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Self-assembly of novel molecular complexes of 1,10-phenanthroline and 5-amino-1,10-phenanthroline and evaluation of their *in vitro* antitumour activity

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ABSTRACT

Novel molecular complexes of 1,10-phenanthroline (phen) and 5-amino-1,10-phenanthroline (5-NH_2-phen) [(5-NH_2-phen)$_2$(phen) (H_2O)$_3$ (**1**), (phen)$_2$(imidazole) (H^+) (BF_4^-) (**2**), (phen)$_2$(benzimidazole) (H^+) (BF_4^-) (**3**), (5-NH_2-phen)$_4$(H_2O)$_3$ (**4**), and (phen)$_3$ (indole) (H^+) (BF_4^-) (**5**)] were synthesized via self-assembly processes and their *in vitro* anticancer activity was investigated. The structures of the compounds were confirmed by UV, FTIR, CIMS(CH_4) and elemental analysis. The crystal structure of **2** was determined by X-ray diffraction. Cytotoxicity of the substances was measured using the cultivated human tumour cell lines HepG2, HEp-2, and 8-MB-GA. The tested substances showed different activity depending on the cell line and amount used. Substances **2** and **3** were not toxic to the non-tumour cells (Lep-3), but significantly toxic to all tumour ones. This is not the case with compounds **4** and **5**, which are non-toxic towards carcinogenic cell lines, but even stimulate both HepG2 and HEp-2.

1. Introduction

Metal-driven self-assembly is a growing area at the forefront of modern coordination chemistry. It is emerging as one of the most promising approaches to the generation of complex supramolecular architectures. They are prepared when metal-coordinated building blocks interact spontaneously with bridging units which are connected by weak non-covalent bonds. Molecules with a wide variety of topologies and shapes have been constructed in this way including boxes, microcycles, helicates and others [1,2]. Molecules self-assemble into multicomponent complex structures when they are instructed to perform in this way. These instructions are included into the compound during its synthesis [3]. A characteristic feature of self-assembly processes is that a kinetically rapid, reversible thermodynamic equilibrium exists between the starting materials and products at all times and all steps. A bond, which is formed "incorrectly" can dissociate and reassociate correctly. As a result one product, which is substantially most favoured, is generated.

In previous papers, we reported on the synthesis of 1,10-phenanthroline (phen) with cations of alkaline earth metals. Under specific conditions we obtained the compounds Mg(phen)$_3$(NO_3)$_2$.9H_2O [4], [Ca(phen)$_2$(H_2O)$_2$(NO_3)] NO_3, [Sr(phen)$_2$(H_2O)$_2$(NO_3)] NO_3 and Ba(phen)$_2$(H_2O)$_2$(NO_3)$_2$ [5]. Crystal structures of the calcium and strontium complexes were solved by X-ray diffraction [6]. The beryllium complex could not be synthesized. Instead, the attempted reaction afforded the metal-free compound (phen)$_3$(NO_3^-)$_2$(H^+)$_2$ [5], which proved to be non-toxic [7] revealing antibacterial activity [8]. Several new compounds were synthesized by self-assembly of phen and alkaline earth metal cations in the presence of nitrate and tetrafluoroborate anions, which competed with one other. These are Mg(phen)$_4$(BF_4)$_2$(H_2O)$_3$, [Ca(phen)$_2$ (NO_3) (H_2O)$_2$]BF_4, Sr(phen)$_4$(BF_4)$_2$ (H_2O)$_3$ and Ba(phen)$_{3.5}$(BF_4)$_2$(H_2O) [5]. The crystal structure of the calcium mixed ligand complex (NO_3^- and BF_4^-) was determined [6]. The interaction of phen with $NaBF_4$ yielded other metal-free compound, (phen)$_2$(H^+) (BF_4^-) [5], which revealed antibacterial [8] and antitumour activity [9]. Different from metal-directed self-assembly is the self-assembly process in biology where mainly metal-free structures are constructed through weak non-covalent inter- or

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intra-molecular interactions. Because of the lack of literature data about formation of metal-free molecular complexes of 1,10-phenanthroline and its derivatives, we undertook their syntheses via self-assembly processes.

