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Antioxidant activity of some benzimidazole derivatives to definite tumor cell lines

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Abstract

A group of bis(benzimidazol-2-yl) amines have been already evaluated for cytotoxicity *in vitro* to human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231 and normal spleen cells and two of them (B1 and B2) have been taken for the purposes of our present investigations. From the second group of compounds representing 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles two substances (B3 and B4) have been chosen because of their most pronounced anti-proliferative effect to human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231 and normal spleen cells, using the *in vitro* proliferative MTS-test. It was important to estimate the cause for this suppressive activity of the compounds. We proposed that this could be due to their antioxidant capacity. The substances were examined for antioxidant activity against hydroxyl and peroxyl radicals, applying the HORAC and ORAC methods and showed considerable capacity. The scavenging capacity of B2 towards hydroxyl radicals is the highest, followed by B1. It was estimated that B2 has the greatest scavenger capacity of oxygen radicals, emitted by the examined cells followed in descending order by B1, B3 and B4. The observed differences can be considered as impact of their structure on the Me^{+2}-helating activity and effective H-atom donation. A correlation was observed between the structure of the particular substance and the expressed antioxidant potential. The latter correlated also with the effect on the tested tumor cell lines. This result means that tumor cells are accompanied by a measurable emission of ROS which might be regulated by a proper application of antioxidants.

Keywords: aminobenzimidazoles; HORAC; ORAC; antioxidant activity; tumor cells

Introduction

The massive production of ROS in an inflammatory environment (characterized as oxidative burst) plays a key role in defense against environmental pathogens. In an inflammatory environment, activated neutrophils or macrophages produce large quantities of superoxide radical and other ROS via the phagocytic isoform of NAD(P)H oxidase. Various types of nonphagocytic cells involving fibroblasts, vascular smooth muscle cells, cardiac myocytes, and endothelial cells are known to produce ROS by NAD(P)H oxidase to regulate intracellular signaling cascades. The immune response is redox regulated process where interleukin-2 production can be induced by relevant concentrations of superoxide radical and hydrogen peroxide and the programmed cell death (apoptosis) is needed both for proper development and to destroy cells that represent a threat to the integrity of the organism. Another question remains open – which molecules are targets of the stress induced in cells by ROS. ROS certainly oxidize membrane lipids, but the amount of ROS emitted after T cell activation is too small to mediate lipid peroxidation and membrane destruction [1]. It is known that metal-induced generation of ROS results in an attack not only on DNA, but also on other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation. Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed

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to ionizing radiation under conditions where hydroxyl radicals or a mixture of hydroxyl/ superoxide radicals are formed [2]. Most cell types have been shown to elicit a small oxidative burst generating low concentrations of ROS when they are stimulated by cytokines, growth factors and hormones. The abnormal behavior of neoplastic cells can often be traced to an alteration in cell signalling mechanisms, such as receptor or cytoplasmic tyrosine kinases, altered levels of specific growth factors. It has been clearly demonstrated that ROS interfere with the expression of a number of genes and signal transduction pathways and are thus resources in the process of carcinogenesis [3]. The exposure to free radicals from a variety of sources is the reason for development of series of defense mechanisms in organisms against the free radical-induced oxidative stress. A more reducing conditions (maintained by elevated levels of glutathione and thioredoxin) of the cell stimulates proliferation and a slight shift towards a mildly oxidizing environment initiates cell differentiation. A further shift towards more oxidizing conditions in the cell leads to apoptosis and necrosis.

Among the naturally occurring antioxidants as flavonoids quercetin, kaempferol, delphinidin, vitamin E and as well as the synthetic compounds, which are studied for their antioxidant activity, the bezimidazole derivatives occupied essential position [4, 5].

