CYTOTOXICITY OF SOME NITROIMIDAZOLE DERIVATIVES - COMPARATIVE STUDIES ON HUMAN AND RAT HEPATOMA CELL LINES

LIDIA RADKO AND MARIA MINTA

Department of Pharmacology and Toxicology, National Veterinary Research Institute, 24-100 Pulawy, Poland
lidia.radko@piwet.pulawy.pl

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Abstract

The cytotoxic potential of metronidazole, tinidazole, ronidazole, and ornidazole, using human and rat hepatoma cell lines (HepG2 and FaO) in culture was assessed. The cells were treated with drugs for 24, 48 and 72 h at 37 °C in 5% CO2 at concentrations of 0.1 to 200 µg/mL. Following the treatment period, the cells were assayed by four independent assays: MTT reduction, neutral red uptake (NRU), total protein content (TPC), and LDH leakage. The results suggest that nitroimidazoles are of low cytotoxic potential (EC50>200 µg/mL). The exception was ronidazole, which demonstrated a distinct endpoint sensitivity related to the species. EC50 (µg/mL) in human cells were: in MTT assay - 196±5.5 and 122±9.3 at 24 and 48 h, respectively, and in NRU assay - 150±1.25 at 72 h. Based on minimal toxic concentrations (EC50) for ronidazole, determined by all methods used in HepG2 cells, it could be concluded that their sensitivity was as follows: MTT>NRU>LDH>TPC.

Key words: hepatoma cell lines, nitroimidazoles, cytotoxicity.

Several derivatives of nitroimidazole constitute the class of antibiotics that have been used to combat anaerobic bacterial and parasitic infections in humans and pets. In human patients, nitroimidazoles are well tolerated and this fact confirms their broad use. However, this group of drugs has documented mutagenic and carcinogenic properties (15), therefore, the use of these drugs in livestock is banned to avoid residues (metabolites) in food (1). Their acute toxicity is low and vary among animal species. Oral LD50 (mg/kg) of metronidazole is <5,000 (rat), 4,350-5,000 (mouse), >750 (dog); tinidazole - 2,000 (rat); ronidazole - 2,850–3,140 (rat), 2,330–2,440 (mouse), 1,250 (rabbit), and ornidazole - 1,789 (rat), 1,420 (mouse) (1). Toxicity studies in vitro are scanty (7, 9, 16).

The aim of the study was to gain more insight to the toxicity of metronidazole, tinidazole, ronidazole, and ornidazole in human and rat hepatoma cell lines (HepG2, FaO). The HepG2 line has been chosen since it constitutes the best characterised human liver cell line. It is widely used in cytotoxicity experiments because of its higher predictability for humans compared to other cell lines (5, 13, 17-19). The FaO line has been isolated from the H4IIEC3 rat hepatoma cell line. It is well characterised and useful for examining the metabolism of cytotoxic substances (22).

Material and Methods

Reagents. Triton X-100, neutral red (NR), dimethyl sulfoxide (DMSO), foetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), coomassie brilliant blue R-250 dye (CBB), trypsin-EDTA, and antibiotic solution (10,000 U/mL of penicillin, 10 mg/mL of streptomycin) were purchased from Sigma–Aldrich (St. Louis, USA). All other chemicals were purchased from commercial suppliers and were of the highest available purity.

Drugs. Metronidazole, tinidazole, ronidazole, and ornidazole were purchased from Sigma–Aldrich (St. Louis, USA).

Cell lines and culture conditions. HepG2 cells (ATCC HB-8065) were cultured in Minimum Essential Medium Eagle (MEME) medium (ATCC). FaO cells (ECACC 89042701) were cultured in F-12 nutrient mixture (Ham), Kaighn’s modification, with L-glutamine medium (GIBCO). The media were supplemented with 10% foetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 µg/mL). The cells were maintained in 75 cm2 cell culture flasks (NUNC) in humidified incubator at 37°C in an atmosphere of 5% CO2 in air. The medium was refreshed every 2 or 3 d.
and the cells were trypsinised by 0.25% trypsin–0.02% EDTA after the cells reached 70%–80% confluence.

**Exposure and assessment of cytotoxicity.** Single cell suspensions were prepared and adjusted to a density of 2x10^5 cell/mL (HepG2) and 2.5x10^5 cell/mL (FaO). One hundred microlitre of cell suspension was seeded in every well of 96-well microtiter plate and incubated for 24 h. The drugs were dissolved in DMSO. The control cells were treated with solvent (0.1% DMSO) only. Eight concentrations ranging from 0.1 to 200 µg/mL were applied. Viability/cytotoxicity was assessed after 24, 48, and 72 h of exposure using four assays described below.

