Fluorescence of the tri-cyclic adenine and isoguanine derivatives and their ribosides: possible analytical applications*

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Fluorescent tri-cyclic purine analogs, derivatives of iso-guanine and adenine, were examined as potential substrates of purine-nucleoside phosphorylase. It was found previously that etheno-derivatives of both compounds are ribosylated in phosphate-free media, but ribosylation places in some instances differ from purine N9. New ribosides are examined as potential substrates of human blood PNP and indicators of this enzyme. Of these, N6-riboside of 1,N6-etheno-adenine was found the most promising.

Key words: nucleobase/nucleoside analogs; fluorescence; purine nucleoside phosphorylase; enzyme activity, blood lysates

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INTRODUCTION

Tri-cyclic analogs of the canonical purines are frequently used as fluorescent probes in enzymological research. The best known example is 1,N6-etheno-adenosine (εAdo) and analogs (Leonard, 1984; Leonard, 1985), but other fluorescent derivatives are also known (Virta et al., 2004; Wang et al., 2017). Tri-cyclic analogs and their ribosides are characterized by moderate biological activity, but some of them reveal promising anti-viral properties (Janz-Wechmann et al., 2015). They are known to react with many enzymes of purine metabolism (Leonard, 1984), and are important intermediates in the process of chemical mutagenesis induced by vinyl chloride (Singer & Kuśmierek, 1982; Chatterjee & Walker, 2017).

In our laboratory we are working on an enzyme purine-nucleoside phosphorylase (PNP, E.C. 2.4.2.1, see Scheme 1), responsible for the regulation of the nucleoside concentrations within the living cells, and a target of many types of pharmaceutical interventions, including gene therapy of the inherited immunological disorders (Bzowska et al., 2000; Grunebaum et al., 2013). We have shown that PNP isolated from E. coli, which is known to possess a broad specificity toward various base and nucleoside analogs (Bzowska et al., 2000; Yehia et al., 2017) is also active towards tri-cyclic εAdo and its 2-aza analog (Wierzchowski et al., 2017). We have found also that 1,N6-etheno-adenine (εAdo), 1,N6-etheno-guanine and 1,N6-etheno-isoguanine (εisoGua) are good substrates for PNP from E. coli (Fig. 2, left) in the reverse (synthetic) pathway, with catalytic and Michaelis’ constants sometimes comparable to those obtained for the enzymatic ribosylation of the parent guanine (Stachelska-Wierzchowska et al., 2018; Stachelska-Wierzchowska et al., 2019). The ribosides obtained in these reactions are...
not necessarily identical with those produced chemically, with ribosylation sites varying for different PNP forms.

The purpose of the present paper is examination of substrate properties of new fluorescent ribosides toward the human blood enzyme, and their analytical potential in clinical investigations. We have previously identified two such substrates, isomeric ribosides of 2,6-diamino-8-aza purine (Wierczowski et al., 2014), and now extend our work to include tri-cyclic ribosides with different spectral characteristics.

Human PNP, as well as other mammalian forms of this enzyme, belongs to the second (trimeric) class within the broad family of PNP (Bzowska et al., 2000), and its substrate specificity is different than that of the hexameric (bacterial) forms. In particular, the trimeric forms of PNP are inactive towards adenosine and some derivatives (Bzowska et al., 2000; Yehia et al., 2017). We therefore considered only those ribosides, which were previously shown to be substrates for the calf enzyme (Stachelska-Wierzchowska et al., 2018, 2019). We have chosen some non-canonical ribosides of etheno-adenine and etheno-isoguanosine (Scheme 2).

**MATERIALS AND METHODS**

Synthesis of the 1,N6-etheno-isoguanine (base, 4) from isoguanine and chloroacetaldehyde has been described previously (Stachelska-Wierzchowska et al., 2019). This reaction is slow (ca. 7 days at room temperature) but the reaction product crystallized easily from neutralized medium and the reaction yield (~70%) was sufficient. Chemo-enzymatic syntheses and identification of two described ribosides of 1,N6-etheno-adenine and 1,N6-etheno-isoguanine (1 and 2) were described elsewhere (Stachelska-Wierzchowska et al., 2018; Stachelska-Wierzchowska et al., 2019). These compounds were stored as frozen solutions. Recombinant calf spleen PNP was obtained from Prof. Agnieszka Bzowska (Warsaw University, Poland).

