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Tellurium-based cysteine protease inhibitors: evaluation of novel organotellurium(IV) compounds as inhibitors of human cathepsin B

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Abstract—New organotellurium(IV) compounds with specific cysteine protease inhibitory activity were synthesized. Serine and aspartic protease activity were not affected by any of these compounds. All Te(IV) compounds tested exhibited high specific second-order constant for cathepsin B inactivation. Tellurium(IV) compound **6** was the best inhibitor of the series, showing a second-order constant of 36,000 $M^{-1} s^{-1}$. This value is about 100-fold higher than the second-order rate for cysteine protease inactivation shown by the historic Te(IV) compound AS 101 (**1**). The inhibition was irreversible and time and concentration dependent; no saturation kinetics were observed, suggesting a direct bimolecular reaction. The results described in this paper show that the new organotellurium(IV) compounds are powerful inhibitors of cathepsin B, constituting promising potential anti-metastatic agents.
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1. Introduction

Lysosomal cysteine proteases, especially cathepsin B, can participate in tumor invasion by degradation of extracellular matrix components.[1] This can take place either intracellularly, by heterophagosomal activity of tumor cells,[2] or extracellularly, by cathepsin B associated to cell surface.[3] The presence of cathepsin B at the plasma membrane results in local dissolution of extracellular matrix proteins and thus enables the tumor cells to invade.[4,5] A high level of cathepsin B and qualitative changes in cathepsin B protein expression, including abnormal pattern of glycosylation, may be important to maintain the malignant phenotype in carcinoma cells.[6] Alterations in cathepsin B expression, in its processing and in its cellular localization have been observed in several human tumor tissues. Clinical investigations showed that cathepsin B is a highly predictive indicator for prognosis and diagnosis of cancer.[7] Inhibition of cysteine protease family of enzymes is often sufficient enough to abrogate the invasion of several types of human and murine cells. The inhibition of just cathepsin B has been postulated to be directly responsible for the abrogation of the invasion process in several tumor cells lines, as for example in the EJ transitional cell carcinoma of bladder,[8] in HOC-1 ovarian cancer cells,[9] and also in the human prostate cancer cell lines.[10] In view of these facts, the development of protease inhibitors has paramount importance in the search for chemotherapeutic agents.

In 1998, Albeck and coworkers found that tellurium(IV) compounds present protease inhibitory activity.[11] The inhibition was specific for cysteine proteases like papain and cathepsin B from bovine spleen. The most potent inhibitor was ammonium trichloro(dioxyethylene-*O*,*O*′)tellurate **1**, also known as AS-101 (Fig. 1), which showed a second-order inhibition constant of

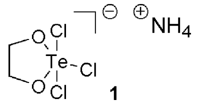

Figure 1. The molecular structure of AS-101.

Keywords: Cysteine protease inhibitor; Cathepsin B; Tellurium(IV) compounds; Organotellurium.

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$37\,M^{-1}\,s^{-1}$. Moreover, among various applications, AS-101 showed immunomodulatory activity[12] conferring protection against deleterious effects of radio-[13] and chemotherapy,[14] and also showed synergistic action with Taxol.[15]

Besides AS-101, the investigation of therapeutic activities of other tellurium(IV) compounds is scarce in the literature when compared to tellurium(II) derivatives.[16] Organotellurium compounds occur naturally as dimethyltelluride and dimethylditelluride just in telluride-resistant microorganism strains.[17] Since no biological function for tellurium is still recognized,[18] this compound is still considered a non-essential element for life.[19] On the other hand, selenium is an essential trace element,[20] but it was earlier considered a non-essential trace element until the discovery of selenium-dependent enzymes like the glutathione peroxidase.[21] The biological importance of organoselenium compounds is much more established than for organotellurium compounds and a large body of information is already available in this field.[22] Following this interest for selenium and the important discovery by Albeck and coworkers for some tellurium(IV) compounds,[11] we can envisage a similar development for the applications of tellurium derivatives in biology and biochemistry.

In view of our long involvement with tellurium chemistry[23] as well as the interest of some of us in cysteine protease inhibition,[24] we initiated work to investigate the inhibitory activity of a number of classes of organotellurium(IV) compounds towards human Cathepsin B. In this paper we describe our first preliminary results on this subject. We choose representative examples of the most common classes of organotellurium(IV) compounds varying the characteristics of the groups bonded to the tellurium atom (Chart 1). The synthetic methodology developed by us to synthesize these classes of tellurium compounds, which involves the interaction of tellurium electrophilic reagents with alkynes or methylaryl ketones, allows the synthesis of a congeneric series. To the best of our knowledge,[25] our group was the first to synthesize a tellurooxetane,[26] the most active compound of the studied series presented in this work.

