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# CONVERSION OF 2-β-D-RIBOFURANOSYLSELENAZOLE-4-CARBOXAMIDE TO AN ANALOGUE OF NAD WITH POTENT IMP DEHYDROGENASE-INHIBITORY PROPERTIES

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Studies on the mechanism of action and metabolism of 2-β-D-ribofuranosylthiazole-4-carboxamide (tiazofurin, TR) by this and other laboratories have established that, in susceptible tumors, this nucleoside is anabolized to an analogue of NAD wherein nicotinamide is replaced by thiazole-4-carboxamide; this nucleotide potently inhibits IMP dehydrogenase leading to a depression of guanosine nucleotide biosynthesis and a cessation of tumor cell proliferation (1-6).

The synthesis of the selenium analogue of TR, 2-β-D-ribofuranosylselenazole-4-carboxamide (selenazofurin, SR) has been reported recently; like TR, this analog exhibits potent antitumor activity against the Lewis lung carcinoma (7). The present studies were undertaken in order to determine whether SR exerts its cytotoxic effects by the mechanisms established previously for TR. Toward this goal, a direct comparison of TR and SR has been carried out in several relevant systems.

P388 cells growing in log-phase culture were exposed to SR or to TR (0.1 μM to 50 mM) continuously for 24 hr, and the drug concentrations inhibiting cell-growth rate by 50% ($IC_{50}$) were calculated (Table 1). SR was found to be ca. 5-fold more potent than TR in inhibiting the proliferation of P388 lymphoblasts. Against a variant of the P388 leukemia rendered resistant to TR (P388/TR), SR also was inert ($IC_{50}$ 5 mM, Table 1), an observation compatible with a common mechanism of resistance.

Table 1

Toxicity of TR and SR to P388 Cells in Culture*

| | $IC_{50}$ | |
|---|---|---|
| Compound | P388/S | P388/TR |
| SR | 0.56 μM | 5 mM |
| TR | 3.0 μM | 16 mM |

*P388/S or P388/TR [a variant of P388 resistant to TR (8)] cells growing in log-phase in RPMI 1640 containing 10% calf serum were incubated with various concentrations of the compounds. Twenty-four hr later cells were counted and the drug concentrations required for 50% inhibition of growth were determined (2). Under the conditions of the culture, these cell lines exhibit a generation time of 13-15 hr.

To determine whether SR forms an analogue of NAD (selenazole-4 carboxamide adenine dinucleotide, SAD) similar to that formed by TR (thiazole-4-carboxamide adenine dinucleotide, TAD), P388 cells in the logarithmic phase of growth were exposed for 1 hr at 37° to [8-$^{14}$C]-adenosine and then incubated further with saline, SR or TR for 2 hr; experimental details are described in the legend to Table 2. These studies established that $^{14}$C-labeled species dependent on the presence of SR or TR were formed in both treated cultures; their elution position on HPLC was coincident with >90% of the IMP dehydrogenase inhibitory activity (data not shown). Under these conditions, the P388 cells formed 2- to 3-fold more SAD than TAD - a capability which might contribute to the increased cytotoxicity of SR. That the labeled peaks in these experiments were, in fact, phosphodiesters was established by exposing them at pH 8.0 to snake venom phosphodiesterase, an enzyme which cleaves phosphodiester linkages but not phosphomonoesters; in both cases, quantitative conversion to radiolabeled AMP and cold TR-5'-P or SR-5'-P was demonstrated by HPLC. In P388/S cultures, both SR and TR caused perturbation of guanosine nucleotide biosynthesis (~75% reduction in the concentration of GTP compared to saline controls, Table 2).

Table 2

Biological Synthesis of TAD and SAD by P388 Cells in Culture*

| Compound | Concentration (μM) | $^{14}$C-TAD/SAD formed (nmoles/g cells) | Titer of biologically formed TAD/SAD producing $ID_{50}$ of IMP dehydrogenase | % Reduction in GTP levels |
|---|---|---|---|---|
| SR | 10 | 96.9 | -- | |
| | 100 | 245.9 | 1:1250 | 75.5 |
| TR | 100 | 106.9 | 1:212 | 74.3 |
| | 1000 | 628.9 | -- | |

*To P388/S cells (60 ml; 3.7 x $10^6$ cells/ml) growing in log phase was added [8-$^{14}$C]-adenosine (30 μCi, sp. act. 57 μCi/μmole) and the cultures were incubated at 37° for 1 hr. Aliquots (10 ml) of the cells were then incubated with saline, TR, or SR at the indicated concentrations for 2 hr. Cells were centrifuged, washed with cold Hanks' balanced salt solution, homogenized in 250 μl of 10% trichloroacetic acid and neutralized, and an aliquot (100 μl) was subjected to HPLC (2). The concentration of GTP in the control cells was 226±15 nmoles/g cells. For determination of IMP dehydrogenase inhibition titer, individual fractions from HPLC were diluted (1:11) with 0.05 M Tris-HCl buffer, pH 7.6, and then tested for their ability to inhibit partially purified (10-fold) IMP dehydrogenase from P388/S leukemia; the fraction corresponding to SAD or TAD was further serially diluted and the titer producing 50% inhibition of IMP dehydrogenase was determined as previously described (3). IMP dehydrogenase activity was determined radiometrically using [$^3$H]IMP as substrate as described earlier (3).