The antioxidative properties of many new benzimidazole derivatives were determined *in vitro* on the rat liver microsomal NADPH-dependent lipid peroxidation (LP) level, the scavenging of superoxide anion and the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), so 5-nitro-2-(phenoxymethyl)benzimidazole invokes strongly inhibition (91%) to lipid peroxidation at 10^{-3} M concentration [6]. 5-Ethoxy-2-ethylthiobenzimidazole hydrochloride known as ethomerzol prevents lipid peroxidation activation and antioxidant system suppression in brain and liver of albino rats at acute hypoxia at concentration25 mg/kg, intraperitaneally, 30 min before hypoxia [7]. Compounds 2-(3-chlorostyryl)-1H-benzimidazole and 2-(4-chlorostyryl)-1H-benzimidazole are found to be moderately active, having IC50 values 99.5 μM/L and 95.5 μM/L respectively as compared to 89.4 μM/L of standard ascorbic acid by nitric oxide scavenging method. 2-Styryl-1H-benzimidazole, 2-(2-chlorostyryl)-1H-benzimidazole and 2-(2-(1H-benzimidazol-2-yl) vinyl)phenol have their IC50 values as 73.2 μM/L, 61.5 μM/L and 61.1 μM/L respectively and are more potent than standard antioxidant ascorbic acid [8] Thiazine derivatives of benzimidazole were found to show potent antioxidant activity [9] and benzimidazoles containing pyridopyrimidine ring as substituent showed the same effect with IC50 value of 10 μg/mL compared to the used commercial ascorbinic acid [10]. A benzimidazole compound containing both tetrahydronaphthalene and 4-phenylpiperazine fragments in molecule displayed scavenging effect at 10-3 M concentration (98%) on superoxide anion radical, a result which is better than that achieved with 30 IU of SOD (76%) [11].

Having in view the above mentioned facts, it is obvious that the study of the antioxidant properties of other benzimidazoles is of pharmacological interest. In this paper we report the examination of antioxidant activity of some benzimidazole derivatives, which were found to display a cytotoxic effect on tumor cell lines (HT-29, MDA-MB-231) respectively proliferating activity against normal spleen cells.

The decision to examine the antioxidant activity of the substances toxic toward tumor cells came from the available data that all living cells (including tumor cells) emit ROS. In the case that cancer cells (like all other living cells) [3] emit ROS (and maybe in greater extent!) and the examined compounds scavenge them, the tumor cells should be suppressed and/or even die! Our preliminary tests (HORAC & ORAC) have shown that these radicals cannot be detected on the tumor cells, maybe because of their low amount and/or very short life. The vice versa way to prove the presence of such radicals was to incubate the tumor cells with antioxidants and to estimate the cellular suppressive or proliferative response.

Materials and methods

The bis(benzimidazol-2-yl)amines (B1 and B2) were obtained by substituition of the sulfuric group in the benzimidazol-2-sulfonic acid through appropriated benzimidazole-2-amines and were discussed and reported by Mavrova et al. [12]. The derivatives of 2,3-dihydro-2-imino-1H-benzimidazoles B3 and B4 were synthesized by nucleophilic substitution of 2-amino-benzimidazoles under solid-liquid phase transfer catalysis conditions in dry acetonitrile as well as in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and detailed description is given in the paper of Mavrova et al. [13].

The chemical structures of the compounds were established by elemental analyses, IR-, ^{1}H NMR and ^{13}C NMR spectra and the results are presented in the Supplementary Material part. The elemental analyses indicated by the symbols of the elements were within ± 0.4% of theoretical values.

Cytotoxicity

All compounds were evaluated *in vitro* earlier [12, 13] for their cytotoxicity to human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231 (American Type Culture Collection, Rockville, MD, USA) and normal spleen cells by using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrasolium inner salt) test. The cell proliferation MTS-assay is based on the fact that the MTS tetrazolium compound is bio-reduced by cells into a colored formazan product that is soluble in the tissue culture medium. This conversion is presumably accomplished by NADPH or NADH, produced by dehydrogenase in metabolically active cells. The greater release amount of formazan indicates to a higher vitality of the cells (proliferation). A low vitality demonstrates a cytotoxic influence of all experimental substances (to all cellular kinds).

