Biological approach of anticancer activity of new benzimidazole derivatives

Katarzyna Błaszczak-Świątkiewicz *, Paulina Olszewska, Elżbieta Mikiciuk-Olasik

Department of Pharmaceutical Chemistry and Drug Analysis, Medical University, Łódź, Poland

A R T I C L E   I N F O

Article history:
Received 30 January 2013
Received in revised form 22 July 2013
Accepted 13 August 2013
Available online 1 February 2014

Keywords:
Anticancer activity
Benzimidazole
Bioreductive prodrugs
Hypoxia
Nitrobenzimidazole

A B S T R A C T

Background: A series of new benzimidazole derivatives, earlier synthesized, was tested in vitro as new bioreductive prodrugs with the potential anticancer activity. Their effect on the DNA destruction and growth inhibition into selected tumor cell lines at normoxia and hypoxia conditions was determined.

Methods: The human lung adenocarcinoma A549 cell line was used to determine the anticancer activity of the analyzed compounds by using WST-1 assay. The apoptosis test (caspase 3/7 assay) was used to define the cytotoxic way of tumor cells death. Additionally test in situ DNA Damage Assay Kit was applied to recognize the DNA destruction.

Results: Four of the examined compounds (1, 3, 7, 9) show a very good antiproliferative effect and three of them are specific for hypoxia conditions (2, 4, 8).

Conclusion: Compound 8 is the most cytotoxic against human lung adenocarcinoma A549 cells at hypoxic conditions. Hypoxia/normoxia cytotoxic coefficient of compound 8 (4.75) is close to hypoxia/normoxia cytotoxic coefficient of tirapazamine (5.59) – reference substance in our experiments and this parameter locates it between mitomycin C and 2-nitroimidazole (misonidazole). The screening test of the caspase-dependent apoptosis proved that the exposure of compounds 1–2 and 7–8 against A549 cells for a 48 h promote apoptotic cell death. Additionally, the test of the DNA damage established that compounds 1, 2, 7, 8 are specific agents for the hypoxia-selective cytotoxicity of nitrobenzimidazoles [6,26].

© 2014 Institute of Pharmacology, Polish Academy of Sciences. Published by Elsevier Urban & Partner Sp. z o.o. All rights reserved.

Introduction

Hypoxia is the leading targeting in cancer therapy. The poor oxygen concentration, which is characteristic for solid tumors affects many processes such as angiogenesis, erythropoiesis and alteration of cellular metabolism at tumor cells [5]. Hypoxia, for sure, can influence the survival of tumor cells by different changes in the gene expression that reduce apoptosis and increase autophagy, vasculogenesis, metastasis, immune reactivity and activity of receptor tyrosine kinase. Generally tumor cells under hypoxia lose genomic stability by generating the reactive oxygen species (ROS) and suppress regulation of DNA repair pathways [8,10,21,23,25].

In order to minimize those survival effects of tumor cells, scientists conduct research into targeted therapy with the use of specific substances which have a bioreductive mechanism of action at hypoxia conditions [1,6,13]. This concept of approaching new chemical classes of pro-drugs activated to selective cytotoxins, was started by using derivatives of aniline nitrogen mustard as the first class of bioreductive prodrugs. Now different chemical bonds such as nitro group, quinones, heterocyclic N-oxides (CB 1954, tirapazamina, AQ4N) are currently radical prodrugs useful for cancer therapy (Fig. 1) [11,12,14,20]. The mechanisms of the metabolic activation of bioreductive prodrugs were shown at Scheme 1 [25]. The common feature of all these new chemical compounds is their ability to generate cytotoxic agents for DNA damage. A new group of benzimidazole derivatives, i.e. potential new agents of the DNA destruction, should be particularly paid attention to [2–4]. These compounds are intensively being worked on as they might have new anticancer properties [7,16,17,22,24]. It was the reason for initiating our experiments in the group of new benzimidazole derivatives and N-oxide benzimidazole derivatives. Therefore, we analyzed a series of benzimidazole derivatives (1–12) to elucidate their contribution to the antyproliferation activity at normoxia and hypoxia conditions. The particularly selective activity of N-oxide benzimidazole derivatives into hypoxia was very interesting for us. Additionally we determined their cytotoxic activity by necrosis or apoptosis. The main reason for our experiments concerned their effect of DNA damage at hypoxia and normoxia cancer cells.
Materials and methods

