Metamizole (dipyrone) – cytotoxic and antiproliferative effects on HeLa, HT-29 and MCF-7 cancer cell lines

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ABSTRACT
Cancer pain treatment is a big challenge for healthcare providers and patients as well. The wide range of non-steroidal anti-inflammatory drugs (NSAIDs) used as painkillers in cancer patients, requires in-depth characterization of their effect on the disease process. The effects of NSAIDs have been widely studied over the last decades as preventive drugs in some oncological diseases. Metamizole is an NSAID belonging to the non-narcotic analgesics group and is highly recommended in oncology either alone or in combinations with opioid analgesics. There is a dearth of information regarding the cytotoxicity profile of metamizole and hence the present study evaluated the potential anticancer activity of metamizole in some permanent human tumour cell lines: HeLa, human cervical cancer cells; HT-29, a human colorectal adenocarcinoma cell line; and MCF-7, human breast adenocarcinoma cells. The studied tumour cells were sensitive to metamizole at doses higher than 25 μg/mL. Metamizole induced a statistically significant decrease in the viability of HeLa, HT-29 and MCF-7 cells in vitro tests as measured by the MTT assay; the highest effect was observed at the 48th hour of the treatment. Metamizole could induce cell death by apoptosis. Metamizole also suppressed the migration of the three tumour cell lines. This was most clearly pronounced in HeLa cells. The results obtained indicate that metamizole is a suitable choice for the treatment of cancer pain and has prospects for further in-depth studies.

INTRODUCTION
Cancer pain is one of the most frightful factors for patients and a big challenge to healthcare providers. More often than not, patients with malignant diseases will experience recurrent episodes of pain. Nonsteroidal anti-inflammatory drugs (NSAIDs) are a first-line approach for mild cancer pain. NSAIDs (including non-opioid analgesics) in combination with opioid therapy are the choice in moderate cases, and opioids in severe ones. On the other hand, numerous non-clinical and clinical studies have demonstrated that NSAIDs have potential as anticancer drugs [1–3]. Epidemiological studies have demonstrated that NSAIDs use is associated with a reduced risk of incidence and mortality from colon cancer [4]. In addition, experimental studies have shown that NSAIDs induce apoptosis [5]. Piazza et al. [6] found that sulindac did not induce differentiation of HT-29 cells, but strongly induced apoptosis. Although the precise mechanism of this action is unknown, Chan et al. [7] proposed that inhibition of cyclooxygenase (COX) stimulates the conversion of sphingomyelin to ceramide, a known mediator of apoptosis. Shiff et al. [8] found that indomethacin, naproxen and piroxicam reduced the proliferation and induced apoptosis of HT-29 colon adenocarcinoma cells.

Metamizole is a popular non-opioid analgesic in many countries, but unknown in many others [9, 10].
Due to its prohibition in the USA in 1977 and many other countries that followed suit, metamizole remains less studied than other NSAIDs. A PubMed search with the key words ‘metamizole’ and ‘apoptosis’ revealed only a few studies. Malsy et al. [11] found that metamizole significantly inhibited cell proliferation and induced apoptosis in pancreatic cancer cells. Metamizole alone or in combination with paracetamol increased apoptosis in colon carcinoma cell lines (SW 480 and HT 29) in concentrations of 1 µmol/L, 250 µmol/L and 1 mmol/L [12]. Shao and Feng [13] reported that metamizole inhibited the proliferation of A549 (human lung carcinoma) cell line in a dose-depending manner, but not of HeLa. The antiproliferative effect was explained by the arrest in G1-phase of the cell cycle. Metamizole may inhibit JNK activation as well as TNF-α production [14]. The treatment of osteosarcoma MG-63 osteoblast-like cells with metamizole showed an increase in apoptotic osteoblasts that later developed into necrotic cells [15]. On the contrary, Zhang et al. [16] demonstrated that metamizole has remarkable neuroprotective effect in cerebral ischemia, identifying metamizole as an anti-apoptotic agent.