Matrix metalloproteinases (MMPs) play a key role in cancer progression. However, clinical trials in which MMP inhibitors were tested on cancer patients have been disappointing. Many reasons have been postulated to explain the failure of clinical trials, the lack of inhibitor selectivity being a major limitation. Thus, despite the consensus, there is an opinion that MMP-mediated proteolysis is essential for cancer progression and that certain MMPs represent important targets for intervention. Their effective and selective inhibition remains a major challenge in drug development. Consequently, several matrix-metalloproteinase inhibitors, including 1,10-phenanthroline, cyclic peptides and others have been found to effectively block migration and invasion of tumour cells [10]. Moreover, specific tumours preferentially express different MMPs. Using various human tumour cell lines, this study is aimed to show the action of the newly synthesized derivatives of 1,10-phenanthroline and 5-amino-1,10-phenanthroline (5-NH_2-phen) as possible inhibitors of MMPs.

2. Chemistry

Molecular complexes of 1,10-phenanthroline [(phen)$_2$(imidazole) (H^+) (BF_4^-) (**2**), (phen)$_2$(benzimidazole) (H^+) (BF_4^-) (**3**) and (phen)$_3$(indole) (H^+) (BF_4^-) (**5**)], and its derivative 5-amino-1,10-phenanthroline [(5-NH_2-phen)$_2$(phen) (H_2O)$_3$(**1**), (5-NH_2-phen)$_4$(H_2O)$_3$ (**4**)] were synthesized via self-assembly processes. The synthesized compounds were characterized by UV, FTIR, CIMS (CH_4), and elemental analysis. The crystal structure of **2** was determined by X-ray diffraction.

3. Citotoxicity assays

Cytotoxicity of the substances was measured *in vitro* using the cultivated human tumour cell lines HepG2 (liver carcinoma), HEp-2 (larynx epidermoid carcinoma), 8-MG-BA (brain tumour, glioblastoma multiforme), and the non-tumour diploid cell line Lep-3 (as a control). For testing toxicity, Mossman's MTT-test [11] was used.

4. Results and discussion

Herein, we report the synthesis and characterization of the following new complexes of phen and 5-NH_2-phen by BF_4^- directed self-assembly: (5-NH_2-phen)$_2$(phen) (H_2O)$_3$ (**1**), (phen)$_2$(imidazole) (H^+) (BF_4^-) (**2**), (phen)$_2$(benzimidazole) (H^+) (BF_4^-) (**3**), (5-NH_2-phen)$_4$(H_2O)$_3$ (**4**), and (phen)$_3$(indole) (H^+) (BF_4^-) (**5**). As was previously mentioned, the interaction of phen with $NaBF_4$ yielded the protonated dimer (phen)$_2$(H^+) (BF_4^-) [5]. Under identical conditions, we reacted (5-NH_2-phen) and $NaBF_4$ expecting to obtain (5-NH_2-phen)$_2$(H^+)(BF_4^-). Surprisingly, the reaction did not proceed in this manner. Instead, beautiful "cactus-like" yellow–brown formations built up of rod-like small crystals of 5-NH_2-phen were observed. In comparison, needle-like crystals spread over the crystallizer were obtained when 5-NH_2-phen was synthesized by reduction of 5-nitro-1,10-phenanthroline. Remarkably, in this case BF_4^- does not mediate the synthesis of (5-NH_2-phen)$_2$(H^+) (BF_4^-) but only directs the shape of the crystallized mass of unreacted 5-NH_2-phen. The first studied compound obtained on interaction of 1,10-phenanthroline, 5-amino-1,10-phenanthroline, and $NaBF_4$ was (5-NH_2-phen)$_2$(phen) (H_2O)$_3$ (**1**). In the UV spectrum of **1** in water there is a band at 229 nm (Σ = 84052 M^{-1} cm^{-1}) and at 278 (Σ = 58720 M^{-1} cm^{-1}) with a shoulder on the left side. For comparison, the UV spectrum of free phen exhibits two bands at 227 and 264 nm. The spectrum of free 5-NH_2-phen is also characterized by two bands at 231 nm (with shoulders on both sides) and at 280 nm, respectively.