Procedures of biochemistry experiments

Cell culture

A549 (human lung adenocarcinoma) cell line was purchased from Health Protection Agency Culture Collections (ECACC, Salisbury, UK), were cultured in F12K medium (HyClone, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml) in 5% CO₂ at 37 °C.

Hypoxic cells were created by culture of A549 cells in hypoxic incubator in 1% O₂ and 5% CO₂ at 37 °C for 24 h before treatment.

DNA damage assay

The effect of compounds on DNA damage was determined based on measuring phosphorylation of histone H2AX on serine 139 using the EpiQuick in situ DNA damage assay kit [Epigentek]. A549 cells were seeded in 96-well plates at a density of 5000 cells/well and cultured in normoxic or hypoxic condition for 24 h before treatment. Next the cells were treated with vehicle or indicated compounds at concentration of IC₅₀ range and were later cultured in the same conditions. DNA damage in normoxic and hypoxic cells was measured after 4 h incubation with tested compounds. After this time, cells were fixed and assay was performed according to the manufacture protocol. The amount of DNA damage was proportional to the intensity of color development. The absorbance

![Scheme 1. One-electron versus two-electron reduction of bioreductive prodrug into the cytotoxic metabolites (in blue) [25]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)](image)

Fig. 1. The known bioreductive prodrugs: (a) CB 1954, (b) AQ4N, and (c) tirapazamine.
was measured at 450 nm using Synergy H1 plate reader [BioTek]. The % of DNA damage was calculated by OD treated sample/OD control × 100% where OD treated samples is the absorbance of the cells treated with compounds and OD control is the absorbance of control cells treated with vehicle.

**Cell viability/cytotoxicity assay**

To determine anticancer activity of the analyzed compounds we evaluated cell viability using WST-1 assay (Millipore) according to the manufacture instruction. The assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. Therefore, the amount of formazan dye formed directly correlates to the number of the live cells in the culture.

A549 cells were seeded in 96-well plates at a density of 5000 cells/well and cultured in normoxic condition. To investigate the effect of compounds on hypoxic cancer cells, A549 cells were exposed to hypoxia (1% O2) for 24 h before treatment. The stock solution of the tested compounds was prepared in DMSO and diluted in complete medium to give the final concentration in the range from 500 μM to 1 μM. Normoxic and hypoxic cells were treated with different concentrations of tested compounds or vehicle (0.2% DMSO) for control cells. Cell viability was assessed after a 48-h incubation with compounds in normoxic or hypoxic conditions. Briefly, WST-1 reagent was added to the cells and the absorbance was determined at 440 nm using a microplate reader (Synergy H1, Bio-Tek) after a 3 h incubation at 37 °C. The percentage (%) of the cell viability related to control cells was calculated by [A] test/[A] control × 100. Where [A] test is the absorbance of the cells treated with compounds and [A] control is the absorbance of control cells. IC50 values (concentration of tested compounds required to reduce cell density to 50%) were calculated by concentration-response curve fitting with the use of a Microsoft Excel-based analytic method.

**Apoptosis assay**
The effect of compounds on the cell apoptosis was determined using the Caspase Glo 3/7 assay [Promega] according to the manufacture instruction. The assay is based on the measurement of caspase-3/7 activity via the proluminescent substrate containing the DEVD (Z-DEVD-aminoluciferin). Following caspase cleavage, a substrate for luciferase is released resulting in the luciferase reaction and the production of luminescent signal.

