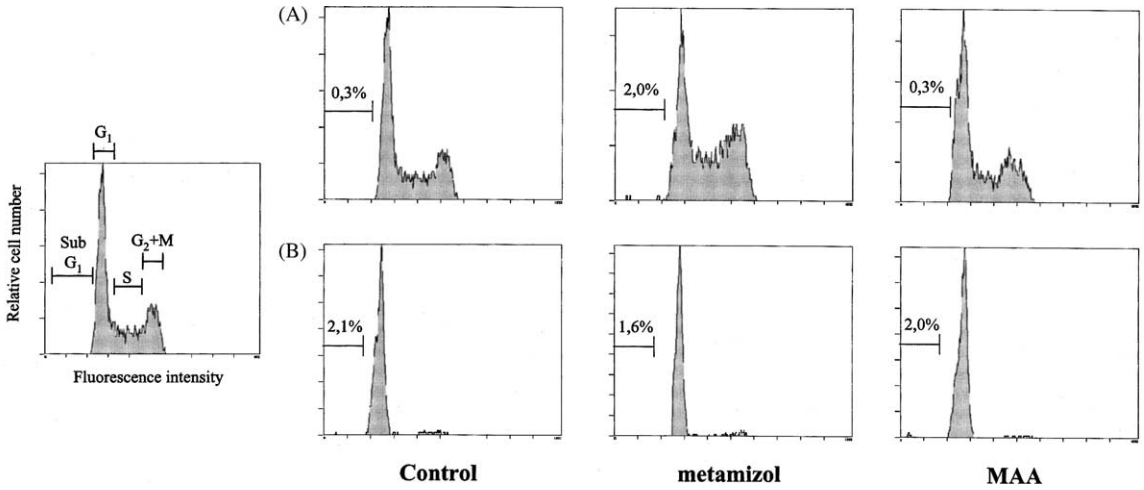

Fig. 2. Effect of metamizol and MAA on HL60 and on DMSO-induced differentiation to HL60-granulocytes. HL60 cells were seeded at 10^4 cells/mL on day 0 alone (A) or with 1.3% (v/v) DMSO to induce granulocytic differentiation (HL60-granulocytes) (B) and cultured in the absence or presence of 75 μM of either metamizol or MAA for 3 days. Cells were then harvested by centrifugation, washed with PBS, fixed, stained with PI and analysed by flow cytometry. The percentage of hypodiploid cells (sub-G_1 compartment) corresponding to apoptotic cells was measured. Histograms represent one of three independent experiments.

apoptosis [29–31]. Consistent with the very low levels of apoptotic cells found upon treatment with metamizol, ASA and diclofenac, we were unable to observe activation of caspases, as the anti-PARP antibody only detected the native form of the enzyme, the 116 kDa band, while the proteolytic PARP fragment of 85 kDa that should be generated upon caspase activation was undetectable (Fig. 4B).

As NSAIDs could cause cell death without inducing apoptosis, we investigated the possible toxic lethal effects of metamizol as compared to similar doses of ASA and equivalent doses of diclofenac on HL60 promyelocytes. Cells were seeded at 10^4 cells/mL and incubated with high pharmacological concentrations of metamizol (75 μM) and equivalent doses of ASA (75 μM) or diclofenac (3 μM) and collected after 1, 2 or 3 days. The effect of these NSAIDs on cell viability was determined by flow cytometry using two complementary criteria, cell morphology and PI passive cell permeability as described in Section 2. Consistent with the effects of metamizol on apoptosis of HL60 cells, the viability was $88.6 \pm 0.67\%$ after 24 hr of metamizol treatment, while viability of control cells was $97.0 \pm 0.73\%$. The viability of metamizol-treated cells increased with time to reach values similar to those observed in control cells. The cell viability of HL60 treated with ASA or diclofenac were similar to control cells (Table 1).