Blood samples were obtained as leftovers from glucose-monitoring. 10 µL blood samples were lysed in 0.5 mL of 2 mM phosphate buffer, pH 7, containing 0.5 mM EDTA and an aliquot (~0.2 mM) of dithiothreitol. The hemolysates were kept at 5°C.

Fluorescence spectra were measured on a Varian Eclipse instrument (Varian Corp., Palo Alto, CA, USA). Spectral resolution was typically 5 nm (emission path) and 2.5 nm (excitation path). UV absorption and kinetic experiments were performed on a Cary 5000 (Varian) thermostated spectrophotometer. Enzymatic reactions were carried out at 25°C. Fluorescence yields were determined relative to tryptophan (0.15) or 1,N6-ethenoadenosine in water (0.56; Leonard, 1984). Spectra were measured in semi-micro 1 mL cuvettes, pathlength 4 mm, to diminish the inner-filter effect and hemoglobin absorption. Substrate/product concentrations were calculated using known molar extinction coefficients: 8200 M⁻¹·cm⁻¹ for etheno-adenine (274 nm) and 7000 M⁻¹·cm⁻¹ for etheno-isoguanine at 291 nm (Stachelska-Wierzchowska et al., 2018; Stachelska-Wierzchowska et al., 2019). Typically, substrate concentrations in fluorescence measurements were 5-fold lower than those measured by UV absorption.

**RESULTS AND DISCUSSION**

It has been shown previously, that enzymatic ribosylation of some nucleobase analogs with PNP as a biocatalyst leads to non-typical ribosides, with ribose moiety attached not necessarily to purine N9, but also to other nitrogen atoms (Stachelska-Wierzchowska et al., 2013; Stachelska-Wierzchowska et al., 2016; Stachelska-Wierzchowska et al., 2018; Stachelska-Wierzchowska et al., 2019). In particular, while the E. coli PNP directs the ribosyl group predominantly to the N9 of etheno-adenine, the calf enzyme leads to almost exclusively N9-riboside (Stachelska-Wierzchowska et al., 2018). In 1,N6-ethenoisoguanine, the situation is even more complex, since typically mixtures of various ribosides are produced (Stachelska-Wierzchowska et al., 2019).

Spectral properties of new ribosides were presented in previous papers (Stachelska-Wierzchowska et al., 2018; Stachelska-Wierzchowska et al., 2019) and those of three 1,N6-ethenoisoguanine ribosides are summarized in Fig. 1.

The most interesting, from analytical point of view, were those ribosides which were generated by calf spleen PNP, that is, N9-β-D-ribosides. We have shown that these ribosides (see Scheme II, above) were also excellent substrates for the E. coli PNP, and phosphorolytic...
Figure 2. Spectral (left) and fluorescence (right) changes observed during the phosphorolysis of N<sup>6</sup>-β-d-ribosyl-1,N<sup>6</sup>-etheno-adenine (1) with *E. coli* PNP as a catalyst, in the phosphate buffer, pH 7, at 25°C. Initial substrate concentration in UV experiment was 66 µM, and for emission 13.2 µM. Cuvettes of a reduced pathlength (4 mm) were used. Fluorescence excitation was at 275 nm. Time intervals: 5 min for UV absorption, 10 min for fluorescence. Final curves are drawn in red.

Figure 3. Spectral (left) and fluorescence (right) changes observed during the phosphorolysis of N<sup>6</sup>-β-d-ribosyl-1,N<sup>6</sup>-etheno-isoguanine (2) in the phosphate buffer, pH 7. Initial substrate concentrations: 68 µM for UV, 14 µM for emission. Fluorescence was excited at 310 nm. Time intervals are as in Fig. 2. Final curves are drawn in red.