A549 cells were seeded in white 96-well plates at a density of 5000 cells/well and cultured in normoxic or hypoxic conditions for 24 h before the treatment with vehicle or selected compounds. Caspase 3/7 activity in normoxic and hypoxic cells was measured after 4 h, 24 h and 48 h incubation with tested compounds. Luminescence values were measured by a microplate reader (Synergy H1, Bio-Tek) at gain 135.

**Cell morphology**
The effects of tested compounds at normoxia and hypoxia on cell morphology after 48 h treatment were evaluated with the phase-contrast microscope (OptaTech).

**Statistical analysis of the data**
The results are expressed as mean ± SD. The statistical analysis was made by using Student’s t-test; p < 0.05 was considered significant.

**Results**

**Chemistry**

The structure of the new benzimidazole derivatives and N-oxide benzimidazole derivatives is shown in Fig. 2. The cyclocondensation of diamine with aldehydes was prepared according to the known and described in literature method [9,18,19]. We worked out the conditions for obtaining new benzimidazole derivatives and N-oxide benzimidazole derivatives as we described earlier [26]. Structure of new benzimidazole derivatives was established by X-ray crystal structure analysis [6].

**Biological activities**

Human lung adenocarcinoma A549 line was used for investigating in vitro anticancer activity of newly synthesized benzimidazole derivatives (1–12). The antiproliferative activity of the compounds was examined by the WST-1 assay after a 48 h exposure. The results were expressed as a relative number of viable cells. The effects of compounds on cell apoptosis were determined by caspase 3/7 activity assay. The effects of the DNA damage were approached by using the EpiQuick in situ DNA damage assay kit [Epigentek]. The changes in cell morphology induced by compound

Fig. 2. Formula structure of potential benzimidazoles bioreductive agents [6].
treatment were visualized with a phase-contrast microscope. Moreover, we evaluated anticancer effects of the tested compounds in hypoxic cells as well. Tirapazamine and Etoposide were used as a reference drugs.

Effect of compounds on DNA damage in normoxic and hypoxic cancer cells

Phosphorylation of histone H2AX is one of the earliest chromatin modification events in response to DNA damage and can be used as a sensitive marker for DNA damage. To evaluate the effects of tested compounds on DNA damage we measured the phosphorylation of H2AX at Ser139 in normoxic and hypoxic A549 cells exposed to selected compounds (1, 2, 7, 8) and tirapazamine at concentration of IC50 range for 4 h.

We did not observe the increase of DNA damage when cells were cultured in the presence of tested compounds in normoxic condition (Fig. 3A). However, the tirapazamine treatment induced DNA damage approximately 80% over control normoxic cells (Fig. 3A).

In contrast to normoxic cells, the treatment of hypoxic cancer cells with compounds 2, 7 and 8 resulted in increased phosphorylation levels of H2AX (Fig. 3B). Compound 8 was the most potent and increased DNA damage in hypoxic cells by ~2.5-fold compared to control cells. The treatment of hypoxic cells with compounds 2 and 7 induced DNA damage at similar levels (~1.8-fold). In contrast, compound 1 had no significant effect on H2AX phosphorylation. The exposure of hypoxic cells to tirapazamine also increased ~1.8-fold DNA damage as compared to control cells (Fig. 3B).

In addition, we used etoposide, a known anticancer drug to induce DNA breakage, as positive control. Etoposide increased the levels of H2AX phosphorylation ~1.5-fold and ~2.5-fold in normoxic and hypoxic cells compared to control sample, respectively.

Our results showed that tested compounds 2, 7 and 8 at concentration of IC50 range caused DNA damage only in hypoxic cancer cells. In contrast, compound 1 had no significant effect on H2AX phosphorylation level either in normoxic or hypoxic cells. Moreover, tirapazamine and etoposide treatment increased DNA damage in normoxic as well in hypoxic A549 cells.