Since the DMSO-differentiated HL60-granulocytes may have a different sensitivity to NSAID than human granulocytes from peripheral blood, freshly prepared buffy

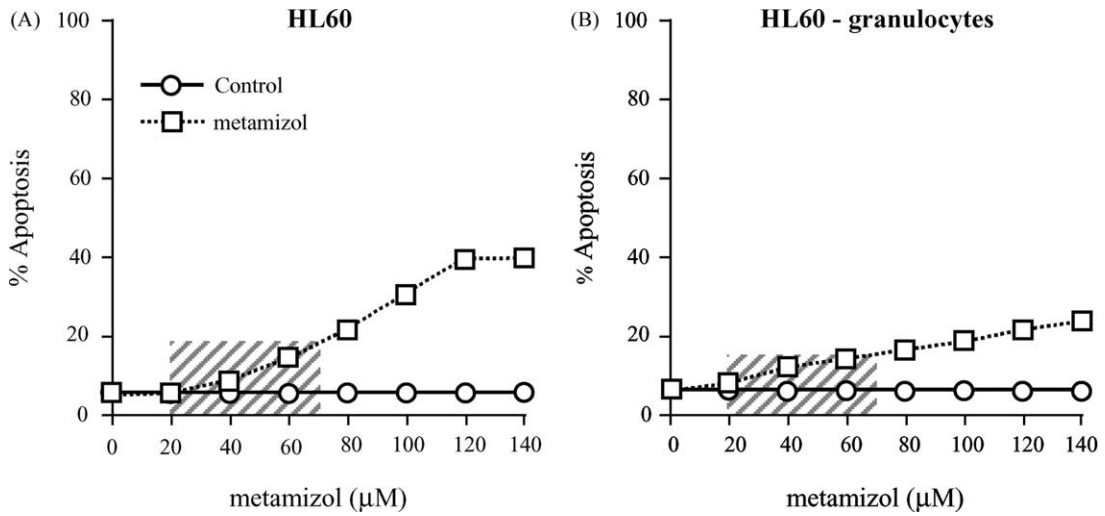

Fig. 3. Dose–response of metamizol-induced apoptosis. (A) HL60 cells were seeded at 10^4 cells/mL and incubated with increasing concentrations of metamizol for 3 days. (B) HL60 cells were treated with 1.3% (v/v) DMSO for 3 days (HL60-granulocytes) and then incubated for 24 hr with increasing concentrations of metamizol. Cells were harvested, fixed, and stained with PI and analysed by flow cytometry. The percentage of cells in the sub-G_1 compartment, corresponding to apoptotic cells, was determined. Similar results were obtained in three independent experiments.