Figure 4. Spectral (left) and fluorescence (right) changes observed during the phosphorolysis of N<sup>6</sup>-β-d-ribosyl-1,N<sup>6</sup>-etheno-adenine (1) with *blood lysate* as a catalyst, in the phosphate buffer, pH 7, at 25°C. Experimental conditions were exactly as in Fig. 2, above. Final curves (points) were obtained by spiking blood sample with the purified *E. coli* PNP. Black curves refer to purified substrate (1) spectra. Time intervals were 5 minutes for UV absorption, 10 minutes for fluorescence. Fluorescence minimum at 410 nm is due to light re-absorption by hemoglobin (Slater band). Note that the isosbestic and isoemissive points are strictly maintained.
reactions were easily followed by UV absorption of fluorescence spectroscopy (Figs. 2 and 3). The new ribosides are fairly stable, and could be stored for months in stock solutions at −5°C (not shown).

Human blood is particularly rich in PNP activity, which is located mainly in erythrocytes (Bzowska et al., 2000). We have shown previously that some phosphorolytic reactions are easily observed spectrally or fluorimetrically using 1000-fold diluted whole blood lysates as a catalyst in ca. 50 mM phosphate buffer (Wierzchowski et al., 2002; Wierzchowski et al., 2014). We have therefore examined the new ribosides as potential substrates for the human PNP, using the same methodology (except optical pathlength of the cuvettes, reduced now to 4 mm).

Experiments with 1000-fold diluted blood lysates have shown that only one of the examined substrates, of N-β-D-ribofuranosyl-1,N-etheno-adenine (1), was readily phosphorysylated (Fig. 4) with calculated reaction rate of ~0.45 µM/min at substrate concentration 66 µM. Phosphorolysis was easily observed spectrophotometrically as well as fluorimetrically. Hemolysate optical background does not interfere with the measurements, except visible re-absorption near the Slater band of hemoglobin at 410 nm (Fig. 4). Blood proteins contribute somewhat to the overall fluorescence with excitation at 275 nm, but with 1000-fold sample dilution this fluorescence, visible as short-wavelength inflection on Fig. 4, right panel, is low and negligible at λ ~ 430 nm, where the measurements are the most accurate.

The second substrate, the highly fluorescent N-β-D-ribofuranosyl-1,N-etheno-isoguanine (2), was apparently inactive (rate <0.01 µM/min) with the same blood sample, although it was rapidly phosphorysylated by the purified E. coli PNP (Fig. 3) as well as by the calf enzyme. This result was somewhat surprising, since the human enzyme belongs to the same class of trimeric PNP, and shows homology of >70% with calf PNP (Bzowska et al., 2000). At present, we are unable to explain this phenomenon.

The experimental conditions of the presented reactions need to be optimized, for applications to clinical analyses. This refers particularly to buffer pH, substrate concentration (the apparent K_m for the human enzyme) and excitation wavelength. With conditions fulfilled, the proposed assay will be probably much more sensitive than those described previously.

CONCLUDING REMARKS

We have described two novel, both fluorescent and fluorogenic, substrates for PNP. One of these, N-β-D-ribofuranosyl-1,N-etheno-adenine (1), can be used to quantitate PNP activity in human blood. Possible applications include early detection of immunological deficiencies (Grunebaum et al., 2013). The second substrate, N-β-D-ribofuranosyl-1,N-etheno-isoguanine (2), can be used to selectively detect bacterial PNP activity in biological samples, with possible use in the investigations of the suicidal gene therapy of cancer, utilizing bacterial PNP to generate in situ cytotoxic nucleobase analogs (Karjoo et al., 2017).

Various assays, including fluorimetric, were previously proposed for this enzyme (Bzowska et al., 2000; Wierzchowski et al., 2002; Wierzchowski et al., 2014), but their sensitivity was not always satisfactory, mostly because of slow reaction rates of the artificial substrates. Therefore search for new, more sensitive substrates is continued.

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