Antioxidant Activity of a Series of Fluorinated Pyrano-nucleoside Analogues of N^4-benzoyl Cytosine and N^6-benzoyl Adenine

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Abstract: Fluorinated nucleoside analogues are known as antitumor, antiviral and chemotherapeutic agents, although the antioxidant activity of this kind of molecules is not yet investigated. In this study we have tested the antioxidant activity of a series of modified pyrano-nucleoside analogues using three in vitro assays. Firstly, the antioxidant capacity of the products was assessed using the DPPH assay and secondly, in order to examine the ability of the products to protect DNA from the activity of reactive oxygen species (ROS), a peroxyl radical (ROO•) and a hydroxyl radical (OH•) induced DNA strand scission assay were used. None of the molecules showed the ability to scavenge DPPH radical and prevent OH• induced DNA strand breakage. Although, most of the tested nucleoside analogues, had the ability to prevent ROO• induced DNA damage.

Keywords: fluorinated nucleosides, antioxidants, DPPH, DNA damage, ROS.
1. Introduction

Nucleoside analogues display a wide range of biological activities as antitumor, antiviral and chemotherapeutic agents [1-4]. The last decades, nucleosides with a six-membered carbohydrate moiety have been evaluated for their potential antiviral [5-8] and antibiotic [9] properties and as building blocks in nucleic acid synthesis [10,11]. One series of uncommon six-membered nucleoside analogues, the unsaturated ketonucleosides, are well established for their antineoplastic activity and immunosuppressive effects [12-16]. It appeared that these nucleosides not only exhibit growth inhibitory activity against a variety of tumor cells [17,18] in vitro and L1210 leukemia [19,20] in vivo, but they also may constitute important synthetic intermediates in the nucleoside field owing to their chemical reactivity in various media [21,22]. It was also proved that the introduction of a fluorine atom in the sugar moiety of the unsaturated nucleosides increases the activity [23], raises the lipophilicity, and makes the penetration of the drug through the cell membrane easier [24-27].

Based on the above, unsaturated 3'-fluoro-2'-ketonucleosides of N^4-benzoyl cytosine have been prepared and showed to have a promising potential in combating the rotaviral infections and in the treatment of colon cancer [28]. Furthermore, a new class of unsaturated 3'-fluoro-4'-ketonucleosides, that of N^4-benzoyl cytosine and N^6-benzoyl adenine, respectively, was synthesized and compared to 5FU, showed to be more efficient as antitumor growth inhibitors and exhibited direct antiviral effect toward rotavirus [29]. In extending these studies, we decided to investigate the antioxidant activity of a series of the aforementioned modified nucleoside analogues. In order to examine their antioxidant activity we tested them using three in vitro assays. Firstly, the antioxidant capacity of the products was assessed using the DPPH radical scavenging assay. Afterwards, in order to examine the ability of the products to inhibit DNA strand cleavage induced by reactive oxygen species (ROS), a peroxyl radical (ROO•) and a hydroxyl radical (OH•) induced DNA strand scission assay were used. To the best of our knowledge the antioxidant activity of this kind of molecules is reported for the first time.

2. Results and Discussion

2.1. DPPH radical scavenging activity

All the tested compounds had no ability to scavenge DPPH radical. This activity may be due to the fact, that they are large molecules and they can’t approach the radical site or due to their steric inaccessibility [30].

