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Synthesis, antioxidant and antitumoral activities of 5'-arylchalcogeno-3-aminothymidine (ACAT) derivatives†‡

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This article presents the preparation and *in vitro* biological activities of new 5'-arylchalcogeno-3-aminothymidine derivatives as antioxidants (inhibition of lipid peroxidation, scavenging of the free radical 2,2-diphenylpicrylhydrazyl and demonstration of a thiol peroxidase-like activity) as well as antitumoral agents against bladder carcinoma 5637. The chalcogeno-aminothymidines presented prominent activity in the tests for both biological properties, showing a direct relation with the chalcogenium atom.

Introduction

In recent years, nucleosides have been a subject of intense investigation. A variety of new nucleosides have been fashioned, mainly in the search for compounds with antiviral or antitumoral properties.^{1–4} Generally, modifications in the base or in the carbohydrate portion have been made to access some key factors for increasing the biological potentiality of these new nucleosides. These modifications may include a variety of reactions such as nucleophilic addition and oxidation or reduction reactions, which may afford new functionalities and modify the biological behavior of the new analogues. On the other hand, nucleoside analogues represent important drugs for different cancer treatments. Currently, approved antitumoral nucleoside analogues include cytarabine, fludarabine, cladribine, gemcitabine, clofarabine, capecitabine, floxuridine, deoxycoformycin, azacitidine and decitabine.⁵ Intracellularly, these nucleosides are converted to their respective nucleotide analogues, which inhibit DNA synthesis through the inhibition of DNA polymerase and/or ribonucleotide reductase.⁶ However, resistance to nucleoside analogues is a common problem in cancer treatment and the resistant profile can be

caused by poor conversion of the parent nucleoside to its active nucleoside monophosphate, diphosphate and triphosphate. Another reason for the decreased activity of a nucleoside analogue is the limited uptake by tumor cells, due to decreased expression of nucleoside transporter proteins.^{5,7,8}

On the other hand, organochalcogenium compounds have been receiving special attention from the scientific community, especially due to their biological properties. For instance, antiviral, antimicrobial, antioxidant and antitumoral are some of the properties related to molecules containing a chalcogenium atom.^{9–12} Despite the prominent importance of nucleosides and chalcogenium compounds, methodologies for preparing chalcogeno-nucleosides are rarely reported and still very limited compared to their isolated counterparts. Consequently, protocols seeking chalcogenium introduction in nucleosides or selective modifications of chalcogeno-nucleoside molecules are a component of an important research field for the preparation of new libraries of organochalcogenium molecules with potential biological effects. The pharmacological effects of nucleosides rely greatly on their metabolism to nucleotides in the carbon 5'. However, they can also have other effects not necessarily mediated by nucleotide formation. Thus, the investigation of nucleoside analogues with substituents in the 5' position can be a tool to determine the extent that the “non-nucleotide pathway” can contribute to the pharmacology of nucleoside analogues. Therefore, the synthesis and biological evaluation of organochalcogenides with substituents in the 5' position are warranted. The study and comprehension of the biological behavior of these new compounds may help in the design of new classes of non-metabolizable nucleoside analogues. In this context, recently, our group has reported on the introduction of a

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Scheme 1 Synthesis and biological evaluation of the 5'-arylchalcogeno-3-aminothymidine derivatives.

chalcogenium moiety in the zidovudine nucleus and evaluated the antitumoral and antioxidant activity of the new compounds formed. The addition of a chalcogenium portion increased the antitumoral and antioxidant activities of the standard zidovudine.¹² In connection with our interest in the development of new nucleosides containing chalcogenium, the preparation of a series of 5'-arylchalcogeno-3-aminothymidine (ACATs) and studies involving the antitumoral and antioxidant activities of these new organochalcogenium compounds are presented here, as depicted in Scheme 1.