In the IR-spectrum, the stretching vibrations (symmetric and asymmetric) of $-NH_2$ group are clearly outlined at 3237 and 3346 cm^{-1}, respectively. They are shifted compared to the corresponding vibrations of the $-NH_2$ group in free 5-NH_2-phen (3222 and 3341 cm^{-1}). An intense peak, which is assigned to the bending vibration of water, appears at 1651 cm^{-1}.

In the (CI,CH_4) mass spectrum a peak appears at m/z = 181, which corresponds to protonated 1,10-phenanthroline. The molecular peak of 5-NH_2-phen is absent, indicating instability of the molecule. Compound **2** was obtained on interaction of phen and imidazole directed by BF_4^-. The self-assembly reaction leads to (phen)$_2$(imidazole) (H^+) (BF_4^-). It is a result of reversible and dynamic multistep process which proceeds for almost one month. The crystal structure of **2** was solved by X-ray structural analysis (Fig. 1).

The aromatic molecules of phen are parallel to each other being involved in π–π interactions. The two pairs of nitrogen atoms are in trans position which permits the local dipoles to cancel.

The imidazolium cation is separated from two phen molecules. It is very strange how the BF_4^- anion holds three aromatic moieties together. The X-ray diffraction studies also show that the imidazolium cation is at a comparatively long distance from the BF_4^- anion and is not involved in π–π interactions. Nevertheless, in the FTIR spectrum many of the characteristic intense frequencies of the free imidazole molecule disappear in the spectrum of the imidazolium complex (Fig. 2). The vibration of BF_4^- in **2** appears as a broad peak centered at 1057 cm^{-1}.

The next molecular complex, synthesized by us, was (phen)$_2$(benzimidazole) (H^+) (BF_4^-) (**3**). Unfortunately, we could not obtain a single crystal suitable for X-ray analysis. It is quite possible, however, that its crystal structure is analogous to that of **2**. Two phen molecules are likely to be involved in π–π stacking interactions, the benzimidazolium cations being situated at a long distance from them. The BF_4^- anion probably serves as a bridging unit.

As in **2**, the IR-spectrum of **3** differs substantially from the spectrum of free benzimidazole. In the course of this study, we carried out a competitive reaction of phen in the presence of a mixture of imidazole, benzimidazole and $NaBF_4$ taken in same molar ratio as in the previous experiments. The substance, which crystallized, proved to be absolutely pure imidazolium molecular complex **2**. This

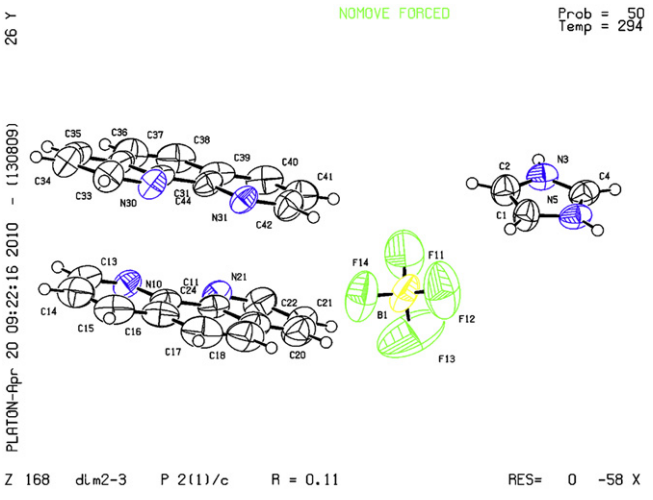

Fig. 1. X-ray crystal structure of complex **2**.

