Biochemical Pharmacology, Vol.31, No.11, pp.2133-2136, 1982.
Printed in Great Britain.

0006-2952/82/112133-04$03.00/0
Pergamon Press Ltd.

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

David A. Cooney, Hiremagalur N. Jayaram, Gulilat Gebeyehu, Charles R. Betts, James A. Kelley, Victor E. Marquez and David G. Johns

Laboratory of Medicinal Chemistry and Biology, DTP, DCT
National Cancer Institute, NIH
Bethesda, MD 20205, U.S.A.

(Received 8 March 1982; accepted 23 March 1982)

Work in this and other laboratories has established that exposure of murine tumor cells to the oncolytic C-nucleoside, 2-β-D-ribofuranosylthiazole-4-carboxamide (TR), results in a notable depression of the concentration of all guanine nucleotides, consequent to inhibition of IMP dehydrogenase (IMPD) (1,2). Extracts of the cells so exposed contain a potent inhibitor of this, a key step in purine nucleotide biosynthesis (3).

Inasmuch as kinetic analyses strongly suggested that the parent drug and its 5'-monophosphate were unlikely to be responsible for this metabolic effect, we have carried out the following studies aimed at uncovering the identity of the proximate inhibitory species.

Male BDF_1 mice bearing subcutaneous nodules of the P388 leukemia were given an intraperitoneal injection of TR at a dose of 800 mg/kg; 2 hr later the tumors were excised and homogenized in 5% perchloric acid (PCA); the supernatant fraction from this homogenate was neutralized with KOH, and examined for its ability to inhibit a partially purified preparation of IMPD (2); 50% inhibition was observed at a titre of 1:128. On HPLC chromatography (Whatman Partisil-10 SAX resin; ammonium phosphate buffer) (Fig. 1), the greater part (>70%) of the

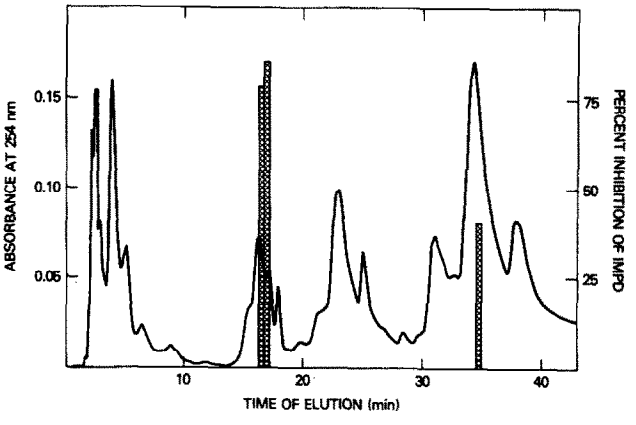

Fig. 1. Chromatographic resolution of IMPD-inhibitory activity in P388-tumor extracts from TR-treated mice. Groups of five mice received TR, 800 mg/kg i.p. Two hours later, tumors were excised, frozen on dry ice, and homogenized in PCA (5%), and the resulting extracts were neutralized with KOH, 40%. After centrifugation, an aliquot was loaded on a radial compression column of Partisil-10 SAX resin (Waters Associates Inc.), pre-equilibrated with ammonium phosphate buffer, 0.05 M, pH 2.88. After sample loading, the column was isocratically eluted with the starting buffer for 10 min, and then developed with a linear gradient of ammonium phosphate, 0.05 M, pH 2.88, and ammonium phosphate, 0.25 M, pH 4.5, for 30 min. One-minute (2 ml) samples were collected. Fractions were diluted (1:11, v/v) with Tris-HCl buffer, 0.05 M, pH 7.6, and examined for their ability to inhibit partially purified IMPD from P388 tumor cells (2), with percent inhibition being indicated by cross-hatched vertical bars (see figure). In control experiments, HPLC fractions from saline-treated P388-tumor-bearing mice failed to show inhibitory activity.

IMPD-inhibitory activity was found to be attributable to a single peak eluting at 16.5 minutes; experimental conditions are described in greater detail in the figure legend.