Results and discussion

Chemistry

Initially, the preparation of 3-azidothymidine mesylate **2** was carried out. For this, derivative **2** was obtained by reacting

the respective zidovudine **1** with mesyl chloride in THF at room temperature for 2 h using Et₃N as a base, affording compound **2** in a 92% yield.⁸ With the respective mesylate in hand, the preparation of compounds **3** began *via* introduction of a chalcogenium moiety and azide reduction. For this, NaBH₄ was employed as a reducing agent, diphenyl diselenide as a dichalcogenide source and azidothymidine mesylate **2** in a 10 mL mixture of THF/EtOH (7:3) as a solvent system. Initially, five eq. of the reducing agent was employed for reflux for 5 h, affording compound **3a** in a 38% yield. When seeking for an increment in the efficiency of this transformation, the reaction time was extended to 12 and 24 h and more effective transformations were consequently obtained, affording the desired product **3a** in 66% and 78% yields, respectively. Additionally, increasing the amount of NaBH₄ to 10 eq. or the reaction time to 36 h made the preparation of the compound in the same efficiency level possible. With the optimized reaction conditions in hand, the reaction was extended to other dichalcogenides in order to obtain a small library of 5'-arylchalcogeno-3-aminothymidine (ACATs) **3a-m** using THF:EtOH (10 mL, 7:3), NaBH₄ (5 eq.), the respective dichalcogenide (0.5 mmol) and azidothymidine mesylate **2** (1 mmol) by refluxing for 24 h, as depicted in Fig. 1.

When analyzing Fig. 1, for instance, in terms of the chalcogenium atom, it is possible to verify that all compounds were obtained in satisfactory yields, with a slight

Fig. 1 Synthesis of 5'-arylchalcogeno-3-aminothymidines **3a-m**.

Fig. 2 Compound **3j** (A) induces higher antiproliferative rates in 5637 cells than commercial AZT (B). Cell proliferation was investigated by the MTT assay. Data are expressed as means $\pm$ SEM from three independent experiments. The different uppercase letters (A and B) indicate significant differences between treatment times and the lowercase letters (a–c) indicate significant difference between treatment concentrations. Differences were considered significant at $P < 0.05$.

advantage of selenium over sulfur (Fig. 1, entries **3a**, **3c–e** and **3i**, **3k–m**, respectively). This may be due to the higher nucleophilicity of selenium than that of sulfur. Tellurium afforded the respective compound **3j** in a lower yield than sulfur and selenium, especially due to the instability of tellurostate in the re-

action system. The use of other ditellurides with activating or deactivating groups (*p*-OMe-C₆H₄ and *p*-Cl-C₆H₄) did not allow the preparation of the respective telluro-nucleosides. In terms of the electronic effects of the substituents attached to the aryl ring of the chalcogenium moiety, electron-donating groups in general allowed the preparation of compounds **3** in better yields than the deactivating ones. A plausible explanation for this may be the higher nucleophilicity of chalcogenolates with these groups attached to the aromatic ring. The higher yields observed in the examples without substituents in the aryl ring of the chalcogenium source (**3a** and **3i**) may be related to the balance between activating/deactivating groups. For instance, activating groups can increase nucleophilicity; however, they may make the cleavage of the diselenide (thus generating the anion) difficult. And on other side, deactivating groups can facilitate dichalcogenide cleavage but decrease nucleophilicity. The neutral substituents do not possess these electronic drawbacks and provide compounds with better efficiency. It is important to highlight the green chemistry aspect of the methodology, where two transformations are involved in a one-pot reaction: the nucleophilic substitution of the mesylate by the chalcogenolate and the reduction of the azide to the corresponding amine. Therefore, fewer reaction steps as well as lower consumption of solvents, energy and chemicals for additional purification are necessary.

Antitumoral studies

5'-Arylchalcogeno-3-aminothymidines **3a–e** and **3i–m** were tested against the 5637 bladder cancer cell line at concentrations from 0.78 to 100 μ M for 24 and 48 h. Additionally, DMSO alone was used as a vehicle for drug dilution and untreated cells were used as negative controls. The antitumoral studies showed that the compound **3j** significantly decreased 5637 cell viability *in vitro* in a time-dose-dependent manner with a cell growth inhibition over 50% from 6.5 μ M in both 24 and 48 h of treatment (Fig. 2A). The

Fig. 3 Antiproliferative effect of 5'-arylchalcogeno-3-aminothymidines on 5637 cancer cells in 48 h of treatment. ACATs **3a** (A), **3b** (B), **3c** (C), **3d** (D), **3e** (E), **3i** (F), **3j** (G), **3k** (H), **3l** (I), and **3m** (J). Cell proliferation was investigated by the MTT assay. Data are expressed as means $\pm$ SEM from three independent experiments.