2.2. Protecting effect of the tested nucleoside analogues against peroxyl radical-induced DNA strand scission

These measurements were performed using a free radical-induced DNA strand scission assay according to the procedure described in the Experimental section. Thirteen pure compounds have been tested and their chemical structures are given in Table 1. From the results obtained in the present study, it is evident, that the role of nucleobase in the chemical structure of the tested compounds is crucial for their antioxidant activity, since compounds 1 and 2 had no inhibitory activity. As can be seen from Table 1, in the case of nucleoside analogues which do not possess a α,β-unsaturated keto system in
their chemical structures, compounds 3, 4, 5, 6, 7 and 8, no or small inhibitory activity was observed at the highest tested concentrations (50 and 100 µM). On the contrary, nucleoside analogues with the α,β-unsaturated keto system, compounds 9, 10 and 11 were potent against the activity of ROO• radicals. It appears that the presence of a carbon-carbon double bond in α,β-disposition to the keto group causes a radical stabilization and increases the observed antioxidant activity. Especially, compound 10 was the most potent molecule, which showed 18 %, 19 %, 25 % and 26 % inhibition at 10, 20, 50, 100 µM respectively (Figure 1). However, compounds 12 and 13, despite the fact that they have a α,β-unsaturated keto system, no inhibitory activity was observed. Probably, this type of behavior is caused by structural prerequisites as well as mechanistic principles that may be due to the large size [30] and to the hydrophobic nature of the Triphenylmethyl group (Tr). Furthermore, a very important observation is that all the molecules had no effect in plasmid conformation when they were tested alone at the highest concentration.

Based on the above findings, it would be interesting and worthy to further investigate the potential effectiveness of this type of molecules in prevention and probably in treatment of diseases caused by the overproduction of radicals.

2.3. Protecting effect of the tested nucleoside analogues against hydroxyl radical-induced DNA strand scission

According to the results obtained, none of the tested compounds had the ability to prevent ΟΗ• radical induced DNA strand breakage. This behavior may be due to their very low solubility in the reaction media.

2.4. Conclusions

This study reports for the first time the antioxidant activity of a series of modified nucleoside analogues of N4-benzoyl cytosine and N6-benzoyl adenine. It is noteworthy that most of the tested compounds showed significant activity to protect DNA from the strand breaking activity of ROO• radicals. What is also important is that the molecules with a α,β-unsaturated keto system were the most potent against the activity of the ROO• radicals which can be explained by a radical stabilization resonance effect. On the contrary, none of the tested products had the ability to scavenge DPPH radical. The different nature of the two radicals may explain that the tested compounds are inert to DPPH radical. DPPH is a stable nitrogen radical that bears no similarity to the highly reactive and transient ROO• radicals. For that reason, many antioxidants that react quickly with ROO• radicals may react slowly or may be inactive to DPPH radical due to their steric inaccessibility [30]. As for the fact that the molecules were unable to inhibit OH• radical induced DNA damage, their low solubility in the reaction media may be responsible for that behavior.

Peroxyl radicals are involved as a major initiator factor of lipid peroxidation chain reactions [31]. Thus, the ability of the tested molecules to protect DNA strand breakage by scavenging peroxyl radicals could suggest that these products may also prevent lipid peroxidation.
Table 1. Percent inhibition of thirteen tested molecules against peroxyl radical-induced DNA strand scission.

| Products | % Inhibition | Products | % Inhibition |
|----------|--------------|----------|--------------|
|          | µM           |          | µM           |          |
|          | 5  | 10 | 20 | 50 | 100 |          | 5  | 10 | 20 | 50 | 100 |
| 1        | NI | NI | NI | NI | NI |         | NI | NI | NI | NI | NI |
| 2        | NI | NI | NI | NI | NI |         | NI | NI | NI | NI | NI |
| 3        | NI | NI | NI | 11±1 | 24±1 |         | NI | NI | NI | 10±5 | 11±1 | 27±4 |         |         |
| 4        | NI | NI | NI | 10±5 | 11±1 | 27±4 |         | NI | NI | NI | 10±5 | 11±1 | 27±4 |         |         |
| 5        | NI | NI | NI | NI | NI |         | NI | NI | NI | NI | NI |
| 6        | NI | NI | NI | 7±2 | 7±3 | 11±3 |         | NI | NI | NI | 16±4 | 20±2 |         |         |         |
| 7        | NI | NI | 16±5 | 27±3 |         |         |         | 9±3 | 18±1 | 19±1 | 25±3 | 26±2 |         |         |         |
| 8        | NI | NI | NI | 16±5 | 27±3 |         |         |         | 9±3 | 18±1 | 19±1 | 25±3 | 26±2 |         |         |         |
| 9        | NI | NI | 16±5 | 27±3 |         |         |         | 9±3 | 18±1 | 19±1 | 25±3 | 26±2 |         |         |         |
| 10       | NI | NI | 16±5 | 27±3 |         |         |         | 9±3 | 18±1 | 19±1 | 25±3 | 26±2 |         |         |         |
| 11       | NI | NI | 16±5 | 27±3 |         |         |         | 9±3 | 18±1 | 19±1 | 25±3 | 26±2 |         |         |         |
| 12       | NI | NI | 16±5 | 27±3 |         |         |         | 9±3 | 18±1 | 19±1 | 25±3 | 26±2 |         |         |         |
| 13       | NI | NI | 16±5 | 27±3 |         |         |         | 9±3 | 18±1 | 19±1 | 25±3 | 26±2 |         |         |         |