In the context of the scarce and partly conflicting available data, the main purpose of this study was to investigate the cytotoxic potential of metamizole in HeLa, HT-29 and MCF-7 human tumour cell lines.

**Materials and methods**

Metamizole sodium (Analgin 500 mg/mL amp.; 2 mL, Sopharma; Batch No. 61116) was supplied by a local pharmacy. The solutions were diluted *ex tempore*. Acridine orange (Cat. N. A 6014), ethidium bromide (Cat. N. 46065) and penicillin–streptomycin (P 4333-100 mL) were purchased from Sigma-Aldrich Chemie GmbH (Darmstadt, Germany). Fetal bovine serum (FBS) was supplied from Gibco (Austria). Dulbecco’s Modified Eagle’s Medium with 4.5 g/L glucose, without L-glutamine was purchased from LONZA, Belgium, and DAPI (4′,6-diamidino-2-phenylindole) was supplied from AppliChem (Darmstadt, Germany).

**Cell lines, maintenance and treatment**

HeLa human cervical cancer cells, HT-29 human colorectal adenocarcinoma cells and MCF-7 human breast adenocarcinoma cells were obtained from the American Type Cultures Collection (ATCC). The cells were maintained in DMEM containing penicillin (100 units/mL), streptomycin 100 µg/mL, 2 mmol/L L-glutamine and 10% FBS in T-75 cm² culture flasks at 37°C in an incubator with 5% CO₂ supply and 95% humidity (CO₂-incubator, model 3111; Thermo Fisher Scientific USA).

The cells (HeLa, HT-29 and MCF-7) were plated at a density of 1 × 10⁵ cells per well in polystyrene, flat bottom 96-well microtiter plates (Corning Costar, Rochester, NY, USA) in DMEM containing 10% FBS and allowed to adhere overnight in a CO₂ incubator at 37°C. At the end of the incubation, the cells were treated with different concentrations of metamizole (0, 15, 90, 150, 300 and 400 µg/mL) in a final volume of 100 µL/well in triplicate wells for each treatment for 24 h or 48 h at 37°C in a 5% CO₂ incubator. All studies were repeated at least twice. Control cells were grown in DMEM containing 10% FBS and allowed to stabilize overnight in a CO₂ incubator at 37°C and left without treatment.

**MTT test**

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) test is colorimetric assay first described by Mosmann [17]. It is based on the reduction of yellow tetrazolium salt MTT to purple formazan by the mitochondrial enzymes of viable cells. The amount of the formed formazan was measured spectrophotometrically at 540 nm and 620 nm (as a reference) wavelength by an ELISA spectrophotometer (TECAN, SunriseTM, Grödig/Salzburg, Austria) and the cell vitality was calculated using the following formula: CV (%) = [OD540 (test)/OD540 (control)] × 100.

**Wound-healing assay (scratch assay)**

The wound-healing assay (scratch assay) was used for evaluation of the effect of metamizole on the tumour cell migration [18]. The wound-healing test analyses the filling of a wound (created mechanically by making a scratch) in the cell monolayer. The filling of the cut with proliferating cells migrating from the periphery to the centre was observed at equal time intervals and was captured by a camera. The area was quantified. For the evaluation of cell migration, we used concentrations of metamizole (HeLa - 90 µg/mL, HT-29 - 150 µg/mL and MCF-7 - 15 µg/mL) that are approximately equal to half of the IC₅₀ value (half maximal inhibitory concentration) measured at the 48th hour by the MTT test.

Twenty-four hours before the experiment, the three cell lines were grown in 24-well cell culture plates at a concentration of 2.5 × 10⁵ cell/mL (1 mL/well) in DMEM medium supplemented with 10% FBS, at 5% CO₂, 37°C and 95% humidity to form a monolayer. In
the day of the experiment, a vertical wound down through the cell monolayer (volume 100 µL) was made and the cultures were treated with metamizole (1 mL of DMEM plus 10% FBS with the desired concentration of metamizole for each cell line). The cell cultures were then incubated under standard conditions as described above. At several time points (0 h, 24 h, 48 h and 72 h), snapshots were taken with an inverted microscope Olympus with a digital camera to evaluate the degree of cell migration (based on the measured area). Control cells from the corresponding cell line were grown without treatment.