Four-hour exposure of the tumor extract from TR-treated animals to either acid (pH 5) or alkaline (pH 9) phosphatase (Sigma Chemical Co.), each at a concentration of 0.01 mg/ml at 37° (followed by 1 min at 95° to destroy the enzymes), wholly failed to eliminate the inhibition of IMPD; the cyclic phosphodiesterase from beef heart (Boehringer-Mannheim Biochemicals) was similarly inert. By contrast, venom phosphodiesterases (Boehringer-Mannheim, Worthington Biochemical Corp.) abrogated >90% of the inhibitory activity under comparable conditions.

These chromatographic and enzymic inactivation results, considered together, led to the suggestion that the inhibitory principle was a phosphodiester bearing a rather strong net negative charge. Since NAD is quantitatively the predominant phosphodiester in intracellular fluids and is, in addition, the obligatory cofactor for the IMPD-catalyzed conversion of IMP to XMP, we then examined the hypothesis that the inhibitory species was an analogue of NAD in which thiazole carboxamide replaced nicotinamide.

To determine whether both TR and adenosine were incorporated into the inhibitor, P388 cells were exposed for 2 hr to either [^{3}H]-TR (10 μM) or [^{14}C]-adenosine (10 μCi, 0.17 μmole) in the absence and presence of unlabeled TR (1 mM); experimental details are described in the legend to Fig. 2. Panel A of Fig. 2 demonstrates that P388 cells converted [^{3}H]-TR to an acidic, tritium-labeled species whose elution position was coincident with the IMPD-inhibitory activity described above, while panels B and C show that a ^{14}C-labeled species was present at this position when [^{14}C]-adenosine was incubated with P388 cells in the presence of TR, but that such a species was absent when TR was omitted. Exposure of the ^{14}C-labeled peak from Fig. 2C to venom phosphodiesterases under the conditions described above resulted in the formation of a ^{14}C-labeled HPLC peak with the elution position of AMP, while alkaline phosphatase was inactive under these conditions.

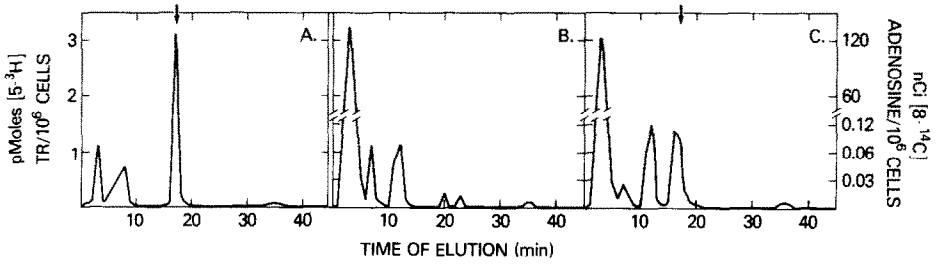

Fig. 2. Chromatographic resolution of radioactivity after exposure of P388 cells to [^{3}H]-TR and to [^{14}C]-adenosine. A: To 50 ml of P388 cells (5 x 10^7 cells) in RPMI medium with 3% L-glutamine, 10% donor calf serum and 5 μM 2-mercaptoethanol, 50 μl of [5-^{3}H]TR (267 nmoles, 34 μCi) was added and incubated at 37° in an atmosphere of 5% CO_2 and 95% air for 2 hr. Cells were then separated by centrifugation at 3,000 g for 2 min and washed twice with 1 ml of Hanks' balanced salt solution. PCA, 0.2 ml of a 5% solution, was then added and the cell pellet mixed and centrifuged at 12,000 g for 2 min. The PCA extract was neutralized with KOH, 40%, recentrifuged, and an aliquot was chromatographed on Partisil-10 SAX resin as described earlier. B and C: To 10 ml of P388 cells (10^8 cells) in RPMI medium containing L-glutamine, donor calf serum and 2-mercaptoethanol (see above), 10 μCi of [8-^{14}C]adenosine was added. Thirty minutes later, saline (panel B) or TR, 10 μmoles (panel C), was added and incubated for 2 hr. Cells were separated by centrifugation at 3,000 g for 2 min, homogenized in 1 ml water and dialyzed against 10 ml of water overnight (16 hr) at 4°. The outer bath was chromatographed on HPLC as detailed earlier.