**MTT reduction assay.** The cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). MTT was dissolved in phosphate buffered saline (5 mg/mL), sterilised by filtration through a 0.22 µm Millipore® filter. A volume of 10 µl was added to every well of microplate and cultures were allowed to incubate for further 4 h at 37°C in 5% CO2 humidified atmosphere. The medium was removed and the intracellular formazan crystals were dissolved in 100 µl of DMSO. The plate was shaken for 15 min at room temperature and transferred to a microplate reader (Multiscan RC Labsystems) to measure the absorbance at 570 nm. The mean optical density (OD) was used to calculate the percentage of cell survival for each dilution of test sample.

**Neutral red uptake (NRU assay).** Medium containing drug was removed and cells were washed with PBS-Ca^2+. Then 100 µL/well of NR solution (50 µg/mL in PBS) was added for 3 h. After this time, the cells were washed again with PBS-Ca^2+. The dye from viable cells was released by extraction with a mixture of acetic acid, ethanol, and water (1:50:49, v/v). After 10 min shaking, the absorbance of the dissolved NR was measured at 540 nm.

**Total protein content (TPC assay).** Medium containing drug was removed and 100 µl of coomassie brilliant blue R-250 dye (CBB) was added to each well. The plate was shaken for 10 min. Then, the stain was removed and the cells were rinsed twice with 100 µl of washing solution (glacial acetic acid/ethanol/water – 5/10/85, v/v). After that, the washing solution was replaced with 100 µl of the desorbing one (1 M potassium acetate) and plates were shaken again for 10 min. The absorbance was measured at 595 nm in microplate reader.

**LDH leakage assay.** The release of LDH from the cytoplasm into the surrounding culture medium was monitored using the commercially available cytotoxicity detection kit (LDH) (Roche Diagnostics, Poland). Medium (100 µL/well) without cells was transferred into corresponding wells of an optically clear 96-well flat bottom microplate and 100 µl of reaction mixture (freshly prepared) was added to each well. Then plates were incubated for 30 min at room temperature in dark conditions. After that time, to stop reaction, 50 µL/well of 1M HCl was added. The absorbance was measured at 492 nm in a microplate reader. The positive control constituted wells with cells and culture medium with 2% Trixon X-100.

**Statistics.** The results (percentage of control values) are expressed as mean ±SD (standard deviation) of at least three independent experiments. One way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test was applied.

Values indicating minimal and mean cytotoxicity (EC_{20} and EC_{50}) at three time points (24, 48, and 72 h) were calculated according to the Hill’s equation (sigmoidal model of concentration-response curve) and expressed as mean ±SEM of three independent experiments. Statistical evaluation was performed using ANOVA followed by Tukey’s post-hoc test. P≤0.05 were considered statistically significant.

**Results**

The response of HepG2 and FaO cells to four nitroimidazole derivatives was assessed by enzymatic conversion of MTT in the mitochondria, determination the accumulation of the neutral red dye in the lysosomes of viable cells, release of the lactate dehydrogenase into the culture medium after cell membrane damage, and protein measurement. The results obtained for both cell lines revealed that all investigated drugs were of low toxicity.

Out of four methods used, MTT assay was the most sensitive. Data obtained from the measurement of the optical density in this assay were transformed as percentages in relation to the control group, considered to be 100% (Fig. 1 and 2). The viability percentages were mostly greater than 50% even with the highest concentrations used. In HepG2 cells, the significant (P<0.05) effects were observed starting from the concentration of 50 µg/mL for ronidazole (24 h), tinidazole (48 h), and ornidazole (72 h) then metronidazole at the concentration of 100 µg/mL (72 h) (Fig. 1). In FaO cells, the significant (P<0.05) effects were observed after 48 h exposure to the concentrations of 10 µg/mL for metronidazole and ornidazole, and 50 µg/mL tinidazole and ronidazole (Fig. 2).

Minimal cytotoxicity concentrations (EC_{20}) are presented in Tables 1 and 2. The values depended on the drug concentration, duration of exposure, assay, and cell line used. In MTT assay, EC_{20} values were calculated for all drugs in both models.

In HepG2 minimal cytotoxicity concentrations were calculated for ronidazole in all assays used, but significant differences were found between each other (Table 1). EC_{20} values obtained in NRU, TPC, and LDH assays were 4 to 5 fold higher than in MTT assay (Table 1). Toxic potential based on the EC_{20} values was as follows: MTT>NRU>LDH>TPC. Ronidazole was also the only drug for which EC_{50} values could be estimated in two assays: MTT (196 and 122 µg/mL after 24 h and 48 h exposure, respectively) and NRU (150 µg/mL after 72 h exposure) (Table 1).
In FaO cells in any case EC<sub>50</sub> value was achieved up to the highest dose used i.e. 200 µg/mL. In the study ornidazole was the only drug for which EC<sub>20</sub> values could be calculated in two assays i.e. MTT - 72.5±8.4 µg/mL (48 h) and 92.9 ± 8.1 µg/mL (72 h) and additionally in LDH - 91.6 ± 7.3 µg/mL (48 h) (Table 2).