**Fluorescence microscopy detection of apoptosis**

**Acridine orange/ethidium bromide staining**

The cytomorphology changes in the tumour cells cultured in the presence of metamizole were studied after double intravital staining with fluorescence acridine orange (AO) and ethidium bromide (EtBr) according to the standard procedure [19].

The tumour cells in a concentration of 1 × 10^5 cells/mL were seeded on sterile glass lamellas placed on the bottom of 24-well plates in DMEM supplemented with 10% FBS and were grown for 24 h in CO_2 to form a monolayer. On the next day, the cells were treated with metamizole in concentrations equal to I_{C50} measured at the 24th hour in the MTT test (400 µg/mL in HeLa, 300 µg/mL in HT-29 and 400 µg/mL in MCF-7) and were grown in CO_2 in standard conditions for another 24 h. Control cells from the corresponding cell line were grown without treatment. After the incubation period, the cell lines were washed twice with phosphate buffered physiological solution (PBS) to remove the non-adherent cells. Equal volumes of fluorescence dyes, prepared ex tempore containing acridine orange (10 µg/mL) and ethidium bromide (10 µg/mL) were used. The freshly stained tumour cells were evaluated and photographed for a period of 10 minutes under a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany) before the fluorescence staining started to fade.

Acridine orange stains both live and dead cells, emitting strong yellow-green fluorescence, as a result of the intercalation in the double-strained DNA, as well as red-orange fluorescence resulting from the binding with single-strained RNA. EtBr is a fluorochrome that passes through intact cytoplasmic membrane, and after intercalation in DNA emits orange fluorescence. EtBr stains dead cells and late apoptotic cells, which have altered cell permeability.

**DAPI staining**

The cell lines were stained with the fluorescent dye DAPI to assess the nuclear morphology. DAPI staining is a reliable method to observe apoptotic changes in the cell nucleus. DAPI is highly selective for binding with the clusters of DNA rich in adenine and thymine but has lower binding affinity to RNA. The molecule of DAPI can pass through intact cytoplasmic membrane, which makes it a useful agent for studying the nucleus morphology of live as well as fixed cells. Typical signs of apoptosis of the nucleus are condensation and margination of nuclear chromatin, fragmentation of the nucleus and the formation of apoptotic bodies.

Cell lines in a concentration of 1 × 10^5 cells/mL in DMEM supplemented with 10% FBS, seeded in sterile glass lamellas placed on the bottom of 24-well plates were grown for 24 h to form a cell monolayer. On the next day, the cells were treated with metamizole in concentrations of 400 µg/mL in HeLa, 300 µg/mL in HT-29 and 400 µg/mL in MCF-7 cells, and were grown for another 24 h in standard conditions. Control cells from the corresponding cell line were grown without treatment. After 24 h, any non-adherent cells were removed by washing with PBS. The staining with DAPI was done after fixing the cells with methanol according to the manufacturer’s protocol. The glass lamellas with the stained cells were mounted with Mowiol medium on microscope slides and were stored in darkness until they were observed under a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany).

**Statistical analysis**

The statistical significance of the results was assessed by analysis of variance (ANOVA), followed by post-hoc comparison test (Bonferroni) with the use of GraphPAD PRISM software, version 5 (GraphPad Software Inc., San Diego, USA). The data are presented as mean values with standard deviation (±SD). Probability values of \( p < 0.05 \) were considered statistically significant.