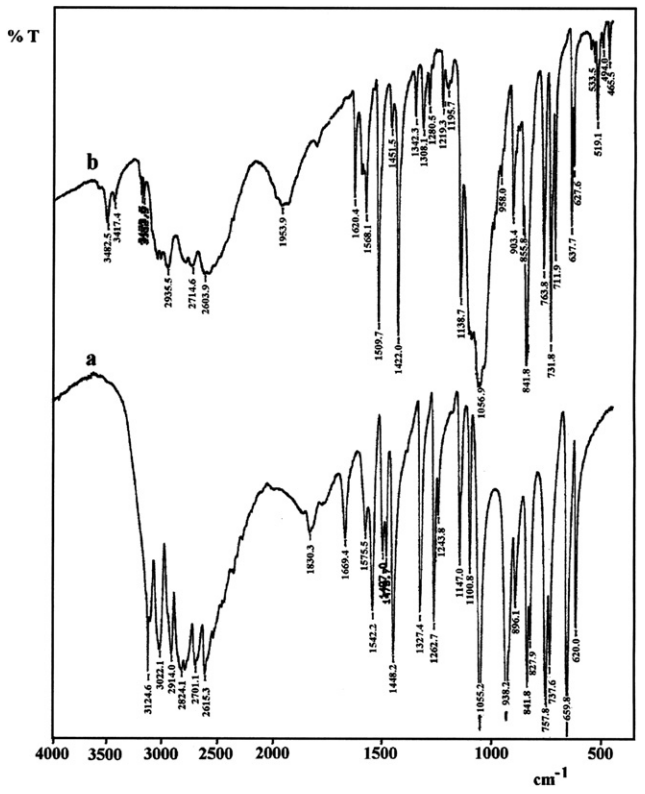

Fig. 2. FTIR spectra of imidazole (a) and complex **2** (b).

result demonstrates the exclusive specificity of self-assembly process. The same observation was previously reported [5].

In the next experiment, we reacted 5-NH_2-phen, imidazole, and $NaBF_4$ attempting to obtain (5-NH_2-phen)$_2$(imidazole) (H^+) (BF_4^-). Elemental analysis and IR-spectroscopy, however, showed that the structure was completely different. It proved to be a tetramer with three molecules of water, (5-NH_2-phen)$_4$(H_2O)$_3$ (**4**).

The IR-spectrum of **4** shows a substantial similarity with the spectrum of **1**, which contains two molecules of 5-NH_2-phen. Both substances contain three molecules of water whose deformation frequency appears as an intense peak at 1652 cm^{-1}. In this case, most probably water performs the role of a bridging unit holding together the aromatic molecules of the complexes via hydrogen bonds.

The last compound studied by us was obtained on interaction of phen, indole and $NaBF_4$. The self-assembly process produced the

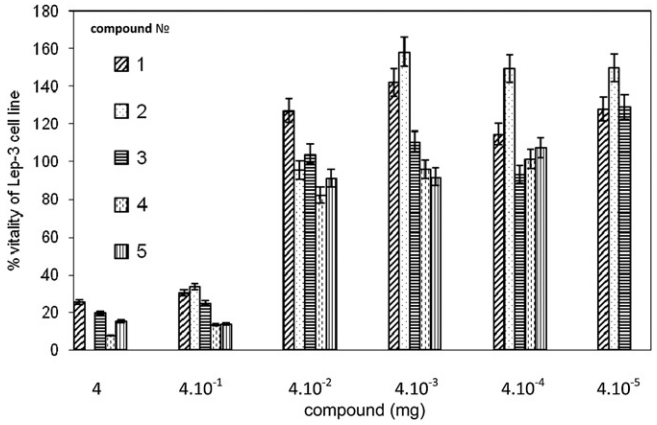

Fig. 3. Vitality (%) of non-tumour human diploid cells Lep-3, treated with substances **1**–**5**.