Effect of newly synthesized compounds on cancer cell viability in normoxia

The concentration–response analysis was performed to determine compound concentrations required to inhibit the growth of cancer cells by 50% (IC50) after incubation for 48 h. Synthesized compounds were tested in a wide range of concentrations, from 1 μM to 500 μM. The relative number of live cells after treatment was evaluated by WST-1 assay.

Four out of 12 tested compounds showed IC50 values below 100 μM in cells cultured in the normoxic condition (Table 1: compounds 1, 3, 7, 9). For comparison, in the same experimental conditions 162 μM tirapazamine was required to inhibit the growth of A549 cells by 50%. Compound 1 and 7 were found to have a higher anticancer activity, with an IC50 value of 36.6 ± 1.8 μM and 24.4 ± 2.6 μM, respectively. The culture of A549 cells in the presence of compound 1 at concentration ca. 30 μM caused approximately 35% inhibition of cells growth compared to control cells. However, the treatment of cells with compound 7 at the same concentration resulted in decreased cell viability by 87% (Fig. 4). These results demonstrated a much higher effectiveness of compound 7 compared to compound 1 at low concentration (30 μM). A lower effect on the cell growth inhibition was observed after treatment with benzimidazole derivatives 3 and 9 (IC50 value 73.4 ± 1.6 μM and 72.0 ± 1.7 μM, respectively). Other tested benzimidazole derivatives were less effective and showed a much higher value of IC50 over the range 160–460 μM (Table 1).

The results showed that benzimidazole derivatives which have a substituent of chlorophenyl (compounds 1, 7) or piperonyl (compounds 3, 9) inhibited the growth of A549 cells more potent than analogous benzimidazole derivatives which contain nitrophenyl (compounds 2, 8) or napthyl (compounds 4, 10).

These results are very similar to our earlier results of cytotoxic activity of new benzimidazole derivatives tested against the cells of human malignant melanoma WM 115 [15,26].

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 [μM] A549</th>
<th>Differential cytotoxicity O/H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>1</td>
<td>36.6 ± 1.8</td>
<td>56.8 ± 1.5</td>
</tr>
<tr>
<td>2</td>
<td>169 ± 1.5</td>
<td>97.4 ± 1.4</td>
</tr>
<tr>
<td>3</td>
<td>73.4 ± 1.6</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>379.5 ± 1.6</td>
<td>254 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>181.7 ± 1.9</td>
<td>165.0 ± 1.6</td>
</tr>
<tr>
<td>6</td>
<td>293.2 ± 1.2</td>
<td>270.0 ± 0.8</td>
</tr>
<tr>
<td>7</td>
<td>24.4 ± 2.6</td>
<td>56.0 ± 2.5</td>
</tr>
<tr>
<td>8</td>
<td>460.5 ± 1.5</td>
<td>96.8 ± 1.9</td>
</tr>
<tr>
<td>9</td>
<td>72.0 ± 1.7</td>
<td>350.0 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>279.0 ± 2.2</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>164.4 ± 2.1</td>
<td>190 ± 1.1</td>
</tr>
<tr>
<td>12</td>
<td>300.0 ± 1.8</td>
<td>398 ± 0.2</td>
</tr>
<tr>
<td>Tirapazamine</td>
<td>162.2 ± 0.6</td>
<td>29.0 ± 1.5</td>
</tr>
</tbody>
</table>

WST-1 assay was used to determine the inhibition of the cell growth after a 48-h incubation with tested compounds. IC50 values (concentration of tested compounds causing 50% inhibition of the cell growth compared to control cells) were calculated and expressed as the mean ± SD, n = 3. No – indicates no effect or less than 50% inhibition of the cell growth of the tested compound.
Effect of new compounds on viability of hypoxic cells