**Results and discussion**

**Evaluation of the cell viability by MTT test**

Several studies have shown that NSAIDs and COX-2 inhibitors suppress the proliferation and induce cell death in various cultured cell lines, as well as potentiate the cytotoxic effects of some antineoplastic drugs through COX-dependent and COX-independent mechanisms [20–31]. In the present study, the results from
the MTT test showed a concentration- and time-dependent antiproliferative effect of metamizole against HeLa, HT-29 and MCF-7 tumour cells (Figure 1). There was a statistically significant decrease \((p < .01)\) in the cell viability of the treated tumour cells compared to the untreated group at the 24th hour in concentrations higher than 25 \(\mu\)g/mL. On the 48th hour, however, a decrease in the cell viability of the HeLa cell line was noticeable in metamizole concentrations higher than 12.5 \(\mu\)g/mL. In HT-29 and MCF-7 cells, the effect of metamizole at the 48th hour was evident in all the tested concentrations. The IC50 values of metamizole on the 24th and 48th hour for the three cell lines were as follows: HeLa – 472.475 \(\mu\)g/mL and 183.401 \(\mu\)g/mL; HT-29 – 314.750 \(\mu\)g/mL and 316.765 \(\mu\)g/mL; MCF-7 – 561.792 \(\mu\)g/mL and 29.297 \(\mu\)g/mL, respectively.

Figure 1. Effect of different concentrations of metamizole on the proliferation of HeLa, HT-29 and MCF-7 cells measured on the 24th and 48th hour using MTT test. The results are mean values ± SD. *\(p < .05\); **\(p < .01\); ***\(p < .001\)
The wound-healing assay studies the migration of a cell population through the speed of proliferation and gap closure. McDonald et al. [32] measured celecoxib motility with the scratch assay on HT-29 cells, showing no significant percent of wound closure, whereas Li et al. [33] indicated that celecoxib can cause a dose-dependent inhibition of the migration of the human pancreatic carcinoma, epithelial-like cell line PANC-1. Wynne and Djakiew [34] demonstrated that decreased cell migration in a prostate cancer cell line (PC-3) treated with enantiomer R-flurbiprofen and ibuprofen is mediated by NSAID-activated gene-1 (Nag-1) protein and neurotrophin receptor p75NTR. Aspirin treatment also delayed the healing after scratching in a concentration-dependent manner in murine breast cancer cells (4T1) [35]. Similar results were obtained with ibuprofen about the migration of human coronary artery smooth muscle cells (HCASMCs) [36].

**Wound-healing assay (scratch assay)**

Based on the migration activity measured with the scratch wound-healing assay, the lowest migration potential was observed in the HT-29 cell line compared to HeLa and MCF-7 tumour cells. This study showed for the first time that metamizole affected the migration of HeLa, HT-29 and MCF-7 human tumour cells. Metamizole demonstrated statistically significant inhibition of the migration compared to the corresponding control at the 24th hour of treatment in HeLa cells (60.79 ± 1.21% for metamizole-treated cells and 88.92 ± 2.69% for the control); at the 48th hour in HT-29 cells (17.87 ± 1.07% for metamizole-treated cells and 25.81 ± 1.79% for the control) and at the 48th and the 72nd hour in MCF-7 cells (82.86 ± 1.29% and 95.96 ± 1.81% for metamizole-treated cells and 86.49 ± 1.29% and 100% for the control). The migration of HeLa cells at the 48th and 72nd hour, that of HT-29 cells at the 24th and the 72nd hour, and of MCF-7 cells at the 24th hour was not significantly reduced compared to the controls (Figure 2). Overall,
metamizole inhibited the migration of the three tumour cell lines, but the effect was statistically significant compared to the control in the HeLa cells at the 24th hour, in the HT-29 cells at the 48th hour and MCF-7 at the 48th and 72nd hour of treatment (Figure 2).

**Fluorescence detection of apoptosis**

Apoptosis of mammalian cells is accompanied by various morphological changes including cell shrinkage, cytoplasm and chromatin condensation, degradation of nuclear DNA into oligonucleosomal fragments and release of signalling molecules that attract phagocytes [37–39]. Apoptosis can be visualized using different dyes. Acridine orange is an acidophilic dye that accumulates in areas of high lysosomal and phagocytic activity, whereas ethidium bromide is an intercalating agent used to visualize DNA [39].