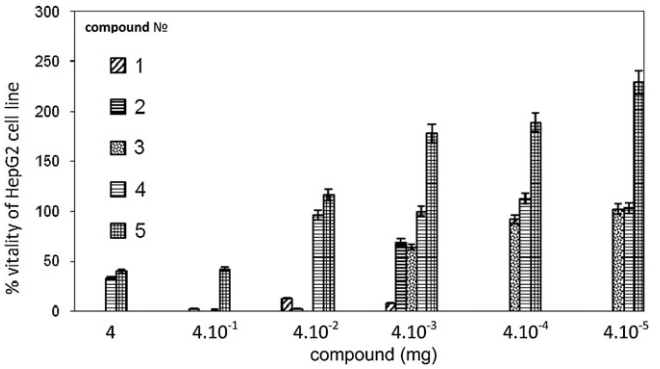

Fig. 4. Vitality (%) of human liver carcinoma cells HepG2, treated with substances **1**–**5**.

compound (phen)$_3$ (indole) (H^+) (BF_4^-) (**5**). In contrast to **2** and **3**, it contains three molecules of phen. In the IR-spectrum of **5** three intense peaks are present at 840 cm^{-1}, 1422 cm^{-1} and 1510 cm^{-1}. The same peaks appear in the spectra of **2** and **3**. A common feature of the three compounds is the presence of the anion BF_4^-. This suggests a similarity in the crystal structure. In analogy with the structure of **2**, it is reasonable to assume that in **3** and **5** two phen molecules are involved in a $\pi-\pi$ stacking interaction. The other aromatic moiety is probably separated from the phenanthroline molecules with BF_4^- anions placed between them.

Furthermore, we carried out a series of experiments in which phen was reacted with $NaBF_4$ in the presence of pyridazine, triazole, pyrimidine, thiazole, thiophene and tryptophane, respectively. It was found that none of the listed compounds was included in a molecular complex of phen. Instead, in all of the cases a competitive reaction led to the previously described substance (phen)$_2$ (H^+) (BF_4^-) [5].

The calculated vitality of different cell lines is illustrated in Figs. 3–6. It can be realized, that toxicity of the substances depends not only on amount applied, but on the type of the cell line as well.

Figs. 3–6 presents the sensitivities of various cell lines to different amounts of the applied substances. The non-tumour human diploid cells (Lep-3) (Fig. 3) are sensitive to all compounds at their highest amounts, but below 4.10^{-2} mg they show vitalities around 160%. Because the aim of the experiment was to obtain a negative toxic effect to normal cells, this is a very satisfying result.

Substances **2** and **3** influence HepG2 cells even at amounts as low as 4.10^{-5} mg where they do not reach 100% vitality insignificantly (Fig. 4). Substances **4** and **5** have an optimal cytotoxic effect at 0.4 mg, which stimulates further use. From Fig. 5 one can realize that all substances are toxic to HEp-2 cells with exception of **4**. The very cancerous cell line 8-MG-BA, although reaching 100% vitality for some of the compounds at amounts below 0.4 mg, they were in

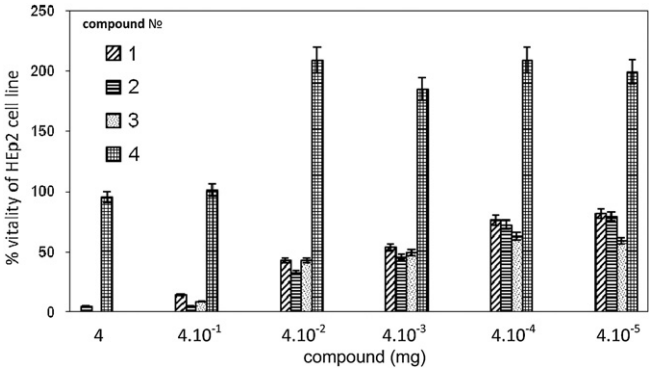

Fig. 5. Vitality (%) of human larynx carcinoma cells HEp-2, treated with substances **1**–**4**.