**Discussion**

Investigation of *in vitro* toxicity appears to be very important in many contexts. *In vitro* systems are used principally for screening and ranking chemicals and for generating more comprehensive toxicological profiles. They are also of a potential use for studying local or tissue- and target-specific effects. Numerous suitable methods, each with advantages and limitations, were developed in the last years to evaluate cytotoxicity (6). The combined use of four methods, which estimate metabolic and lysosomal activity, membrane integrity, and proliferation, is at present the most practical approach for evaluating basic cellular structures and function (3, 4, 11, 12, 21).

It exists the opinion that the assay selection is the most important factor governing the uniform quality of the data; however, the origin of cell lines used is also of big importance (14). At present, most studies are performed on permanent cell lines (13, 17-19). To assess the impact of the assay selection, estimation of EC<sub>50</sub> is valuable (14).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Time</th>
<th>Metronidazole</th>
<th>Tinidazole</th>
<th>Ronidazole</th>
<th>Ornidazole</th>
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<td>EC&lt;sub&gt;20&lt;/sub&gt;</td>
<td>EC&lt;sub&gt;20&lt;/sub&gt;</td>
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<td>ND</td>
<td>150 ± 4.0</td>
<td>34.3 ± 9.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>122 ±9.3</td>
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<td></td>
<td>72</td>
<td>47.6±6.3</td>
<td>87 ± 1.3</td>
<td>28.0 ± 4.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>ND</td>
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<td>149 ± 6.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>72</td>
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± SEM (n=3). ND – not determined. The different superscripts (A, B, C, D) within columns indicate significant differences between assays at the corresponding time of exposure (P<0.05)

<table>
<thead>
<tr>
<th>Assay</th>
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<th>Metronidazole</th>
<th>Tinidazole</th>
<th>Ronidazole</th>
<th>Ornidazole</th>
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<td>MTT</td>
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<td>48</td>
<td>8.9 ± 0.8</td>
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<td>72</td>
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± SEM (n=3). ND – not determined.
**Fig. 1.** The cytotoxicity of nitroimidazoles after 24, 48, and 72 h exposure of human (HepG2) hepatoma cell lines. The results are expressed as mean ± SD of three independent experiments. *P*≤0.05 in comparison to control.
Fig. 2. The cytotoxicity of nitroimidazoles after 24, 48, and 72 h exposure of rat (FaO) hepatoma cell lines. The results are expressed as mean ± SD of three independent experiments. * P ≤ 0.05 in comparison to control.
In the study, in most cases EC50 values were not determined up to the highest concentration used, i.e. 200 µg/mL. Thus, differences between sensitivity of assays could be assessed on the basis of minimal cytotoxic concentration, i.e. EC20. Minimal toxic concentration values (EC20, µg/mL) ranged from 0 to 200 µg/mL in HepG2 cells and from 0 to 100 µg/mL in FaO cells. The results showed that the ranking of assays sensitivity was as follows: MTT>NRU>LDH>TPC.

The exception was ornidazole for which EC50-MTT (µg/mL) in HepG2 cells was determined (196 ±5.5 and 122 ±9.3 at 24 h and 48 h, respectively) and EC50-NRU (150 ±1.25 at 72 h). Moreover, its minimal toxic effects were noted in all assays used.

In the literature, there are few studies on evaluation of the cytotoxic potential of nitroimidazoles. Ferreira et al. (7) demonstrated that metronidazole at the concentration of 50 µg/mL decreased viability of human fibroblasts (FMM1) by 30% after 24 h exposure, and approximately by 40% after 48, 72, and 96 h treatment. Metronidazole was not cytotoxic in the concentration range from 2.5 to 250 µM (0.43-42.75 µg/mL) in MCF-7 cells, during 48 h incubation, but 30% inhibition was observed after exposure to 250 µM (42.75 µg/mL) (16). Hausen et al. (9) presented that concentration of 84.8 µM (14.5 µg/mL) inhibited viability of IEC-6 cells by 8% (24 h).

Overall, toxic potential of nitroimidazoles in vitro is low and this is in line with the low acute toxicity in vivo (1). Moreover, as regards the hepatotoxicity, rare cases caused by nitroimidazoles have been reported in humans (2, 8, 10, 20).

References