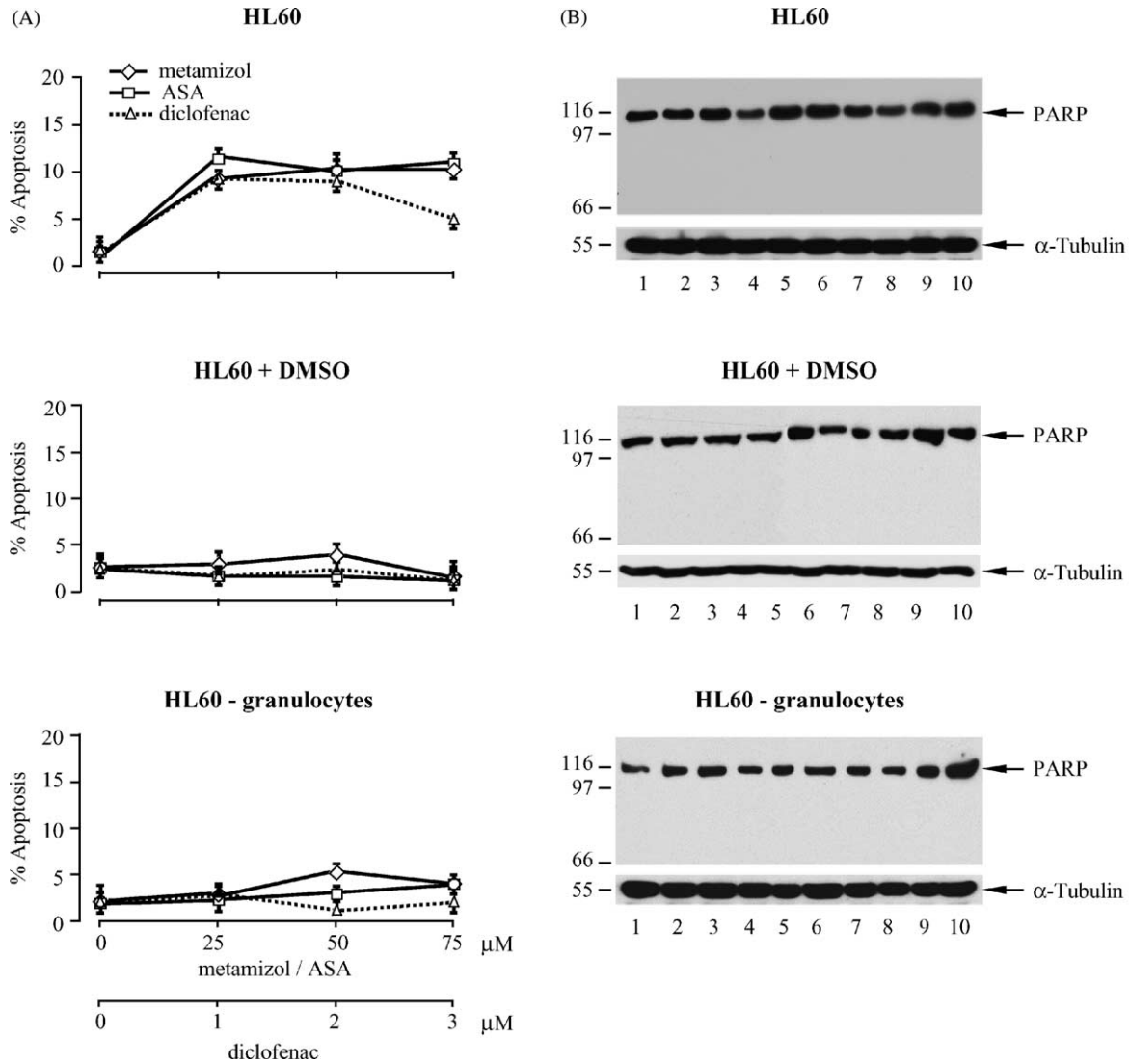

Fig. 4. Analysis of the apoptosis induced by metamizol, ASA or diclofenac on HL60, HL60 + DMSO and HL60-granulocytes. HL60 cells were seeded at 10^4 cells/mL, and incubated in absence (HL60) or presence of 1.3% (v/v) DMSO (HL60 + DMSO) for 3 days with different concentrations of metamizol, ASA or diclofenac. Another aliquot of cells was incubated for 3 days with 1.3% (v/v) DMSO (HL60-granulocytes), then the culture was resettled at 10^4 cells/mL and cells were maintained for another 24 hr in the presence of different concentrations of metamizol, ASA or diclofenac. (A) All cultures were harvested, fixed, stained with PI and analysed by flow cytometry DNA content as described in Section 2. Data represent the mean ± SD of three independent experiments carried out in triplicates. (B) Cell extracts were prepared, and the equivalent of 20 µg of protein was subjected to SDS-PAGE and Western blotted with anti-PARP antibody or with anti-α-tubulin, as loading control. Samples of cells without NSAID treatment (lane 1) or treated with metamizol (25, 50 or 75 µM, lanes 2, 3 and 4, respectively), ASA (25, 50 or 75 µM, lanes 5, 6 and 7, respectively) or diclofenac (1, 2 or 3 µM, lanes 8, 9 and 10, respectively) were analysed. This is representative of three independent experiments.