These results were compatible with the hypothesis that the IMPD inhibitor was the thiazole carboxamide analogue of NAD. The following preparative-scale enzymic synthesis of this analogue (hereafter referred to as TAD) was therefore undertaken to characterize its physico-chemical and biological properties more fully and to establish its identity with the inhibitor generated in tumor cells. Ten milliliters of a neutralized 0.01 M solution of TR-5'-monophosphate was admixed with 10 ml of 0.01 M ATP-$MgCl_2$ in 0.1 M Tris-HCl, pH 7.8, and 5 mg NAD pyrophosphorylase (Boehringer-Mannheim Biochemicals). After 16 hr at 37°, the entire mixture was loaded onto a 1 x 100 cm column of Hamilton HA-X4 resin previously converted to the formate form and washed exhaustively with deionized water. The column was developed with a linear gradient of ammonium formate (0-1 M); the major product, which eluted at 0.7 M formate, was pooled and repeatedly lyophilized. Residual ADP and ATP, when present, were removed by a second chromatography under identical conditions. Five milligrams of product was obtained which was homogeneous on HPLC under the conditions described above and which co-eluted with the IMPD-inhibitory activity generated in TR-treated P388 tumor cells. In addition, the compound was a potent inhibitor of IMPD (K_i ca. 0.5 μM) and showed identical susceptibility to phosphodiesteratic cleavage to that seen with the material from P388 cells. The λ_{max} at pH 7.0 was 252 nm.

Positive and negative ion mass spectra of TAD were obtained on VG Micromass 7070 mass spectrometers equipped with VG fast atom bombardment (FAB) ion sources (4) operated at an accelerating voltage of 4 kV. Both mass spectra convincingly support a dinucleotide structure of MW 669 in which the nicotinamide of NAD has been replaced by thiazole-4-carboxamide. The fragmentation pattern for the negative ion FAB mass spectrum is illustrated in Fig. 3. Molecular weight is indicated by a M-H peak occurring at m/z 668 and a peak due to the anion of a dipotassium adduct at m/z 746. Ions at m/z 346 and 426 suggest a nucleotide base of mass 134, i.e. adenine, while the corresponding cleavages of the phosphodiester linkage from the other end of the molecule produce fragments at m/z 339 and 419 indicating a second nucleotide base with mass 127, i.e. thiazole-4-carboxamide.

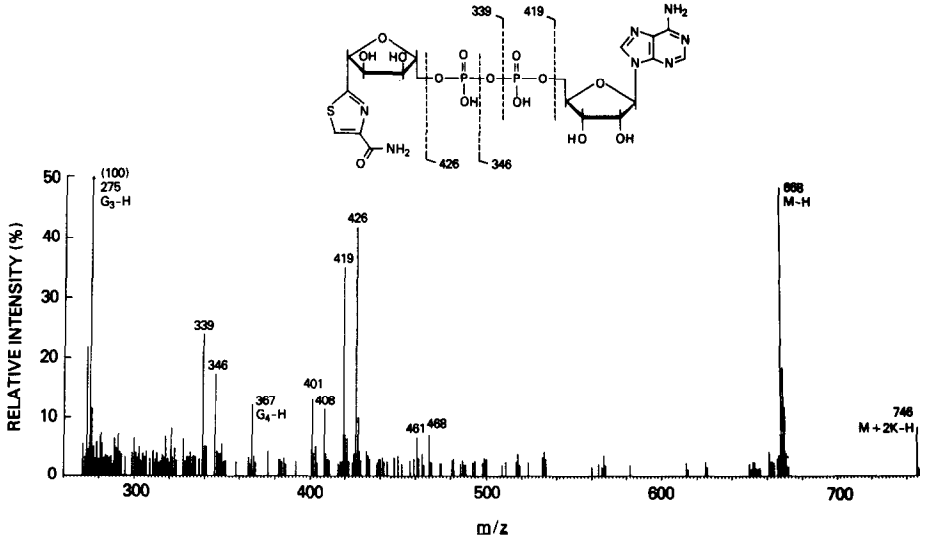

Fig. 3. Fragmentation pattern for TAD obtained by negative ion FAB mass spectroscopy. Ionization was effected by a beam of argon atoms derived by neutralizing argon ions accelerated through 4 kV. Glycerol was used as the sample support medium. Spectra were recorded at a scan speed of 20 sec/dec using an oscillographic recorder. Peaks designated with G correspond to glycerol adducts, e.g. G_3-H denotes the anion resulting from proton abstraction from the glycerol trimer.