Fig. 4 Number of 5637 human bladder carcinoma cells in each phase of the cell cycle (Sub G0, G0/G1, S, and G2/M) after 24 hours of treatment with compound **3j**. Results are expressed as mean $\pm$ SEM of the mean from three independent experiments. Data were analyzed by two-way ANOVA followed by Bonferroni test. ** $\neq$ G0/G1 control and * $\neq$ S control ($p < 0.05$).

vehicle alone showed no cytotoxicity or antiproliferative activity at 24 and 48 h of treatment. **3j** showed an *in vitro* antitumoral activity with IC_{50} values of $6.61 \pm 2.02 \mu\text{M}$ in 24 h and $7.94 \pm 1.92 \mu\text{M}$ in 48 h of treatment. Commercial AZT showed IC_{50} values of $128.82 \pm 1.43 \mu\text{M}$ in 24 h of treatment (Fig. 2B).

Data from compounds **3a–e** and **3i, k–m** demonstrated that they have no antiproliferative activity against 5637 bladder cancer cells showing IC_{50} values $>100 \mu\text{M}$ (Fig. 3). Since compound **3j** has tellurium (Te) in its structure, the selectivity observed among **3a–m** compounds in terms of *in vitro* growth inhibition of 5637 cells may be related to the chalcogenium atom. Both organic and inorganic Te derivatives presented antimicrobial, antihelminthic, antioxidant, immunomodulatory, and anticancer activities.^{13–18} A study from Abondanza *et al.*¹⁷ demonstrated that an organotellurium compound was able to induce apoptosis in HL60 human promyelocytic leukaemia cells by down-modulation of Bcl-2 expression. Additionally, small inorganic Te complexes displayed excellent safety profiles and promising anti-cancer therapeutic potential.¹³ Having the prominent antitumoral activity for tellurium-aminothymidine **3j** over its

Fig. 5 Effect of azidothymidine derivatives (200 μM) on the inhibition of lipid peroxidation induced by iron. Diphenyl diselenide ((PhSe)₂) and α -tocopherol (200 μM) were used as positive controls. a $\neq$ DMSO; b $\neq$ (PhSe)₂; and c $\neq$ α -tocopherol.

Fig. 6 Concentration dependent inhibition of lipid peroxidation by compound **3j**, (PhSe)₂ and α -tocopherol. Results are expressed as mean $\pm$ S.E.M. of three determinations. * $\neq$ 0 μM . Data were analyzed by one-way ANOVA followed by Newman–Keuls test ($p < 0.05$).

selenium and sulfur counterparts, additional studies for this compound were performed in order to evaluate its toxicity and antioxidant activity.

Cell cycle analyses

The number of cells in the different phases of the cell cycle was detected by flow cytometry after treatment with compound **3j**. The results in Fig. 4 show that a significant reduction of cells in the G1 phase was observed in cells exposed to **3j** compared to untreated cells. A higher number of cells was detected in the S phase of the cell cycle but apoptosis was not observed in 5637 cells in response to a dose of 12.5 μM of **3j**. The cell cycle arrest in the S phase (DNA synthesis) can be related to the fact that azido-thymidine is an analogue of thymidine. When in its active form, zidovudine triphosphate is a substrate, together with natural thymidine triphosphate, which can be incorporated into the DNA chains.²⁰ Once incorporated, it stops the growth of the DNA chain, blocking cellular replication.²⁰ The study of Olivero *et al.*²¹ demonstrated that AZT is responsible for the accumulation of cells

Fig. 7 DPPH scavenging activity of azidothymidine derivative compounds (1 mM) after 90 min of incubation. a $\neq$ DMSO. Butylated hydroxytoluene (BHT, 0.5 mM) was used as a positive control.