**Acridine orange/ethidium bromide staining**

The control cells developed normal morphology and monolayer growth typical of the corresponding cell type (Figures 3(a), 3(c), 3(e)). Treatment with metamizole (400 μg/mL for HeLa, 300 μg/mL for HT-29 and 400 μg/mL for MCF-7 cells) resulted in the appearance of early apoptotic cells with bright green nuclei with chromatin condensation in the form of intense green areas and late apoptotic cells with orange nuclei with chromatin condensation. Typical signs of apoptosis were observed, such as cell shrinking, cell wrinkling, nucleus fragmentation and apoptotic bodies (Figure 3).

**Figure 3.** Fluorescence microscopy of human tumour cells after 24 h treatment with metamizole. HeLa control (a); HeLa metamizole 400 μg/mL (b); HT-29 control (c); HT-29 metamizole 300 μg/mL (d); MCF-7 control (e); MCF-7 metamizole 400 μg/mL (f). Magnification 40X; AO/EtBr staining.
Metamizole induced marked alterations in the growth and morphology of the HeLa cells. The cell monolayer was damaged; the cells were oval to polygonal and early apoptotic cells (shrunk oval cells with condensation of the chromatin) were observed. Those at late apoptosis were predominant, characterized with cell blebbing (blistering of the cell membrane), chromatin condensation and margination, nuclear fragmentation and apoptotic bodies. The presence of dead and destructed cells with pycnotic nuclei was indicative of late apoptosis and significant metamizole-induced cytotoxic effect (Figure 3(b)). The antineoplastic effect of metamizole was less pronounced in HT-29 compared to HeLa cells. There were morphologically altered cells with signs of early apoptosis (bright green nuclei with chromatin condensation in the form of dense green areas) and single cells with signs of late apoptosis (a bright orange coloured nucleus with condensed, aggregated and fragmented chromatin) (Figure 3(d)). The metamizole treatment of the MCF-7 cells resulted in the appearance of cells in the early stages of apoptosis (green nuclei with chromatin condensation) as well as single cells with signs of late apoptosis (bright orange stained nuclei with margination and chromatin condensation) and cells with typical signs of apoptosis - cell blebbing, fragmentation of the nuclei and apoptotic bodies (Figure 3(f)).

The results demonstrated that the tested HeLa, HT-29 and MCF-7 tumour cells were sensitive to metamizole. They showed different degrees of morphological and growth changes, indicative of an early or late
apoptosis, after metamizole treatment. Some of the signs of metamizole-induced apoptotic cell death included bright green nuclei with condensation of the chromat in appearing as intense green areas (early apoptotic cells) and orange nuclei with condensation and chromat in margination (late apoptotic cells). These findings are in accordance with the MTT test results and indicate that the induction of apoptosis could be one of the antineoplastic mechanisms of metamizole.

**DAPI staining**

We used DAPI staining to analyse the morphological alterations in the nucleus of metamizole treated cells (concentrations of 400 µg/mL, 300 µg/mL and 400 µg/mL, corresponding to HeLa, HT-29 and MCF-7 cells) observed at the 24th hour (Figure 4). The non-treated controls of HeLa, HT-29 and MCF-7 cells had intact nuclei, equal in shape and size, with smooth edges and homogenously spread chromatin (Figures 4(a), 4(c), 4(e)). In the control MCF-7 cultures, cells in the phase of mitosis were also observed (Figure 4(e)). On the contrary, the treatment of HeLa, HT-29 and MCF-7 cells with metamizole led to significant alterations in the nucleus morphology typical of induction of cell death by the pathway of apoptosis: blebbing, pyknosis, nucleus fragmentation and granulated apoptotic bodies.

The observed morphological changes in HeLa cells after the treatment with metamizole had clear signs of apoptosis: different shape and size, with clear fragmentation of the nucleus, condensation of the chromat in and multiple apoptotic bodies (Figure 4(b)). In the HT-29 cells treated with metamizole, there were nuclei with uneven edges, condensed chromat in and single fragmented nuclei (Figure 4(d)). The nuclei of metamizole treated MCF-7 cells were single and fragmented, increased in size with condensed chromat in, and multiple apoptotic bodies (Figure 4(f)).