2. Experimental part

2.1. Materials

Non-glycosylated human cathepsin B S115A, denominated wild-type cathepsin B was sub-cloned, as proenzymes, into the vector pPIC9 and expressed in yeast *Pichia pastoris* as described previously.[27] The concentration of the active enzyme was determined by titration using the cysteine proteinase inhibitor E-64.[28] Papain, the irreversible inhibitor E-64, the fluorogenic amidomethylcoumaryl substrate Cbz-FR-MCA, and DTT were purchased from Sigma. Size exclusion Sephadex G-25 resin was purchased from Amersham Pharmacia Biotech.

2.2. Tellurium compounds

Tellurium tetrachloride was obtained by the reaction of tellurium (230 mesh) and chlorine,[29] *p*-methoxyphenyltellurium trichloride[30] and *p*-methoxyphenyltellurium tribromide,[30] 1-ethynyl-1-cycloheptanol[30] and 1-phenyl-2-propyn-1-ol[31] were prepared according to the known methods. 1-Ethynyl-1-cyclohexanol and 2,2-dimethyl-1-propyn-2-ol were purchased from Aldrich and used without further purification.

Tellurium compound **2** was prepared by the reaction of tellurium tetrachloride with propargyl alcohol in benzene followed by complexation with triethylbenzylammonium chloride.[32] Compound **5** was obtained by refluxing tellurium tetrachloride with 1-phenyl-2-propyn-1-ol in benzene.[33] Compounds **3**, **6**, **8**, and **9** were prepared by refluxing *p*-methoxyphenyltellurium trichloride, respectively, with 2,2-dimethyl-1-propyn-2-ol, 1-ethynyl-1-cyclohexanol, 1-ethynyl-1-cycloheptanol, and phenylacetylene in dry benzene as described previously.[26] Compound **7** was prepared in a similar way as

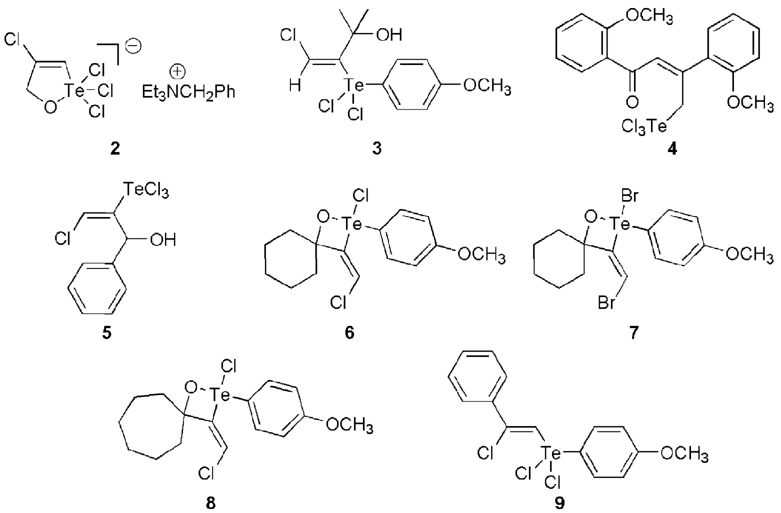


Chart 1. Organotellurium(IV) compounds tested as inhibitors of Cathepsin B.

the corresponding chlorides by reacting 1-ethynyl-1-cyclohexanol with *p*-methoxyphenyltellurium tribromide. Compound **4** was prepared by reacting tellurium tetrachloride with *ortho*-methoxyacetophenone in benzene under reflux.[34] All compounds were fully characterized by spectroscopic methods (^{1}H NMR, ^{13}C NMR, ^{125}Te NMR and IR) and by elemental analysis. In addition, X-ray diffraction analyses were performed for monocrystals of the whole series, confirming the structures proposed by routine spectroscopic methods.