A Values are the means ± SE of the percent inhibition from three independent experiments.

B NI: no significant inhibition (less than 7 %). *p < 0.05 when compared with control (plasmid DNA plus AAPH). Ac: Acetyl, Tr: Triphenylmethyl.
Figure 1. Effect of nucleoside analogue 1-(3-deoxy-3-fluoro-β-D-glycero-hex-2-enopyranolsyl-4-ulose)-N^4-benzoyl cytosine (product 10) on peroxyl radical-induced plasmid DNA stand scission. Bluescript-SK+ plasmid DNA (1 µg/10 µL) was incubated in the presence of 2.5 mM AAPH for 45 minutes in the dark and the reaction products were electrophoresed in 0.8 % agarose gel. Lane 1: negative control; Lane 2: 2.5 mM AAPH; Lanes 3-7: AAPH plus 5 µM, 10 µM, 20 µM, 50 µM, 100 µM of the product respectively; Lane 8: plasmid DNA plus 100 µM of the product.

3. Experimental Section

3.1. Chemicals

1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2’-Azobis(amidinopropane hydrochloride) (AAPH) were obtained from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and solvents used in this study were of the highest purity commercially available.

3.2. Nucleoside analogues and their Preparation

Nucleoside analogues 1-13 were previously synthesized, and freshly prepared in DMSO, or in water.

3.3. DPPH assay

The radical scavenging efficiency of the nucleoside analogues was determined on the basis of the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH'). The method of Brand-Williams et al. was used with slight modifications [32]. Briefly, the reaction was carried out in 1 mL of methanol containing 100 µM freshly made DPPH' in methanol and the tested molecules at various concentrations (5, 10, 20, 50 and 100 µM). The contents were vigorously mixed, incubated at room temperature in the dark for 20 minutes and the absorbance was measured at 517 nm using a Hitachi U-1500 Spectrophotometer. In each experiment, the tested product alone in methanol was used as blank and DPPH' alone in methanol was used as control. All experiments were carried out in triplicate and at least on two separate occasions. The radical scavenging capacity (RSC) of the tested compounds was expressed as percentage of DPPH' elimination calculated according to the following equation:

\[
\% \text{ RSC} = \frac{\text{absorbance of control} - \text{absorbance of tested product}}{\text{absorbance of control}} \times 100
\] (1)
3.4. Evaluation of the protecting effect of the compounds by peroxyl radical-induced DNA strand scission assay

DNA strand breakage was measured by the conversion of supercoiled Bluescript-SK+ plasmid double stranded DNA to open circular and linear forms. The assay was performed according to the method of Chang et al. with some modifications [33,34]. Peroxyl radicals were produced by thermal decomposition of an azo-initiator (i.e. AAPH). AAPH at physiological temperature generates alkyl radicals which react with oxygen and give alkylperoxyl radicals leading to strand scission of plasmid DNA (Reactions 1 and 2).