Fig. 8 Concentration and time-dependent curves of compound **3j** in the DPPH scavenging activity test. Results are expressed as mean $\pm$ S.E.M. of three determinations. * $\neq$ 0 minute. Data were analyzed by one-way ANOVA followed by Newman-Keuls test ($p < 0.05$).

in the S phase, which is in agreement with the data obtained in this work.

Antioxidant evaluation

Generally, antitumoral compounds show drawbacks related to their toxicity, such as side effects related to reactive oxygen species (ROS). In this context, organochalcogenium compounds appear as prominent antioxidants, showing exciting results as radical scavengers. Based on this, the antioxidant potential of the compounds was investigated in three chemical systems, namely, inhibition of iron-induced phosphatidylcholine oxidation (TBARS production),¹⁹ scavenging of the free radical 2,2-diphenylpicrylhydrazyl (DPPH),²² and demonstration of a thiol peroxidase-like activity.²³ From the 10 compounds tested at 200 μ M, only **3j** exhibited a favorable inhibitory effect against Fe(II)-induced lipid peroxidation of phosphatidylcholine (Fig. 5). Compound **3j** was more effective than diphenyl diselenide and presented an effect similar to α -tocopherol (positive control). The inhibitory concentration curve for compound **3j** (Fig. 6) was then formed. The calculated IC_{50} for lipid peroxidation inhibition was 4.67 ± 0.33 μ M (**3j**), 35.3 ± 3.48 μ M (α -tocopherol) and 232.7 ± 15.38 μ M

Fig. 9 Thiol peroxidase-like activity of azidothymidine derivatives (450 μ M). Diphenyl diselenide ((PhSe)₂) was used as a positive control. a $\neq$ DMSO. Results are expressed as mean $\pm$ SEM of three determinations. Data were analyzed by the student's *t* test.

Fig. 10 Concentration curve of compound **3j** in the thiol peroxidase-like activity test. Diphenyl diselenide ((PhSe)₂) and ebselen were used as positive controls. * $\neq$ 0 μ M. Results are expressed as mean $\pm$ SEM of three determinations. Data were analyzed by one-way ANOVA followed by the Newman-Keuls *post hoc* test ($p < 0.05$).

(diphenyl diselenide). The IC_{50} value for TBARS formation of **3j** was significantly lower than those found for diphenyl diselenide and α -tocopherol ($p < 0.05$ in both comparisons).

Notably, only compound **3j** was effective (Fig. 7) in scavenging the colored free radical 2,2-diphenylpicrylhydrazyl by the azidothymidine derivative compounds at 1 mM. Concentration- and time-dependent inhibitory curves were then formed for this compound (Fig. 8). Compound **3j** scavenged 50% of DPPH after approximately 38.7 ± 8.9 min (1 mM), 77.3 ± 10.3 min (0.5 mM), 104.7 ± 10.0 min (0.25 mM), and >180 min (0.1 mM). The activity of all azidothymidine derivatives was lower than the positive control BHT, which scavenged 50% of the DPPH radical in approximately 21.33 ± 0.6 min (0.5 mM) and 22.5 ± 0.87 min (0.25 mM).

Thiol peroxidase-like activities were determined for all compounds. This methodology may indicate the efficacy of azidothymidine derivatives to mimic the antioxidant enzyme glutathione peroxidase (GPx). At 450 μ M, compounds **3a**, **3b**, **3c**, **3e**, **3j**, and **3l** decomposed H₂O₂ more efficiently than the control (DMSO) (Fig. 9). Next, a concentration curve with compound **3j** was formed and ebselen and diphenyl

Fig. 11 Cell viability in human leukocytes after treatment with compound **3j**. *t*-Butyl hydroperoxide (1 mM) was used as a positive control. Results are expressed as mean $\pm$ SEM of three determinations. * $\neq$ DMSO. Data were analyzed by one-way ANOVA followed by Newman-Keuls test ($p < 0.0001$).

