

The Vitality of Tumor Cells and the ROS Emission after Treatment with Benzimidazole Substitutes and Somatostatin Analogs

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ABSTRACT

BACKGROUND: A synthesized 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazole M1 has been chosen because of its most pronounced antiproliferative effect (preliminary studies) to human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231 and not toxic to normal Lep 3, determined using the in vitro proliferative MTS-test. It is believed that this suppressive activity is due to its antioxidant capacity. Other two new linear somatostatin analogs which contain hydrophobic amino-acids 3c (D-Phe-c(Cys-Phe-D-Trp-Lys-Tle-Cys)-Thr-NH₂) and 3L (Pro-Phe-Val-Tyr-Leu-Ile-D-Trp-Lys-Tle-Thr-NH₂) were tested for their toxicity to the same cells and all experimental substances were tested to activated macrophages.

METHODS: The ROS-scavenging ability was examined (by HORAC / Hydroxyl Radical Averting Capacity/ and ORAC / Oxygen Radical Absorbance Capacity) using the same cells and the obtained results confirmed a considerable suppressive capacity of the compounds.

RESULTS: The last two compounds exerted the most pronounced inhibition of the tumor cell vitality (up to 77%) at higher concentrations and were not toxic to normal Lep-3 cells. After similar incubations with the substances, activated human peritoneal macrophages displayed also emission of ROS /reactive oxygen species/ determined by chemiluminescence (CL). Compound M1 showed pronounced activity against activated peritoneal macrophages (PMA) mainly in the 100000× dilution in comparison to the HORAC/ Hydroxyl Radical Averting Capacity/ and ORAC; 3L is most effective only in the 100× concentration.

CONCLUSION: All tested compounds showed different suppressive activity depending on the cell line and on the substances amount applied using HORAC / ORAC and CL.

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Key words: 2-iminobenzimidazoles; Somatostatin analogs; ROS emission of tumor cells

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INTRODUCTION

New C-amide analogs of octreotide (SMS 201–995) modified at positions 5 with Orn, Dab (diaminobutanoic acid) and Dap (diaminopropanoic acid) and at positions 6 with the unnatural amino

acid Tle (tert-leucine) were synthesized. The antioxidant capacity of the compounds was tested by chemiluminescence (CM)^[1] and ORAC (Oxygen Radical Absorbance Capacity) and HORAC (Hydroxyl Radical Averting Capacity)^[2] methods.

The aim of the presented experiments was to verify whether the compounds (having antiproliferative impact and antioxidant activity on examined tumor cells) would exert any antioxidant activity towards activated human phagocytes as well (using both methods).

The antioxidant capacity of proteins is thought to encompass both free radical scavenging by amino acid residues and chelation of pro-oxidative transition metals. Most cell types have been shown to elicit small oxidative burst generating low concentrations of ROS when they are stimulated by cytokines, growth factors and hormones. Detection and quantification of ROS /reactive oxygen species/ can be performed by indirect methods, such as observations of chemical changes caused by ROS or by direct quantification of the amount of ROS^[3,4,5].

The abnormal behavior of neoplastic cells can be often traced to an alteration in cell signaling mechanisms, such as receptor or cytoplasmic tyrosine kinases or 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 instruments in the process of carcinogenesis^[6].

With the discovery that ROS are used as intracellular messengers and regulators, new chemistries were developed with the micromolar detection requirements in mind. These agents are primarily fluorescence based, but recently luminescent based detections have been introduced.

Having in mind the results from the biological effect (suppressive and/or proliferative) on human tumor cells and from the ROS scavenging capacity of several newly synthesized compounds of different origin, the present examinations were directed to estimate their parallel influence on activated human phagocytes using CL.

MATERIALS AND METHODS

Tumor cells: the colon carcinoma HT-29 cells, the breast cancer cells MDA-MB-231 and normal Lep 3 cells (commercial cell lines - American Type Culture Collection ATCC, Rockville, MD, USA) and their examinations are published previously^[7].