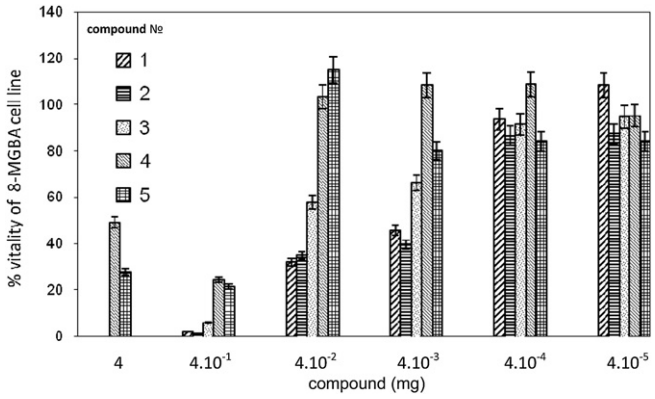

Fig. 6. Vitality (%) of human glioblastoma brain tumour cells 8-MG-BA, treated with substances **1**–**5**.

no case stimulated to proliferation (Fig. 6). It is worth mentioning that at a quantity of 4.10^{-1} mg all tested compounds show an optimal suppressive effect.

In summary, it can be stated that the substances **1, 2** and **3** have a pronounced cytotoxic effect on HEp-2 cells, substances **2**,**3** and **5** on 8-MG-BA cells, and substances **2** and **3** on HepG2 cells. Therefore, molecular complexes **2** and **3** demonstrate the most outstanding toxic effect towards all examined human tumour cell lines although exhibiting no cytotoxicity towards the control at below 4.10^{-2} mg.

1,10-phenanthroline and its derivatives express different activity to metastatic enzymes of the tumour cell matrix (MTT-enzymes). Using quite different tumour cell lines, we can suspect such capacity of action of our newly synthesized compounds. As shown by Gerber et al. [12], phen has enzyme-modulatory properties in addition to its antioxidant activity. In rat hepatocytes phen caused inhibition of respiration and enhancement of cellular ATP content, pyruvate release and CO_2 formation from glycerol. This is a result of the phen modulatory action on various enzymes involved in cellular energy metabolism. Sanchez-Sweatman et al. [13] demonstrated by *in vitro* experiments that prostate PC3 adenocarcinoma cell lines were not subjected to matrix degradation after co-culturing with phen.

5. Conclusion

The following new compounds (5-NH_2-phen)$_2$(phen) (H_2O)$_3$ (**1**), (phen)$_2$ (imidazole) (H^+) (BF_4^-) (**2**), (phen)$_2$(benzimidazole) (H^+) (BF_4^-) (**3**), (5-NH_2-phen)$_4$ (H_2O)$_3$ (**4**), and (phen)$_3$(indole) (H^+) (BF_4^-) (**5**) were synthesized by BF_4^- directed self-assembly. Antitumour investigations of the molecular complexes **1**–**5** were performed. As expected, the substances showed differing activities depending on cell line and amount of the compound used. Pronounced cytotoxic effects on HEp-2 cells were observed for **1**, **2**, **3**, on 8-MG-BA cells for **2**, **3**, **5**, and on HepG2 cells for **2** and **3**.

Therefore, molecular complexes **2** and **3** demonstrated the most outstanding toxic effect towards all examined human tumour cell lines although having no cytotoxicity towards the control at amounts below 4.10^{-2} mg. This is not the case with **4** and **5**, which are non-toxic to the tumour cells, but stimulate both HepG2 and HEp-2.