2.3. Kinetic measurements

Cathepsin B activity was monitored spectrofluorometrically using the fluorogenic substrate Cbz-Fr-MCA on a Hitachi F-2000 spectrofluorometer equipped with a thermostated cell holder. The excitation and emission wavelengths were set at 380 and 460 nm, respectively. Prior to the assay, the enzyme was activated by incubation for 10 min at 25 °C in 50 nM sodium phosphate (pH 6.0), 200 nM NaCl, 1 mM EDTA, and 5 nM DTT, followed by gel filtration in order to remove the excess DTT (see below). The cathepsin B kinetics of Cbz-FR-MCA hydrolysis were performed in the absence or in the presence of different Te(IV) compounds concentrations at 37 °C in 50 mM sodium phosphate (pH 6.0) containing 200 mM NaCl and 1 mM EDTA. The substrate concentration was kept 20-fold below the K_M values. The kinetics of cathepsin B inactivation by Te(IV) compounds were obtained in pseudo-first-order conditions. The inhibition reaction was monitored continuously by the fluorescence of the enzymatic-release product. The obtained exponential decay curves could be better fitted according to the first-order relationship shown in Eq. 1.

$$P = P_\infty[1 - \exp(-k_{\mathrm{obs}}t)], \tag{1}$$

where P and P_∞ are the product concentration at a given time and at infinite time, respectively, and k_{obs} is the observed first-order rate of Te(IV)-induced enzyme inactivation. Irreversible inhibition of cathepsin B by Te(IV) compounds was verified by gel permeation chromatography as described below.

2.4. Gel permeation chromatography

In order to remove the DTT excess, a 1 mL solution of activated cathepsin B was loaded on a 1 × 20 cm Sephadex G-25 column, preequilibrated with 50 mM sodium phosphate buffer (pH 6.0) containing 200 mM NaCl and 1 mM EDTA. The enzyme was eluted with the same buffer, and the cathepsin B activity was monitored as described above. To determine if the inhibition promoted by Te(IV) compounds upon cathepsin B activity was irreversible or not, a 1 mL solution of an inhibition mixture was applied in the same gel permeation approach, and the amount of cathepsin B activity recovered from the column was measured.

2.5. Cell culture and survival assay[35]

Human HL60 promyelocytic leukemia cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 UI/mL). The cytotoxity of compound **6** was assessed using a cell proliferation assay developed by Mosmman, 1983. Briefly, 5×10^5 exponentially growing cells were seeded in 96-well microculture plates with various drugs concentrations in a volume of 100 µL. After 72 h incubation, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye (5 mg/mL) were added to each well and the samples were incubated for 2 h at 37 °C. Formazan crystals were dissolved with 100 µL of 0.04 M HCl–isopropyl alcohol. The optical density (OD) of the wells was measured with a microplate reader at 630 nm. HL60 cells survival was calculated by Eq. 2.

$$\text{HL60 cells survival} = (\text{mean OD treated well} / \text{mean OD control well}) \times 100. \tag{2}$$

3. Results

Te(IV) compounds **2**–**9** were specific to inhibit cysteine protease Cathepsin B. No inhibition was observed for serine proteases, trypsin, and elastase, or for aspartic protease cathepsin D.

A representative time course of cathepsin B inactivation by compound **3** is shown in Figure 2a. As can be observed, compound **3** led to enzyme inactivation in a time- and concentration-dependent manner. The apparent inactivation rate constant was measured in function of compound **3** concentration to determine the second-order constant for the inhibitor (Fig. 2b). The kinetics analysis shows that **3** inactivated cathepsin B by a simple bimolecular reaction, since no saturation kinetics was observed in the inactivation of the protease by this compound.

Similar experiments were performed with all organotellurium(IV) compounds of the series and the same behavior was observed in all cases. The second-order inactivation rates were dependent on the inhibitors structure (Table 1). Compound **6** was the best inhibitor of the series, showing a second-order constant of 36,000 $M^{-1}s^{-1}$. This value of second-order rate of inactivation constants is about two-magnitude order higher than the value exhibited by AS 101, the first tellurium(IV) compound to show protease inhibitory activity.[11]

The inhibition of the cathepsin B by organotellurium(IV) compounds **2**–**9** was found to be irreversible by gel permeation chromatography of the inhibited enzyme. After the chromatography, no enzymatic activity was recovered. On the contrary, the control experiment showed that active cathepsin B was totally recovered under identical chromatography conditions (Fig. 3). The inhibited enzyme could be reactivated by incubation with 2 mM DTT at 37 °C for 10 min. In addition, the enzyme inhibition could be avoided by pretreatment of the enzyme with a thiol derivative such as cysteine, DTT,