Cytotoxicity: All compounds were evaluated *in vitro* by the cell proliferation MTS-assay which 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 a lower vitality of the cells (inhibition). A low vitality demonstrates a cytotoxic impact of all tested substances.

Synthesis of the compounds: 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazole (M1)^[8] and of the linear analogs of octreotide^[9] are described earlier (given in the "Supplementary materials").

Oxidative burst of whole blood phagocytes: Heparinized (50 IU/ml) blood samples were obtained from healthy male volunteers with their informed consent. The sampling procedure was in accordance with the ethical standards of the responsible committee of the Institute of Biophysics on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. The number of leukocytes in the blood and their relative differentiation counts were determined using a Coulter counter STKS (Coulter Corporation, Miami, FL, USA). Luminol-enhanced chemiluminescence of human

phagocytes in the whole blood was measured using an LM-01 microplate luminometer (Immunotech, Prague, Czech Republic). The principle of the method is based on luminol interaction with the phagocyte-derived oxidizing species, which results in strong light emission at 425 nm. Briefly, the reaction mixture consisted of 10 μ l whole blood, 1mM luminol (stock solution of 10mM luminol in 0.2M borate buffer) and 0.81 μ M phorbol-12-myristate-13-acetate (PMA-peritoneal macrophages) as the activator in the case of the activated CL response. The total reaction volume of 200 μ l was adjusted with Hanks balanced salt solution. The same incubations were done under influence with the experimental substances. The assays were run in duplicates. The CL emission expressed as relative light units (RLU) was recorded continuously for 60 min at 37°C.

ROS-measurments

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 hour. This solvent system is widely applied for enhanced extraction of phenolic substances from plant materials, foods etc^[10]. The solutions obtained were applied for determination of the antioxidant capacity immediately.

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

ORAC-Oxygen Radical Absorbance Capacity: The experiment is carried out on the same equipment, in the same buffer and applies also fluorescein-disodium salt as a probe^[10]. The generation of oxygen radical (peroxyl radical, RO $_2\cdot$) 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.

Evaluation of experimental data - 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 sample, standard and the blank sample. The final expression, which describes the antioxidant potential of the sample is:

Antioxidant capacity = (NA_{sample}/NA_{standard})*(C_{eff}-standard/C_{eff}-sample), where.

NA_{sample} = AUC_{sample} – AUC_{blank}; NA_{standard} = AUC_{standard} – AUC_{blank}.

C_{eff} = effective concentration, i.e. the final concentration in the cell.

RESULTS

The experiments undertaken in our previous works^[1,7,8] have shown that the most expressed antiproliferative impact on the mentioned tumor cells *in vitro* had the M1 substance (1,3-disubstituted-2,3-

dihydro-2-iminobenzimidazole), followed by 3c. Exactly these substances were not toxic to the normal cells (IC_{50} was not stated) and because of this effect they were chosen for the present trials (Table 1).

The data for the antioxidant activity of these compounds are shown in Table 2.

M1 showed measurable antioxidant capacity which is definitively influenced by its structural peculiarities. M1 did not show strong scavenging effect towards hydroxyl radical. At the same time it exhibited cytotoxicity against HT-29, but was not toxic to MDA-MB-231 and normal spleen cells. The hydroxyl radical emission of the cells (HORAC) is in any case stronger in comparison to this of the oxygen emission. M1 has a pronounced toxic effect to the HT29 cells (opposite effect in comparison to the cytotoxic effect). 3c and 3L showed most pronounced effect on HT29 and MDA-MB-231 tumor cell lines only at high concentration, which is insufficient for assuming a definite antitumor activity. The HORAC and ORAC were not far different from these results. HORAC revealed best emission by 3c and lowest by M1.

However, the limitation of ROS emission from the tumor cells by means of different substances might not be the only way for suppressing of the cell development.

This situation led us to the decision to use the chemiluminescence method (of human phagocytes under influence of the same substances). Because of the well known fact that activated macrophages do emit ROS^[11,12,13] it was interesting to examine their behavior after exposure to the mentioned experimental substances.