\[
AAPH \xrightarrow{37^\circ C} 2R^* + N_2 \tag{1}
\]

\[
R^* + O_2 \rightarrow ROO^* \tag{2}
\]

The reaction mixture (10 µL) containing 1 µg Bluescript-SK+ plasmid DNA, 2.5 mM AAPH in phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$) and the tested compound at different concentrations (5, 10, 20, 50, 100 µM) was incubated in the dark for 45 min at 37 °C. After incubation, the reaction was terminated by the addition of 3 µL loading buffer (0.25 % bromophenol blue and 30 % glycerol) and analyzed in 0.8 % agarose gel electrophoresis at 70 V for 1 h. The gels were stained with ethidium bromide 0.5 µg/mL, destained with water, photographed by UV translumination using Vilber Lourmat photodocumentation system (DP-001.FDC) and analyzed with Gel-Pro Analyzer version 3.0 (MediaCybernetics, Silver Spring, USA). Also, Bluescript-SK+ plasmid DNA was treated with each compound alone at the highest concentration used in the assay in order to test the effects of compounds on plasmid DNA conformation. Each experiment was repeated three times.

What is important to be noted is that DMSO did not affect the assay in the tested concentrations.

3.5. Evaluation of the protecting effect of the compounds by hydroxyl radical-induced DNA strand scission assay

DNA strand breakage was measured by the conversion of supercoiled Bluescript-SK+ plasmid double stranded DNA to open circular and linear forms as previously. Hydroxyl radical-induced DNA relaxation assays were performed according to the method of Keum et al. [34,35] with some modifications. The reaction mixture (10 µL) consisted of 1 µg Bluescript-SK+ plasmid DNA, 10 mM Tris-HCl – 1 mM EDTA, the tested molecule at different concentrations (5, 10, 20, 50, 100 µM) and 40 mM H$_2$O$_2$. Hydroxyl radicals (OH•) were generated from UV photolysis of hydrogen peroxide (H$_2$O$_2$) after irradiation of the reaction mixture with a 300 W UV lamp (OSRAM) for 3 min at a distance of 50 cm (Reaction 3).

\[
H_2O_2 \xrightarrow{UV radiation} 2OH^* \tag{3}
\]
The reaction was terminated by the addition of 3 μL loading buffer (0.25 % bromophenol blue and 30 % glycerol) and analyzed in 0.8 % agarose gel electrophoresis at 70 V for 1 h. The gels were stained with ethidium bromide 0.5 μg/mL, destained with water, photographed by UV translumination using Vilber Lourmat photodocumentation system (DP-001.FDC) and analyzed with Gel-Pro Analyzer version 3.0 (MediaCybernetics, Silver Spring, USA). Each experiment was repeated three times.

3.6. Percent inhibition of Peroxyl and Hydroxyl radical-induced DNA strand scission

The percent inhibition of radical-induced DNA strand cleavage by the compounds was calculated using the following equation:

\[
\% \text{ Inhibition} = \left( \frac{S_o - S}{S_{\text{control}} - S_o} \right) \times 100
\]

(2)

Where \(S_{\text{control}}\) is the percent of supercoiled DNA in the negative control sample (without tested compound and AAPH or \(H_2O_2\) plus UV radiation), \(S_o\) is the percentage of supercoiled plasmid DNA in the control sample (without tested compound but in the presence of the radical initiating factor, AAPH or \(H_2O_2\) plus UV radiation) and \(S\) is the percentage of supercoiled plasmid DNA in the sample with the tested compound and the radical initiating factor (AAPH or \(H_2O_2\) plus UV radiation). It should be noted that isolated Bluescript-SK+ plasmid DNA contained approximately 10-20 % open circular DNA prior to treatment.

3.7. Statistical analysis

For statistical analysis the one-way ANOVA was applied followed by Dunnett’s test for multiple pair wise comparisons. Dose-response relationship was examined by Spearman’s correlation analysis. Differences were considered significant at \(p < 0.05\).

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