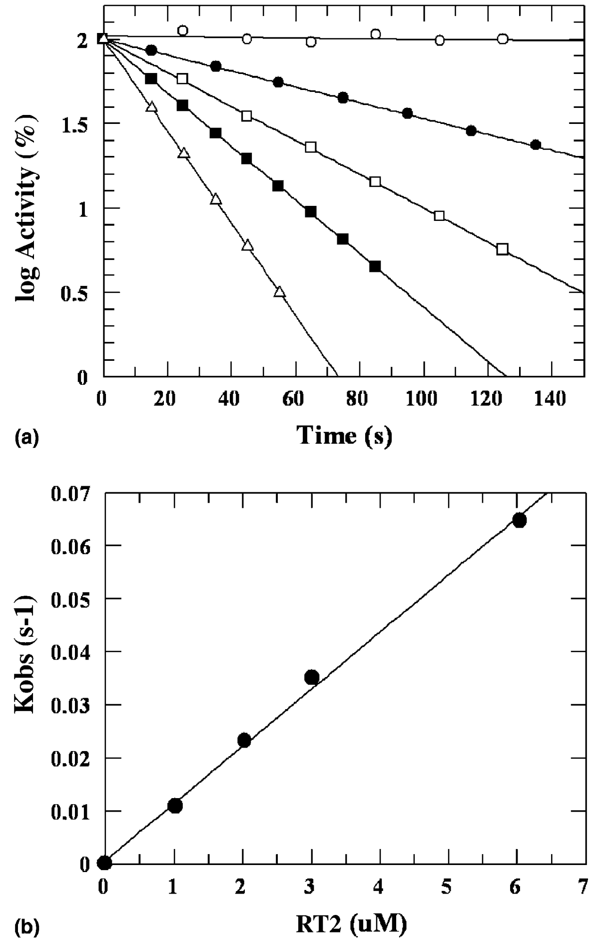


Figure 2. Time course of inactivation of cathepsin B by compound **6**. (a) The kinetics of inactivation of cathepsin B was performed in 50 mM sodium phosphate (pH 6.0) containing 200 mM NaCl and 1 mM EDTA, at various concentration of compound **6**: control (○), 1.0 (●), 2.0 (□), 3.0 (■), 6.0 (△) μM of **6**. The progress of the reaction was monitored continuously by the fluorescence of the released product. (b) Cathepsin B first-order rate inactivation in function of compound **3** concentration.

Table 1. Second-order rate constants for the inactivation of cathepsin B by Te(IV) compounds

Organotellurium(IV) derivative	Cathepsin B Inhibition k_2, $10^3\,M^{-1}s^{-1}$
1	3.7×10^{-2} (Ref. 11)
2	6.0 ± 0.3
3	11 ± 1
4	15 ± 2
5	12 ± 1
6	36 ± 3
7	7.7 ± 0.9
8	1.6 ± 0.1
9	7.9 ± 0.4

Values are means of four experiments.

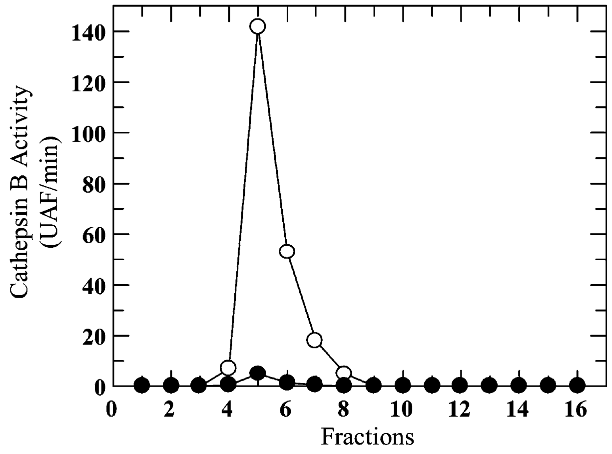


Figure 3. Gel permeation chromatography: Cathepsin B was incubated in 50 mM sodium phosphate (pH 6.0) containing 200 mM NaCl and 1 mM EDTA, in the absence (○) or in the presence of 2.0 μM **6** (●) and then submitted to Sephadex G-25 column.