From the obtained results (Table 3), it is obvious that neither 3C nor 3L affected the CL response of professional (activated macrophages) phagocytes in the whole human blood. Only M1 significantly inhibited the CL response of the phagocytes in the two highest concentrations, i.e. 100x (0.04 mg) and 1000x (0.004 mg) diluted samples.

DISCUSSION

The suspicion that tumor cells emit high values of ROS and because of this they are mostly inhibited by strong helators is not always the reason for the lack of cell vitality. In our results M1 is an example for inhibiting the tumor cell vitality, having low HORAC and ORAC values and this impact concerns only one cell type (e.g. reaction choice). Substance M1 (Suppl.mat.) is not a typical helator and has

bulky substituents, which additionally limit the access to the nitrogen atom. The position and environment of the available nitrogen atoms in M1 are not appropriate for effective complex formation with metal ions thus resulting in quite low HORAC-values.

In comparison to the M1 the somatostatin analogs (3c and 3L) definitely revealed weaker antiproliferation activity and scarce ROS scavenging possibility. It is arguable whether this happens because of the small amount ROS emission by the cells or because of the compound's properties ("Supplementary materials"). Our previous experience of ROS chelating from cancer cells^[2] confirms that tumor cells are accompanied by a measurable emission of ROS which might be regulated by a proper application of antioxidants.

In the early years Hatch *et al.*^[14] found by the CL that the different normal cell types, the peritoneal macrophages reacted best and were strongest chelators against oxygen. In many cases authors have shown the antimicrobial mechanisms of the phagocytes as chelators^[15].

In our CL-examinations the results of the substance chelator properties were not very different from those measured by HORAC and ORAC. M1 showed pronounced activity against activated PMA only in the 100000x(0.0004 mg) dilution. Substance 3L is effective in the 100x (0.04 mg) concentration.

CONCLUSION

In summary it could be concluded: (1) Both methods are much closed to each other with slow variations; (2) The sensitivity of the methods shows different impact of the compounds on the tumor cells and PMA. This result is definitely not only due to the methods used, but to the cell lines as well; (3) The variety of the scavenging capability is mostly due to cell lines, to the methods used and in great extent to the structure of the used compounds.

Table 2 Antioxidant activity (HORAC and ORAC) of the 2,3-dihydro-2-imino-1H-benzimidazole (M1).

Compound	Molecular mass, Da	HORAC, M GAE/M	ORAC, M TrE/M
M1	369.50	1.83	1.95
3c	1043.472	10.56	1.10
3L	1277.7536	3.32	1.22

Table 1 Antiproliferative impact of the synthesized compounds on different tumor cells (nM/ mL).

Compound	IC ₅₀ (μM / nM)			EC ₅₀ (μM / nM)		
	HT 29	MDA-MB-231	Normal Lep 3 cells	HT 29	MDA-MB-231	Normal Lep 3 cells
M1	9.26±0.11	ND	ND	ND	1.39±0.22	14.13±0.59
3L	ND	ND	ND	0.01±0.13	4.11±0.08	ND
3c	3.38±0.11	1.23±0.15	ND	1.23±0.05	4.51±0.11	1.20±0.20

ND: not detectable.

Table 3 Oxidative burst of phagocytes in the whole blood after exposing to the compounds.

Dilution	Compounds (4 mg/ml)					
	M1		3C		3L	
	Spontaneous	PMA-activated	Spontaneous	PMA-activated	Spontaneous	PMA-activated
Control	28009±300	75170±5418	28009±300	75170±5418	28009±300	75170±5418
100x	27914±304	39361±1751*	28071±298	75455±4850	27899±360	77219±5046
1000x	27936±282	61258±3227*	27973±229	75904±4845	28410±296	75889±4952
10000x	28323±296	76264±4936	28535±274	74322±5052	28163±241	74963±4942
100000x	28048±395	77429±4840	27704±366	75827±4847	27681±298	76997±5124
1000000x	28280±309	75519±4986	27670±278	75567±4942	28123±218	75282±4764

SUPPLEMENTARY MATERIAL

(A). New synthesized compound

A compound representing 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles (M1)(Mavrova *et al.*, 2012) has been chosen because of its pronounced antiproliferative effect to the examined tumor cells using the in vitro proliferative MTS-test. It was important to estimate the cause for this suppressive activity of the compound. We proposed that this could be due to its antioxidant capacity.