coats were treated for 24 hr with metamizol, MAA, and ASA (75µM or 10 mM) or with equivalent doses of diclofenac (3 or 400 µM). The granulocytes were purified and their degree of apoptosis determined as before. Metamizol, MAA, and ASA, at the highest pharmacological dose (75 µM), induced 3.9 ± 0.6%, 7.85 ± 1.0%, and 10.0 ± 4.5% of apoptotic granulocytes respectively, while diclofenac treatment provoked about 16.0 ± 1.8% of apoptosis, as compared to 4.3 ± 0.5% of apoptosis in control cells. At concentrations well over the pharmacological range, 10 mM for metamizol, MAA and ASA, or 400 µM for diclofenac, these NSAIDs induced about 90% of agranulocytosis (Fig. 5). Experiments carried out with

Table 1
Viability of metamizol-, ASA-, and diclofenac-treated HL60 cells

	24 hr	48 hr	72 hr
Control	97.0 ± 0.73	97.4 ± 1.27	97.5 ± 0.36
Metamizol	88.6 ± 0.67	91.3 ± 0.23	94.0 ± 0.25
ASA	95.0 ± 0.13	97.0 ± 0.45	97.7 ± 0.16
Diclofenac	96.7 ± 0.32	97.7 ± 0.33	96.8 ± 0.95

Cells were seeded at 10^4 cells/mL and treated with 75 µM of metamizol or ASA, or with 3 µM diclofenac and aliquots of cells were collected after 1, 2 or 3 days. Cells were then harvested by centrifugation, washed with PBS and resuspended in 0.5 mL of PBS containing 10 µg/mL PI. The cell viability was determined by both cell morphology and by their passive permeability to PI, as described in Section 2. The table shows the mean ± SD of three independent experiments, made in triplicates.

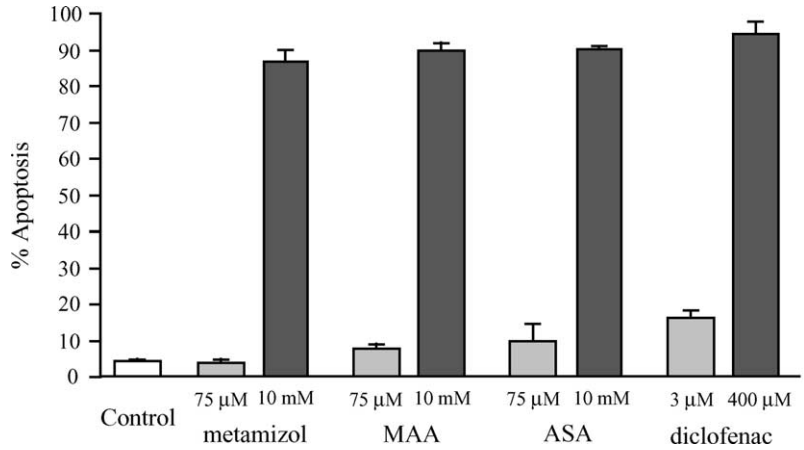

Fig. 5. Quantification of apoptosis induced by metamizol, MAA, ASA or diclofenac on human granulocytes. Freshly obtained human buffy coats were diluted 5-fold in PBS and incubated in absence or presence of metamizol, MAA, ASA (75 µM or 10 mM) or diclofenac (3 µM or 400 µM) for 24 hr at 37°. Then, granulocytes were purified as described in Section 2, fixed, stained with PI and analysed by flow cytometry DNA content as described in Section 2. Data represent the mean ± SD of three independent experiments carried out in triplicates.

purified granulocytes from buffy coats gave similar results (data not shown).