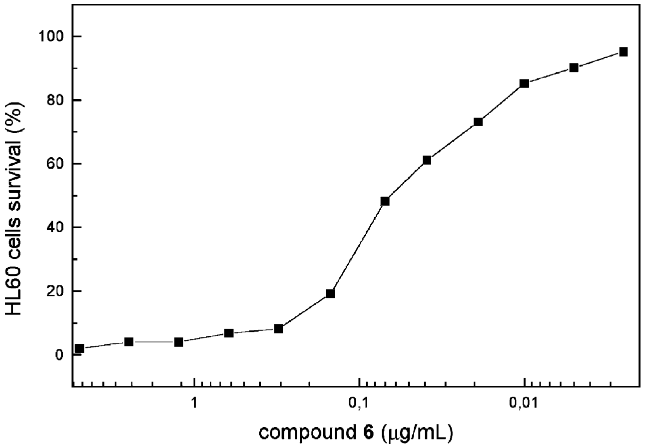


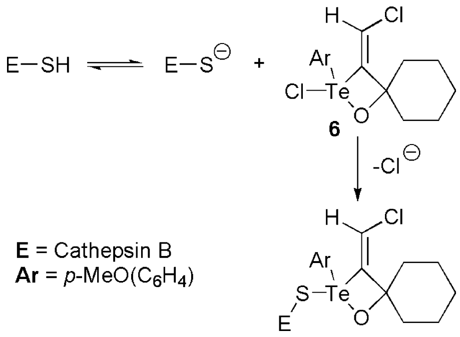
Figure 4. Cytotoxicity of compound **6** towards HL60 cells.

and mercaptoethanol. The inactivation rate was decreased by the previous saturation of the enzyme with its substrates. These results suggest that the irreversible character of the inhibition is related to the functionalization of the thiol group of the enzyme active site, which replace one of the tellurium ligands leading to Enz-S-TeL_3.

Compound **6** was tested for its cytotoxicity against HL60 cells. After 3 days of continuous exposure compound **6** exhibited a marked cytotoxic effect. Its cytotoxicity (IC_{50}, 72 h, MTT assay[35]) was approximately 0.07 μg/mL (Fig. 4).

4. Discussion

The inactivation of cathepsin B by organotellurium(IV) compounds can be due to the high nucleophilic character of the thiol residue at the active site of cathepsin B combined with the electrophilic character of the tellurium atom. The covalent interaction of these organotellurium(IV) compounds and the enzyme could be established by a nucleophilic attack at tellurium atom by the enzyme thiolate followed by the displacement



Scheme 1. Proposed interaction of Cathepsin thiolate with compound **6**.

of the chloride ion or other leaving group linked to tellurium (Scheme 1).

According to this hypothesis, the high reactivity of compound **6** could be associated with the Lewis acidic character of the tellurium atom. The substitution of chloride ion takes preference over the ring opening of the telluroxetane ring as the study of the model reaction with cysteine suggests.[36] Interestingly, the interaction of telluroxetanes with the enzyme is biased by the volume of the cycloalkane ring because telluroxetane **8**, with a cycloheptane ring is 20-fold less active than derivative **6**. Moreover the substitution of chloride by bromide in derivative **7** also led to a 4-fold decrease in the inhibition efficacy. These differences in the inhibition activity of compounds **7** and **8** compared to **6** can only be attributed to the nature of the halogen linked to tellurium, or to the increase of the cycloalkane ring, which modifies the electronic and the steric characteristics of each compound. A more detailed study with model thiols is in course to shed light on this matter. The interesting difference in reactivity between compounds **6** and **7** shows that the synthesis of similar telluroxetane derivatives would be interesting in order to enhance the activity of the organotellurium(IV) compounds by variation of the substituents on tellurium or on the organic chain linked to tellurium.

In conclusion, we described the potent inhibitory activity of novel organotellurium(IV) compounds towards Cathepsin B. Our results indicate a potential therapeutic application for them as anti-metastatic drugs.[37] In this context it must be emphasized that the inhibition of cathepsin B promoted by these compounds is dependent on the absence of reducing agents, since the presence of such agents suppress the inhibition. This fact suggests that the Te(IV) compounds studied by us may be effective only against the extracellular form of cathepsin B, since the cytoplasm of the cells contain a large amount of reducing agents, such as glutatione, cysteine, NADPH, and others. It is well known that in normal cells, cathepsin B is confined to the lysosome, but in cancerous cells a 4–30-fold increase of cathepsin B levels in the plasma membrane of the cell surface is observed.[38] These facts indicate that the Te(IV) compounds **2**–**7** can be effective only on cell surface cathepsin B without interfering with the lysosomal normal forms of the enzyme.

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34. NMR data for compound **4**. ^{1}H NMR (CDCl$_3$, 500 MHz, ppm, J in Hz) δ 7.93 (dd, $J = 1.9$ Hz, 1H), 7.65–7.61 (m, 1H), 7.47–7.42 (m, 3H), 7.09–7.03 (m, 2H), 7.02–6.96 (m, 2H), 5.22 (s, 1H), 3.92 (s, 3H), 3.90 (s, 3H). ^{13}C NMR (125 MHz, CDCl$_3$, ppm) δ 197.2, 160.4, 157.1, 155.8, 137.2, 132.8, 132.1, 130.2, 127.2, 125.8, 121.3, 121.2, 112.2, 111.4, 63.1, 56.1, 55.7.
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