Substance characterization: Melting points (mp) were determined on an Electrothermal AZ 9000 3MK4 apparatus and were uncorrected. The thin layer chromatography (TLC, Rf values) was performed on silica gel 60 plates F254 (Merck, 0.2 mm thickness) using mobile phase n-heptane / ethyl acetate - 2:1 and visualization was effected with ultraviolet light. IR spectra were recorded on a HP-ST-IR spectrophotometer as potassium bromide discs. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance II+ 600 MHz NMR instrument. The spectra are referred to the solvent signal. Chemical shifts are expressed in ppm and coupling constants in Hz. The precise assignment of the ¹H and ¹³C NMR spectra was accomplished by measurement of 2D homonuclear correlation (COSY), DEPT-135 and 2D inverse detected heteronuclear (C-H) correlations (HMQC and HMBC). The microanalyses for C, H, N and S were performed on Perkin-Elmer elemental analyzer.

General procedure for the synthesis of compound M1: To a solution of 1-(un)substituted-2-aminobenzimidazole (0.004 mol) and 0.016 mol of append in 20 ml dry acetonitrile 0.024 mol of the corresponding halogen derivative was dropped by cooling. The solution was stirred for 4 hours at ambient temperature and the obtained precipitate was filtered. Additional quantity of the compounds was received through refluxing the filtrate for 1 hour, removing the solvent and crystallizing the obtained oil product with suitable solvent.

The compound crystallized after addition of ethyl acetate. Yield - 84 % (method B); Mp - 222 - 224 °C; Rf = 0.67, mobile phase: benzene/ethanol - 2:1; ¹H NMR (DMSO-d₆) δ (ppm): 1.990 (m, 4H, 2-CH₂), 2.686 (m, 4H, 3-CH₂), 4.224 (t, J=7.4 Hz, 4H, 1-CH₂), 7.154 (t, J=7.2Hz, 2H, p-Ph), 7.178 (d, J=7.4 Hz, 4H, o-Ph), 7.246 (t, J=7.6 Hz, 4H, m-Ph), 7.32 (AA' part of AA'XX' system, 2H, 5-H and 6-H) and 7.56 (XX' part of AA'XX', 2H, 4-H and 7-H), 8.875 (bs, 2H, NH.HBr). ¹³C NMR(DMSO-d₆) δ (ppm): 29.19(2-CH₂), 31.84(3-CH₂), 42.52 (1-CH₂), 110.37(4-C and 7-C), 123.47 (5-C and 6-C), 125.96 (p-Ph), 128.07 (o-Ph), 128.34 (m-Ar), 129.56 (3a-C and 7a-C), 140.93 (i-Ph), 149.03 (C=N); Analysis: Calc. for C₂₅H₂₇N₃; C, 81.26; H, 7.37; N, 11.37; Found: C, 81.29; H, 7.39; N, 11.35.

(B). Somatostatin analogs - The conventional solid-phase peptide synthesis based on

Fmoc (9-fluorenylmethoxycarbonyl) chemistry was employed to synthesize a series of new analogues of SSAs (3c - and 3L-). Rink-amide MBHA resin and TBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) were used as solid-phase carrier and condensing reagent. Three-functional amino acids were embedded as N^α-Fmoc-Thr(tBu)-OH, N^α-Fmoc-Cys(Acm)-OH, s N^α-Fmoc-Lys(Boc)-OH, N^α-Fmoc-Orn(Boc)-OH, N^α-Fmoc-Dab(Boc)-OH, N^α-Fmoc-Dap(Boc)-OH, N^α-Fmoc-D-Trp(Boc)-OH (3c and 3L) (Staykova *et al.*, 2012).

CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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