The tumor microenvironment characterized by hypoxia is the new target of novel potential anticancer substances which have a bioreductive mechanism of action. For these reasons we also evaluated the effect of our compounds on hypoxic cancer cells. A549 cells were exposed to hypoxia (1% O₂) for 24 h before treatment and cells were maintained in the hypoxic condition during culture in the presence of compounds. As shown in Table 1, the most active agents among the benzimidazole series 1–12 in hypoxic conditions were compounds 1 and 7. In hypoxic conditions, the cells viability was significantly altered when cells were cultured in the presence of compounds 2 and 8. Both compounds at concentration ca. 56 μM inhibited the growth of hypoxic tumor cells by approximately 50% of control (compound 1 – IC₅₀ value 56.8 μM, compound 7 – IC₅₀ value 56.0 μM). The exposure of hypoxic cells to compound 2 and 8 at concentration ca. 97 μM decreased cell survival by 50%. Moreover, compound 8 was more effective in hypoxic cells compared to normoxic cells with IC₅₀ ca. 97 μM and 460 μM, respectively. The same activity is characteristic for compound 2. In contrast, compounds 3 and 10 showed no significant anticancer effect at any doses tested in hypoxic cells (Table 1 and Fig. 4).

Effect of compounds on cell apoptosis

Based on IC₅₀ values, we selected compounds 1, 2, 7 and 8 for further biological evaluation. To evaluate if the inhibition of the cell growth in response to these compounds was due to the induction of apoptosis, caspase 3/7 activity was measured. Apoptosis assay was performed in normoxic and hypoxic A549 cells exposed to selected compounds at concentration of IC₅₀ range for different periods of time. The treatment of normoxic cells with compounds 1, 2, 7 and 8 did not increase the caspase 3/7 activity over the control cell levels either after 24 h and 48 h exposure. Similar results were obtained in hypoxic cells. In contrast, the
treatment of normoxic and hypoxic cells with tirapazamine for 24 h resulted in increased caspase 3/7 activity by 4-fold and 7-fold compared to control cells for each condition, respectively (Fig. 5). These results suggest that these compounds at the tested concentrations inhibited A549 cell growth rather than the inhibition of cell proliferation. Moreover, our results demonstrated that culture of control cells in hypoxia decreased caspase 3/7 activity approximately 2-fold (Fig. 5A) and 7-fold (Fig. 5B) compared to control normoxic cells. These results showed that generally hypoxia contributes to increased cancer cell survival by attenuation of cell apoptosis, not necrosis. The screening test of caspase-dependent apoptosis of tested compounds for a 48 h hypoxic and normoxic exposure showed promoting apoptotic cell death in relation to necrotic death [26].

Discussion

From our screening tests, we found that compounds 1 and 7 were the most effective in the inhibition of the cell growth in normoxic as well as in hypoxic A549 cells. But their cytotoxic activity at hypoxia drew attention too. The compounds 2 and 8 were more potent to specifically inhibit cell viability of hypoxic cancer cells while they were less effective in normoxic cells. Moreover, hypoxic/aerobic cytotoxicity coefficient of compound 8 was 4.75 while for tirapazamine was 5.59. This parameter locates the tested compounds between mitomycin C (cytotoxic coefficient from 1 to 5) and 2-nitroimidazole (misonidazole) with toxicity coefficient from 5 to 15. Additionally we found that the time of compounds exposure at normoxia and hypoxia conditions contribute of cell death by apoptosis not necrosis. The major results proved that compounds 2 and 8 are specific for hypoxia and they possess potential activity as bioreductive pro-drugs activated at hypoxia conditions. It probably depends on the presence of the two very characteristic groups: nitro group and N-oxide group at their structure. Additionally, we observed that compound 8 is more effective in hypoxia than in normoxia environment. We suggest that the presence of the nitro substituent at structure of compound 8 causes the increase of percentage of DNA damage. All these features of the described new benzimidazoles classified them near the known bioreductive prodrugs such as tirapazamine or PR-104A.

Funding

Synthetical and biochemical research is supported by Medical University of Lódź, Poland (503-3015-1, Grant No. 507–13-052).

References


Fig. 5. Influences of the compounds 1, 2, 7, 8 and tirapazamine on cell apoptosis in normoxic and hypoxic conditions.