Fig. 12 Survival rate after subcutaneous (s.c.) injection of 100 $\mu\text{mol kg}^{-1}$ of **3j**. Data were analyzed using the log-rank (Mantel-Cox) test.

diselenide were used as positive controls (Fig. 10). In the presence of thiophenol, compound **3j** enhanced H_2O_2 decomposition with similar potency from 55 to 225 μM . Furthermore, compound **3j** also presented considerably better GPx-like activity than ebselen and diphenyl diselenide (positive controls). In previous studies, few selenium-containing AZT derivatives were observed to have exhibited weak antioxidant properties (e.g., inhibition of lipid peroxidation and Gpx-like activity). Here, none of the selenium compounds tested exhibited appreciable antioxidant activity.²⁴ One plausible explanation for the absence of antioxidant activity of the selenium-containing compounds tested here can be related to the relative stability of the selenium moiety. In general, it is possible to separate organoselenium compounds in two generic groups: those that can be metabolized or chemically reduced to selenol intermediates (a variety of diselenides and ebselen) and those that can be transitorily oxidized to selenoxides (for instance, selenophenes).^{25,26} Although the chemical nature of diselenides normally indicates a region that can be reduced under biological conditions, the oxidation of selenide to selenoxide is less difficult to predict *a priori*. The presence of bulky substituents in the selenium

Fig. 13 Organ-to-body weight ratio of the brain (A), liver (B), kidney (C) and spleen (D) after treatments with **3j** (100 $\mu\text{mol kg}^{-1}$) or DMSO (control group). Data were analyzed by the unpaired *t* test. There was no significant difference between the groups.

Fig. 14 (A) Effect of **3j** (100 $\mu\text{mol kg}^{-1}$, s.c.) on the urea and (B) creatinine levels. Data were analyzed by the unpaired *t* test. There was no significant difference between the groups.

atom and electronic interferences may have hindered the reactivity of Se in the ATZ derivatives studied here.

Cell toxicity

Human leukocyte viability^{27,28} is shown in Fig. 11. Incubation of human leukocytes with *t*-butyl hydroperoxide (positive control) caused a decrease in cell viability (approximately 40%) when compared to the control (DMSO). In contrast, incubation of human leukocytes with compound **3j** (100 μM) did not change cell viability.

Toxicity *in vivo*

Mice were injected with 100 $\mu\text{mol kg}^{-1}$ of compound **3j** or DMSO (control group) subcutaneously (s.c.) and observed for 7 days. No overt sign of toxicity was visualized, except for the one animal injected with **3j** that died after 24 hours (Fig. 12). Food, water and body weight were verified after the treatment and **3j** s.c. injection did not change the parameters (data not shown).

After 7 days, the animals were euthanized and tissues (the brain, liver, kidney and spleen) and blood were collected. Fig. 13A–D depict the organ-to-body weight ratio for the brain, liver, kidney and spleen. By analysing Fig. 13, it is possible to see that compound **3j** did not modify the ratios compared with the standard DMSO.

Urea and creatinine levels are presented in Fig. 14A and B, and **3j** did not change their levels in the plasma. These results indicated that the compound did not exhibit renal toxicity.

Additionally, ALT (Fig. 15A) and AST (Fig. 15B), which are classical markers of liver function, were evaluated. As

Fig. 15 (A) Effects of **3j** (100 $\mu\text{mol kg}^{-1}$, s.c.) on ALT and (B) AST activity. Data were analyzed by the unpaired *t* test. There was no significant difference between the groups.

observed in Fig. 14, these parameters did not change after treatment with 100 $\mu\text{mol kg}^{-1}$ of 3j, indicating that the compound does not have a hepatotoxic effect.

All experiments are in accordance with law 11.794, of October 8, 2008, Decree 6899, of July 15, 2009, with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was approved by the Ethics Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 03/31/2016 (Protocol number CEUA 4622031115).

Conclusion

The synthesis of a new series of 5'-arylchalcogeno-3-aminothymidine and the biological evaluation of the respective compounds are described here. As observed, the antioxidant and antitumoral activities were strictly related to the chalcogenium atom, thus revealing impressive biological responses in the parameters evaluated for the tellurium derivative 3j. Additionally, the toxicities *in vivo* and *in vitro* for the most effective compound were evaluated and no cell damage or overt sign of toxicity was observed, although the dose of 3j tested here caused 25% of mortality. The high lethality of this high dose indicates that a detailed dose-response curve has to be done in mice and rats.

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