Attention was next focused on confirming by chemical synthesis the identity of the active principle responsible for the action of SR. The selenium analogue of TAD (SAD) was synthesized by a route analogous to that used for preparing TAD (4,9), i.e. from adenosine -5'-phosphomorpholidate and the tri-N-octylammonium salt of SR-5'-monophosphate (7). After a 5 hr incubation at 45° with stirring, pyridine was removed and the reaction mixture was loaded onto a Hamilton HA-X4 anion exchange column (formate form, 8 x 280 mm) maintained at 37° and eluted with a linear gradient of water (270 ml) and a mixture of ammonium formate (2 M) and formic acid (2.6 M). Fractions of 8 ml were collected at a flow-rate of 1.54 ml/min and absorbance was monitored at 270 nm. The purity of the fraction corresponding to SAD was confirmed on HPLC, using a Partisil 10 SAX column eluted with ammonium phosphate buffers

(4). In this system, the chromatographic behavior of synthetic SAD was identical to that of SAD formed in vivo, and, coincidentally, to that of TAD. However, the $\lambda_{max}$ of SAD was shifted to 258 nm from the 252 nm observed for TAD.

Structural characterization of SAD was carried out by high resolution (Fourier transform) proton NMR spectrometry and by fast atom bombardment mass spectrometry. The 200 and 500 MHz NMR spectrum obtained in $D_2O$ revealed the expected aromatic singlets in the low field region ($\delta$ 8.52, 8.89 and 9.03), the adenosine anomeric proton doublet ($\delta$ 6.06), and the C-nucleoside anomeric proton appearing as a broad singlet at $\delta$ 4.70. The singlet at $\delta$ 8.52 was assigned to the C-5 proton of the selenium heterocycle. This signal was shifted to the lower field in relation to its equivalent signal in TAD ($\delta$ 8.40), a phenomenon which is in agreement with the greater deshielding effect of selenium as compared to sulfur (10). The adenosine aromatic protons at C-2 and C-8 evidenced a significant downfield shift ($\delta$ 8.89 and 9.03) in comparison to TAD ($\delta$ 7.96 and 8.17). This shift could result from the diamagnetic anisotropy of the Se-containing heterocycle in a conformation for the dinucleotide where base stacking is expected to occur.

Additional evidence of the structure of SAD was obtained by negative ion mass spectral analysis utilizing a VG Micromass 7070E mass spectrometer equipped with a fast atom bombardment (FAB) ion source (11) and operated at an accelerating voltage of 6 kV. The spectrum observed is illustrated in Figure 1. A cleavage pattern similar to that of TAD (4) is seen, but the replacement of S by Se in the thiazole-4-carboxamide ring is clearly evident. Molecular weight is indicated by the M-H peak at m/z 716 which shows a 48 amu increase in mass over the corresponding peak in TAD due to the replacement of $^{32}S$ by $^{80}Se$, the most abundant selenium isotope. In addition, all fragments containing Se possess a characteristic peak cluster encompassing a 7 amu range due to the five major selenium isotopes. This can be seen best in the "A" series ions at m/z 367 and 467 which contain the selenazole-4-carboxamide and are likewise shifted upward in mass by 48 amu over the corresponding fragments in TAD. In contrast, the "B" series fragments containing adenine at m/z 346 and 426 are unchanged.

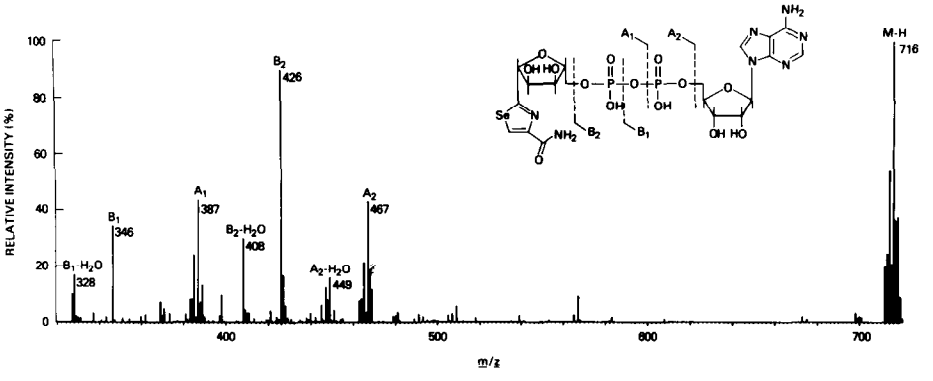


Figure 1: Fragmentation pattern for SAD obtained by negative ion FAB mass spectrometry. Ionization was effected by a beam of xenon atoms derived by neutralizing xenon ions accelerated through 8.2 kV. Glycerol was used as the sample matrix. Spectra were acquired at a scan speed of 10 sec/decade under the control of a VG 2035 data system. The glycerol background has been computer subtracted and the above spectrum is the weighted average of five repetitive scans.

Finally, the kinetic behavior of chemically synthesized SAD was examined versus a partially purified preparation of mammalian IMP dehydrogenase. Like TAD, the selenium dinucleotide inhibited the enzyme in a formally non-competitive manner with NAD as variable substrate; the inhibition, however, was considerably more potent than that seen with the sulfur dinucleotide ($K_i$ = 5.5 x $10^{-8}$M for SAD vs 2.0 x $10^{-7}$M for TAD).

In summary, these studies establish that, although the structural requirements for activity in the thiazole-4-carboxamide series are stringent (2), selenium can replace sulfur in the heterocyclic ring with full retention or even enhancement of biological activity. The greater cytotoxicity of SR compared to TR appears to result from two factors: more extensive formation of SAD than TAD by susceptible tumor cells; and a 4-fold greater affinity of SAD than TAD for its enzymic target, IMP dehydrogenase.

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