4. Discussion

Here we have analysed the effects of metamizol on granulocytes using, as a model system, the HL60 promyelocytic cells alone or in the presence of DMSO, which induces their granulocytic differentiation described [15]. Metamizol is a pro-drug of MAA since it is immediately hydrolysed in solution. According to pharmacokinetic studies in humans, after oral administration of metamizol at high dose (up to 2 g) MAA concentration reaches a plasmatic peak of ~30 µM and only for few minutes since the half-live of MAA is 4 hr [22]. In most of the experiments described here, we used 75 µM metamizol, that is more than 2-fold of the maximal blood concentration of its main metabolite found in humans, to guarantee a maximal exposition to this drug and its main metabolite. Under these experimental conditions, metamizol (75 µM) and/or MAA did not alter the DMSO-induced granulocytic differentiation of HL60 promyelocytic cells. The up-regulated expression of CD11b, CD11c (Fig. 1), CD45 and c-Src, as well as the increased activity of c-Src (p-c-Src) (data not shown) associated with the differentiation process were unaffected by the presence of metamizol or MAA. Cessation of cell proliferation and accumulation of cells into the G_1 phase of the cell cycle was also observed; this event, that is associated with the differentiation of HL60 to granulocytes induced by DMSO, was unaltered by these drugs. Therefore, we can conclude that metamizol and MAA at the highest pharmacological concentrations did not modify the granulocytic differentiation program of HL60-DMSO treated cells. These results also show that HL60 or HL60-granulocytes were not detected at the sub-G_1 phase of the cell cycle, indicating that at this concentration (75 µM) metamizol or MAA did not induce agranulocytosis.

The NSAIDs are known to induce apoptosis, and sometimes necrosis, in a variety of cell types, including chick embryo fibroblasts, HL60, B-CLL and different tumoral cells [5–8,32]. We analysed the apoptotic action of metamizol and the spontaneously generated MAA on HL60 promyelocytic and on granulocytic differentiated cells and found that, as certain NSAIDs, metamizol induces apoptosis but only at concentrations that exceed by far the pharmacological range. Under the normal limits of metamizol concentrations, between 20 and 75 µM, the percentage of apoptotic cells is about 10%, and only at doses which are cytotoxic, over 100 µM, metamizol causes apoptosis in more than 30% of the HL60 population. The action of metamizol was then compared to that of ASA and diclofenac. Since the plasma concentration peaks for analgesic and antipyretic doses of ASA and diclofenac range from 15 to 100 µM and 1 to 3 µM, respectively [23–25], equivalent concentrations of these drugs were used (75 µM of metamizol, MAA or ASA, or 3 µM diclofenac). The results showed that these three compounds have no apoptotic effects on the promyelocytic HL60 cells and on the DMSO-differentiated granulocytic cells. In our experience, flow cytometry detection of apoptotic cells by propidium iodide staining, shows background values ranging between 1 and 10% in many different cell types. HL60 cell line and mature blood derived granulocytes do not exhibit a different behaviour, consequently we have considered as relevant apoptotic cellular response only values over 10%. Sulindac, an aspirin-like compound, that reaches about 10 µM at plasma levels never induce a significant grade of apoptosis on HL60 cells [8].

The data presented here demonstrate that even at a high pharmacological concentration (75 µM) metamizol or MAA do not induce apoptosis or necrosis on HL60 promyelocytic cells or on granulocytic differentiated cells.

Nevertheless, it could be argued that HL60 is a promyelocytic established cell line that may have a different sensitivity to these NSAIDs than freshly derived blood granulocytes. However, when human buffy coats (Fig. 5) or purified granulocytes (data not shown) from peripheral blood were treated with 75 μM of metamizol, MAA or ASA, or 3 μM diclofenac, did not exhibit a relevant degree of apoptosis. Only at toxicological/suprapharmacological concentrations, equivalent to millimolar range, they caused over 90% agranulocytosis. This last observation is consistent with other studies demonstrating the agranulocytotic action of NSAIDs in several cell lines [5–8].

Clinically, agranulocytosis consists of a vast lost of granulocytes from peripheral blood. Here, we found that promyelocytic HL60 cells, HL60-granulocytes, buffy coats of purified granulocytes treated with high pharmacological doses of metamizol, MAA, ASA or diclofenac showed very minor effects on cell viability or apoptosis. Therefore, we propose that metamizol, in the upper range of *in vivo* concentration do not induce apoptosis neither provoke toxicity on granulocytes. These results also indirectly support that the mechanism of metamizol-induced agranulocytosis should be of immunoallergic origin since a toxic effect of this drug is excluded.

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