There is scarce information in the literature on any anticancer properties of metamizole. NSAIDs are best studied as chemopreventive agents in colorectal cancer [40–42]; however, the concentrations of NSAIDs used there are much higher than those required for COX inhibition. Several molecular COX-dependent and COX-independent mechanisms of antineoplastic effects of NSAIDs have been proposed, suggesting that complex cellular and molecular pathways are involved in induction of apoptosis, autophagy, inhibition of proliferation and prevention of cancers [43–45]. The exact mechanism depends on the cancer type and particular NSAID. NSAIDs are a structurally diverse group of chemical compounds. The amphiphilic nature of NSAIDs allows them to interact with lipid membranes, modulate the membrane biomechanical properties and cell signalling pathways [46].

There are literature data suggesting that NSAIDs could reduce the risk of several common cancers [47–51]. However, there is no definite evidence that the pre-diagnostic use of NSAIDs is associated with higher survival of cancer patients [52–55].

To date, several pyrazolone derivatives have been shown to possess promising antiproliferative activity in human cancer cell lines [56–61]. Metamizole is pyrazolone derivative with pronounced analgesic, antipyretic, spasmylytic properties and favourable gastro-intestinal safety profile [62,63]. The drug also possesses weak anti-inflammatory and mild antiaggregant activities [64]. However, metamizole is banned in many countries due to bone marrow toxicity [65].

Herein, the antitumour effects of metamizole on three types of human tumour cell lines (HeLa, HT-29 and MCF-7) were investigated. Metamizole has central as well as peripheral mechanisms of action that do not solely rely on cyclooxygenase (COX) inhibition. In the literature there are diverse data considering COX expression in different cell lines [66–69]. HeLa, HT-29 and MCF-7 cell lines have high expression of COX-1, but relatively low of COX-2 [70–72]. Metamizole and its metabolites have been shown to inhibit COX-1 and COX-2 activity by a different pathway than the classical aspirin-like NSAIDs mechanism [73]. The complex mechanism of action of metamizole is still obscure, as it is not a typical NSAID, but is rather classified as a non-opioid analgesic.

**Conclusions**

In the present study, HeLa, HT-29 and MCF-7 human tumour cells were sensitive to metamizole as observed after the 24th and the 48th hour of the study. Concentrations higher than 25 µg/mL significantly decreased the viability/proliferation of HeLa, HT-29 and MCF-7 human tumour cells in in vivo tests as measured by the MTT assay, with a better effect observed at the 48th hour of treatment. Based on the IC50 values at the 48th h, the MCF-7 cells (human breast adenocarcinoma cells) were most sensitive, followed by HeLa (human cervical cancer cells) and HT-29 (human colorectal adenocarcinoma cell line). The antiproliferative effect of metamizole was achieved at least partly by induction of apoptosis as observed by fluorescence. Dual AO/EtBr staining enabled the observation of cells in early and late apoptosis (cell
membrane blebbing with condensation and margination of chromatin, nuclear fragmentation and apoptotic bodies) after metamizole treatment. DAPI staining also indicated morphological changes typical of apoptotic cell death (aggregated and fused chromatin, fragmented nuclei). Metamizole induced significant suppression of cell migration in HeLa, HT-29 and MCF-7 tumour lines. According to the migration activity in the scratch wound-healing assay, HeLa cells had the highest migration potential, it was medium for MCF-7 and low for HT-29. Our study demonstrated a high anti-proliferative effect of metamizole achieved by induction of cell death via the apoptotic pathway. There was decreased migrability in all three types of studied human tumour cell lines. The obtained results warrant further research on the anticancer properties of metamizole to unravel the biological targets of carcinogenesis and mechanisms for the tumour suppressing activity of NSAIDs.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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