6. Experimental protocols

6.1. Chemistry

6.1.1. Chemicals and apparatus

1,10-Phenanthroline.H_2O and $NaBF_4$ were obtained from Merck, Darmstadt, Germany. Imidazole, benzimidazole and indole were purchased from Fluka Chemie AG, Buchs, Switzerland. 5-Amino-1,10-phenanthroline.H_2O was prepared in our laboratory from 5-nitro-1,10-phenanthroline by reduction with hydrazine hydrate using Raney-nickel as a catalyst. All other chemicals used were of the highest available quality. Composition of the products was determined by elemental analysis on Vario ELV5.18.018 (Elementar Analysensysteme GmbH, Hanau, Germany) performing in CHNS mode. FAB-mass spectra were taken on a Thermo DFS High Resolution Magnetic Sector MS Direct Probe - $CI(CH_4)$. Infrared spectra were recorded on a Perkin–Elmer 1600 Series FTIR spectrophotometer in the range of 4400–450 cm^{-1}, resolution 4 cm^{-1}, in KBr pellets. UV spectra were recorded on UV–VIS Cary 100 instrument. X-ray diffraction intensities were collected on a Siemens P4 diffractometer.

*6.1.1.1. Preparation of (5-NH_2-phen)$_2$(phen) (H_2O)$_3$ (**1**).* 172 mg (0.87 mmol) phen.H_2O, 185 mg (0.87 mmol) (5-NH_2-phen).H_2O, 274 mg (2.5 mmol) $NaBF_4$ and 5 ml of 96% EtOH were dissolved in 30 ml distilled water at 70 °C. Then the solution was brought to ambient temperature, and distilled H_2O added to 50 ml. The solution was kept in a refrigerator at 2–3 °C for 30 d. The formed yellow–brown crystals were filtered off, washed with distilled water and ethyl ether and dried in a desiccator over P_4O_{10}.

Yield: 50.6%.

Anal. calcd. for $C_{36}H_{32}N_8O_3$ (C, H, N):
Calculated (%): C, 69.15; H, 5.12; N, 17.92.
Found (%): C, 69.23; H, 5.19; N, 18.36.
FTIR (cm^{-1}): 625, 737, 843, 1409, 1488, 1618, 1651, 3237, 3348.
$CIMS(CH_4)$: 181 $[phenH^+]^+$.

Compounds **2**, **3**, **4** and **5** were prepared under identical conditions to **1**.

*6.1.1.2. (phen)$_2$(imidazole) (H^+) (BF_4^-) (**2**).* Yield: 50.2%.
Anal. calcd. for $C_{27}H_{28}N_6BF_4$ (C, H, N):
Calculated (%): C, 62.75; H, 4.06; N, 16.27.
Found (%): C, 62.93; H, 4.19; N, 16.34.
FTIR cm^{-1}: 731, 841, 1056(BF_4^-), 1422, 1509.
$CIMS(CH_4)$: 181 $[phenH^+]^+$, 196 $[phen(CH_4)]^+$.

*6.1.1.3. (phen)$_2$(benzimidazole) (H^+) (BF_4^-) (**3**).* Yield: 38.7%.
Anal. calcd. for $C_{29}H_{31}N_6BF_4$ (C, H, N):
Calculated (%): C, 65.74; H, 4.06; N, 14.83.
Found (%): C, 65.31; H, 3.84; N, 14.30.
FTIR cm^{-1}: 729, 839, 1058(BF_4^-), 1422, 1510, 1619.
$CIMS(CH_4)$: 117 $[bimid]^+$, 181 $[phenH^+]^+$, 196 $[phen(CH_4)]^+$

*6.1.1.4. (5-NH_2-phen)$_4$(H_2O)$_3$ (**4**).* Yield: 49.7%.
Anal. calcd. for $C_{48}H_{42}N_{12}O$ (C, H, N):
Calculated (%): C, 68.98; H, 5.03; N, 20.12.
Found (%): C, 68.53; H, 4.91; N, 20.14.
FTIR cm^{-1}: 738, 1409, 1488, 1618, 1652, 3235, 3345, 3437.