ROS-measurements

Preparation of the samples

Approximately 2 mg of each sample were dissolved in 2 mL acetone:distilled water: conc. acetic acid = 70:29.5:0.5 at room temperature for 1 h. This solvent system is widely applied for enhanced extraction of phenolic substances from plant materials, foods etc. [14]. According to a preliminary experience, a more appropriate solvent of the tested substances is DMSO. It is a free radical scavenger, especially in the case of hydroxyl radical [14]. Samples B1, B2 and B4 do not dissolve entirely and were sonicated additionally (20 kHz, 30 W, 1 min at room temperature). The suspensions obtained were applied for determination of the antioxidant capacity immediately.

Determination of the antioxidant capacity

Hydroxyl radical averting capacity (HORAC)

The method is based on *in situ* generation of hydroxyl radicals (OH*) by catalytic decomposition of hydrogen peroxide by divalent metal salts ($Co2^{+}$) at 37°C and pH 7.4 (75 mM sodium phosphate buffer) [15]. The reaction is performed in 10 mm light path quartz fluorescence cell on Perkin Elmer LS5 fluorometer equipped with thermo stated cell holder. Fluorescein-disodium salt was used for monitoring of the free radical generation and their scavenging by the tested substances (λex=493 nm; λem=518 nm, observation period 30 min). Gallic acid (GA) was used as a standard. The results are expressed micromole GA equivalents/mol substance.

Oxygen radical absorbance capacity (ORAC)

The experiment is carried out on the same equipment, in the same buffer and applies also fluorescein-disodium salt as a probe [16, 17]. The generation of oxygen radical (peroxyl radical, RO2*) is achieved by thermal decomposition of AAPH (2,2'-azobis-2-methyl-propanimidamide dihydrochloride, purchased from Cayman Chemical Co.) at 37°C and pH 7.4 in the above mentioned phosphate buffer. TROLOX® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble derivative of vitamin E, purchased from Sigma-Aldrich) was used as standard. The results are expressed as micromole TROLOX equivalents/mol substance.

Treatment of experimental data

For both standard substance and running sample sigmoid fluorescence decay curves were obtained. The antioxidant potential of the sample is evaluated by comparison of its net area (NA) with that of the standard. For its determination is used the so called "Area Under Curve" – AUC, calculated for both sample and standard

$$AUC = \sum_{2}^{n-1} \frac{I_i}{I_1}$$

-by subtracting the AUC of the blank (buffer) we obtain the NA-values for both sample and standard

The final expression which describes the antioxidant potential of the sample is:

$$\text{Antioxidant potential} = \frac{AUC_{\text{sample}} - AUC_{blank}}{AUC_{\text{standard}} - AUC_{blank}} * \frac{C^{eff}_{\text{standard}}}{C^{eff}_{\text{sample}}},$$

Where the effective concentrations (in the photometric cell) of sample and standard are used. The final result is in mol standard equivalents/mol sample.

Results

The synthesis of compounds B1-B4 was performed as illustrated in Figure 1:

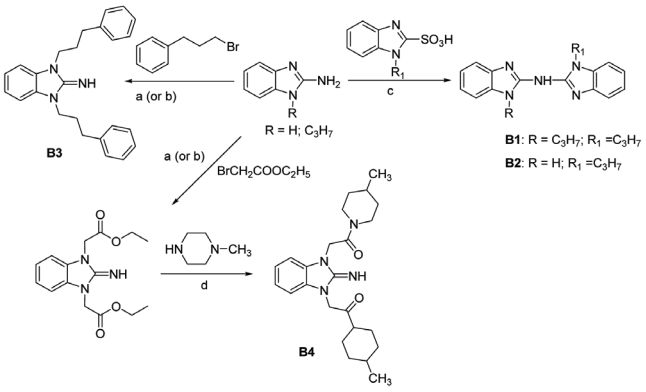

Figure 1 Schemeof theof 2-aminobenzimidazole derivatives B1-B4 synthesis. (a) DBU acetonitrile, halogen derivative, 20 °C; (b) acetonitrile, TBAB, dry K2CO3; 20 °C; (c) 180 °C for 30 min; (d) ethanol, 1-methylpiperazine, refluxing, 4 h

Relative cell viability of the tested by MTS-test compounds, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration [12, 13]. All data points represent an average of three independent assays and the obtained results were plotted and IC50 and EC50 were calculated. The data are given in Table 1 and Table 2.