In proton magnetic resonance studies of TAD in D_2O, carried out using a Varian XL-200 instrument with Fourier transform capability, the low field end of the FT-200 MHz spectrum showed three singlets (δ 7.80, 7.56 and 7.32) (Fig. 4). The δ 7.80 singlet was assigned to the C-5 proton of the C-nucleoside moiety, based on comparisons with the spectra of the individual fragments. For the remaining two singlets, if one follows the analogy of the assignments made for NAD (5,6), the δ 7.56 singlet should correspond to the C-8 proton and the δ 7.32 singlet to the C-2 proton of the adenosine component.

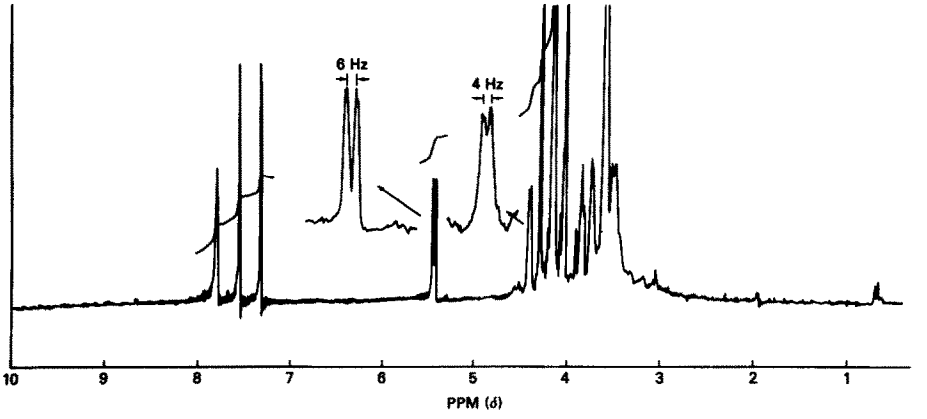

Fig. 4. FT-200 MHz NMR spectrum of TAD in D_2O. Chemical shifts are given with reference to TMS in the absence of the standard based on the lock solvent at 24.2°.

At higher field, the relative position of the C'1-H signals of both nucleoside components reflected the greater deshielding effect of the adenosine nucleoside fragment in relation to the C-nucleoside. The anomeric proton of the adenosine component appeared as a doublet centered at δ 5.44 with a coupling constant of 6 Hz. The corresponding anomeric proton for the C-nucleoside appeared as a doublet at δ 4.40 with a coupling constant of 4 Hz. All these features of the NMR are in agreement with the proposed structure of TAD as an analogue of NAD in which the nicotinamide of NAD has been replaced by thiazole-4-carboxamide.

To confirm the structure of the enzymically prepared material described above, TAD was synthesized from adenosine-5'-phosphomorpholidate and tri-n-octylammonium TR-5'-monophosphate according to the general procedure for the preparation of unsymmetric nucleoside pyrophosphates developed by Roseman et al. (7). After workup, a similar isolation and purification methodology to that described above for biosynthetic TAD afforded a compound with NMR-spectral, chromatographic and IMPD-inhibitory properties identical to the latter.

Acknowledgements - We thank Dr. Ivor Lewis, VG Analytical, Altrincham, Cheshire, England for running the positive ion FAB mass spectrum and Dr. Carter Cook, University of Illinois, Urbana, IL for obtaining the negative ion FAB mass spectrum.

REFERENCES

1. D.G. Streeter and J.P. Miller. Biochem. biophys. Res. Commun. 103, 1409 (1981).
2. H.N. Jayaram, R.L. Dion, R.I. Glazer, D.G. Johns, R.K. Robins, P.C. Srivastana and D.A. Cooney. Biochem. Pharmac. (in press).
3. R.C. Jackson, G. Weber and H.P. Morris. Nature 256, 331 (1975).
4. M. Barber, R.S. Bordoli, R.D. Sedgwick and A.N. Tyler. J. chem. Soc. Chem. Commun. 325 (1981).
5. O. Jardetzky and N.G. Wade-Jardetzky. J. biol. Chem. 241, 85 (1966).
6. R.H. Sarma and N.O. Kaplan, J. biol. Chem. 244, 771 (1969).
7. S. Roseman, J.J. Distler, J.G. Moffatt and H.G. Khorana, J. Am. chem. Soc. 83, 659 (1961).