*6.1.1.5. (phen)$_3$(indole) (H^+) (BF_4^-) (**5**).* Yield: 21.9%.
Anal. calcd. for $C_{44}H_{31}N_7BF_4$ (C, H, N):
Calculated (%): C, 70.82; H, 4.29; N, 13.14.
Found (%): C, 70.78; H, 4.09; N, 12.63.
FTIR cm^{-1}: 715, 776, 841, 1060(BF_4^-), 1421, 1596, 1616, 3161, 3386.

Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values.

6.1.1.6. X-ray structural analysis. Compound 2 crystallizes in the monoclinic space group *P*2(1)/*c* with $a = 9.359(5)$, $b = 18.578(10)$, $c = 13.651(8)$ A, beta = 93.01(65)°, V = 2416(2) $Å^3$. Intensity data were collected for a crystal of 2 up to theta = 25° on a Siemens P4 diffractometer at 294 K using omega scans and Mo Kalpha radiation

(0.71073 Å). Semi-empirical absorption corrections were applied using psi scan data. The structure was solved by direct methods (SHELXS) and refined with the program SHELXL to $R1 = 0.109$ for 1780 reflections with $I > 2\text{sigma}(I)$ and $wR2 = 0.288$ for all 4032 independent reflections. Anisotropic temperature factors were introduced for the non-hydrogen atoms and protons were refined at geometrically calculated positions as riding atoms. The relatively high R1 and wR2 values were due to the poor crystal quality.

6.1.2. Pharmacology

Cytotoxicity of the substances was measured *in vitro,* using the following cultivated human tumour cell lines:

6.1.2.1. HepG2. Hepatocellular carcinoma derived from liver tissue of 15-year-old male with differentiated hepatocellular carcinoma, epithelial in morphology (American Type Culture Collection ATCC, Rockville, MD, USA).

6.1.2.2. HEp-2. Tumour cells from epidermoid carcinoma, larynx, HeLa markers (ViroMed Laboratories, Minnetonka, MN, USA). This cell line was established in 1952 [14] from tumours that had been produced in irradiated-cortisonized weanling rats after injection with epidermoid carcinoma tissue from the larynx of a 56-year-old male [15];

6.1.2.3. 8-MB-GA. Human glioblastoma multiforme, brain tumour cells, epithelial-like, adherent cells growing as monolayer, established from the frontal brain lobe of a 54-year-old woman with glioblastoma multiforme in 1989 [16].

6.1.2.4. Lep-3. Non-tumour human diploid cell line (as a control).

Cells were cultivated with different amounts of the substances (ten-fold dilutions from 4 mg/ml solution, in the interval from 4 to 4.10^{-5} mg) and kept in 5% humidified atmosphere of CO_2 for 24 h. Toxicity was determined by the MTT- Mossman's test [11]. Cells were seeded in 96-well flat-bottomed microplates (Orange scientific) at a concentration of 2.10^4 cells/well. At the 24th h cells from monolayers were washed and covered with media modified with different amounts of compound tested. Each amount was applied in 4 wells. Samples of cells, grown in non-modified medium, served as a control. After 24 h of incubation, the solutions were removed from the plates. After 3 h incubation with MTT solution (5 mg MTT in 10 ml D-MEM) at 37 °C under 5% carbon dioxide and 95% air, then extracted with a mixture of ethanol and DMSO (1:1, vol/vol). The absorbance of each well at 540 nm was read by an automatic microplate reader (Absorbance Reader Tecan). Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each amount. Applied amount response curves were constructed manually for each experiment. All data points represent an average of three independent assays.

6.1.3. Statistical analysis

Statistical deviations were calculated automatically by Excel 2007 software program.

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