Table 1 *In vitro* biological effect compounds B1 and B2

Comp	*IC50 ± SE (μM)*			*EC50 ± SE (μM)*		
	HT-29	*MDA-MB-231*	*Normal spleen cells*	*HT-29*	*MDA-MB-231*	*Normal spleen cells*
B1	1.92 ± 0.08	0.006 ± 0.27	ND[a]	ND	ND	$0.5.10^{-4}$ ± 0.6
B2	0.91 ± 0.11	0.135 ± 0.06	0.11 ± 0.62	ND	ND	ND

[a] not detected

The data for the expressed antioxidant capacity towards hydroxyl radical (HORAC) and peroxyl radical (ORAC) (made separately from the MTS-experiment!) are summarized in Table 3 and the graphical presentation of the data obtained is shown in Figure 2. It is evident that all tested samples show measurable antioxidant capacity which is definitively influenced by their structural peculiarities. According to the hydroxyl radical scavenging capacity the investigated substances can be ordered as follows: B2>B1>>B3>B4 (B3~B4).

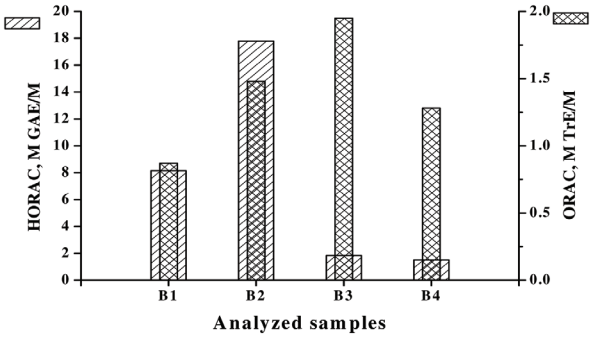

Figure 2 Graphical presentation of the data obtained about the ROS-scavenger activity of the examined substances

Table 2 *In vitro* cytotoxic and proliferative effect against HT-29, MDA-MB-231 and normal spleen cells

Comp	*IC50 ± SE (nM)*			*EC50 ± SE (nM)*		
	HT-29	*MDA-MB-231*	*Normal spleen cells*	*HT-29*	*MDA-MB-231*	*Normal spleen cells*
B3	9.26 ± 0.11a	ND	ND		1.388 ± 0.22	14.13 ± 0.59
B4	0.013 ± 0.81	ND	1.38 ± 0.04	ND	1.59 ± 0.12	

[a]Statistical significant differences in the level of cells in both control and experimental groups were determined ($p \leq 0.05$).

Table 3 Antioxidant activity (HORAC and ORAC) of the bis(benzimidazol-2-yl)amines (B1 and B2) and 2,3-dihydro-2-imino-1H-benzimidazoles (B3 and B4)

Sample index	*Molecular mass, Da*	*HORAC, mM GAE/g*	*HORAC, M GAE/M*	*ORAC, mM TrE/g*	*ORAC, M TrE/M*
B1	333.43	24.43	8.15	2.62	0.87
B2	291.35	61.10	17.80	5.10	1.48
B3	369.50	4.96	1.83	5.27	1.95
B4	413.52	3.62	1.50	3.09	1.28

Discussion

As it can be seen from Table 1 compound B1 showed relative high cytotoxicity against HT-29 and MDA-MB-231 cells (IC50-0.006μM) but in the same time that compound revealed proliferative effect toward normal spleen cells (EC50-0.5.10^{-4} μM). Respect to the results shown by the other 2-aminobezimidazole derivatives (Table 2) it may be pointed that B3 exhibited cytotoxicity against HT-29, but is not toxic against MDA-MB-231 and normal spleen cells, while compound B4 showed the highest toxicity against HT-29 (IC_{50}-0.013 nM). If the results, obtained for compound B1 are taken in consideration it should be noted that 1-propyl-N-(1-propyl-1H-benzimidazol-2-yl)-1H-benzimidazol-2-amine (B1) revealed proliferative effect against normal spleen cells at lower concentration in comparison to the concentration at which the compound exerted cytotoxic effect to HT-29 cells. Besides it manifested cytotoxic effect to MDA-MB-231 cells and proliferative activity to normal spleen cells at low concentration.

In the same time its antioxidant capacity towards hydroxyl radicals however is definitely low (Figure 2). The scavenging capacity of B2 towards these radicals is the highest, followed by B1. The hydroxyl radical emission of the cells (HORAC) is in any case stronger in comparison to this of the oxygen emission. It was estimated that B2 has the greatest scavenger capacity of oxygen radicals followed in descending order by B1, B3 and B4. B3 has a pronounced toxic effect to the MDA-MB-231 cells, whereas it has no effect to other cell species. We could assume that the antioxidant activity of B2 correlates well with its cytotoxicity, because it can scavenge the oxygen radicals from the tumor cells better, than from the normal cells. Our suggestions were in the direction whether the free radical scavenging ability of the tested substances may stimulate their suppressor activity. And not only this, we expect that tumor cells emit higher amounts of the mentioned radicals and depending on their structure, the examined substances would serve as effective scavengers and would suppress the tumor cells' development, but in different manner and extent.

On the base of the promising screening results it might be concluded that the selectivity of compound B2 could be essential for anticancer drug discovery.

According to the observed hydroxyl radical scavenging capacity (i.e. effective helating of Me^{2+}), the investigated substances can be ordered as follows: B2>B1>>B3>B4 (B3~B4). Sample B2 might be considered as a multi-center complex forming agent (multidentate ligand). The additional alkylation in substance B1 significantly limits its complex formation ability resulting in approximately two times lower HORAC-value. Substances B3 and B4 are not typical helators and have bulky substituents, which additionally limit the access to the nitrogen atom. The carbonyl groups in B2 may form internal adducts with the imino-group thus preventing its interaction with metal ions. In other words, the position and environment of the available nitrogen atoms in B3 and B4 are not appropriate for effective complex formation with metal ions, and these results in quite low HORAC-values.

The observed ORAC (oxygen radical absorbance capacity) values of the tested substances raise the question for their HA-donation capability. We have two pairs either substituted to a different extent – B2 vs B1 or differing in symmetrically situated bulky substituents – B3 vs B4. Interesting fact is that these structural peculiarities affect almost equally the observed ORAC-values, e.g. Δ B2/B1 = 1.48–0.87 = 0.61 M TrE/M and Δ B3/B4 Δ 1.95–1.28 = 0.67 M TrE/M. In the case B2 vs B1 there is a change from 2 HA-donation sites to a single one. In the case B3 vs B4 the substituents differ in their structure and a possible HA-donation site is the imino-group. In B3 it may still be effective, but in B4 the spatially close carbonyl groups may form internal adduct(s) by means of H-bond(s) thus reducing the HA-donation capability.

Conclusion

In conclusion we have to note that substance B2 is essential in prevention of hydroxyl radical generation. Substance B3 is essential in HA-donation ability thus limiting the effect of peroxyl radicals, but the capacity of the other substances is of a comparable magnitude, even exceeding the capacity of TROLOX, a water-soluble analog of the well-known lipophilic physiological antioxidant Vitamin E. In this sense, although a little bit speculatively, the peroxyl radical scavenging capacity seems to be a dominating feature of the tested set of substances.

Conflict of interest

All the authors declare that they have no conflict of interest.

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