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Novel Inhibitors of Poly(ADP-Ribose) Synthetase

Michael R. PURNELL and William J. D. WHISH
Department of Biochemistry, University of Bath, Claverton Down, Bath, Avon BA2 7AY, U.K.

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In a search for new inhibitors of the nuclear enzyme poly(ADP-ribose) synthetase, it was found that various benzamides substituted in the 3-position were the most inhibitory compounds found to date. Two of the benzamides, 3-aminobenzamide and 3-methoxybenzamide, were found to be competitive inhibitors, with K_i values of less than $2\,\mu$M.

Despite a large amount of research, the function fulfilled by the modification of nuclear proteins by mono- and poly-(ADP-ribose) remains unknown (Hilz & Stone, 1976; Hayaishi & Ueda, 1977; Purnell *et al.*, 1980). Workers have suggested putative roles in the regulation of DNA synthesis, transcription and repair, cell-cycle events and cellular differentiation and development (for a summary, see Purnell *et al.*, 1980). One approach to determine the function is to inhibit the enzyme responsible for ADP-ribosylation, poly(ADP-ribose) synthetase (EC 2.4.99.–), *in vivo* and look for cellular dysfunction. Various compounds are known to inhibit poly(ADP-ribose) synthetase *in vitro*. Unfortunately, the results obtained from the study of cells treated with any of the inhibitors used to date are of limited value, because these compounds lack physiological specificity. Nicotinamides (Clark *et al.*, 1971) affect the synthesis of NAD and may deplete cellular phosphoribosyl diphosphate pools (Leiber *et al.*, 1973), resulting in a decrease in nucleotide synthesis. Thymidine (Preiss *et al.*, 1971) is known to inhibit DNA synthesis by depleting dCTP concentrations in the cell (Meuth *et al.*, 1976). Methylated xanthines and cytokinins, also inhibitors of poly(ADP-ribose) synthetase, are known to affect cyclic phosphodiesterase (EC 3.1.4.17) (Levi *et al.*, 1978). Thus any alteration of cellular processes observed on treatment of cells with the above compounds cannot be ascribed directly to inhibition of poly(ADP-ribose) synthetase.

In this laboratory, a search has been made for physiologically specific inhibitors of the enzyme that can enter the cell and affect only poly(ADP-ribose) synthetase. Presented below are the results obtained from the study of various benzamides and structurally related compounds on poly(ADP-ribose) synthetase activity *in vitro*.

Materials and Methods

Nicotinamide, nicotinic acid and 2- and 4-aminobenzoic acids were obtained from BDH Ltd., Poole, Dorset, U.K. 6-Aminonicotinamide, thymidine and NAD^+ were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 3-Nitrobenzoyl chloride, 3-acetylpyridine, 3-nitrobenzamide, 3-nitroacetophenone, 3-cyanophenol, 3-hydroxybenzoic acid and 3-methoxybenzamide were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. 3-Bromobenzoyl chloride was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

3-Aminobenzamide was synthesized from 3-nitrobenzamide by catalytic hydrogenation, and 3-amino-*N*-methylbenzamide by hydrogenation of 3-nitrobenzamidomethane (made from 3-nitrobenzoyl chloride and methylamine). 3-Acetamidobenzamide and 3-succinylaminobenzamide [3-(3-carboxypropionyl)aminobenzamide] were prepared from 3-aminobenzamide and the corresponding acid anhydride. 3-Bromobenzamide was prepared from the acid chloride and ammonium acetate in acetone (Finan & Fothergill, 1961), and 3-hydroxybenzamide from 3-cyanophenol by treatment with slightly alkaline H_2O_2 by the method of Radziszewski (1885).

Isolation of nuclei and extraction of poly(ADP-ribose) synthetase

Nuclei were isolated from thymus of a freshly slaughtered pig as described by Khan & Shall (1976). The nuclei were resuspended in 0.5M-NaCl/100mM-triethanolamine (adjusted to pH 8.2 with conc. HCl/10mM-$MgCl_2$/2mM-dithiothreitol. After 10min on ice, the suspension was centrifuged for 60min at 100000g. The supernatant, containing the enzyme, was immediately frozen and used for subsequent enzyme assays.

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Poly(ADP-ribose) synthesis was measured by the incorporation of [*adenine*-^{3}H]NAD^{+} (synthesized from [^{3}H]ATP; The Radiochemical Centre) into acid-insoluble material. The assay mixture consisted of 100mM-triethanolamine/HCl, pH 8.2, 10mM-$MgCl_2$, 2mM-dithiothreitol and 50μM-[^{3}H]NAD^{+} (2μCi/nmol), in 180μl. The reaction was started by the addition of 20μl of the 0.5M-NaCl extract of nuclei. After 5min at 26°C, a 20μl portion was applied to a filter disc (Whatman grade 1; 2.0cm diam.), which had been presoaked in 20% (w/v) trichloroacetic acid in diethyl ether and dried at room temperature. The disc was left in aq. 20% trichloroacetic acid for 30min on ice, washed four times with 1% trichloroacetic acid, once with ethanol and once with diethyl ether. After the disc had dried at room temperature, the radioactivity was determined by counting in 0.5% (w/v) 2,5-diphenyloxazole in toluene, by using a Packard liquid-scintillation counter.

Protein was determined by the method of Sedmak & Grossberg (1977), with bovine serum albumin as standard.

Results and Discussion

Benzamide, a close analogue of nicotinamide, was first shown to be an inhibitor of poly(ADP-ribose) synthetase by Shall (1975). It has the major advantage over other analogues of nicotinamide in that, since it lacks the ring nitrogen of nicotinamide, it cannot be metabolized by NAD-biosynthetic enzymes. The major drawbacks to its use as an inhibitor for physiological studies are its extremely low solubility and very hydrophobic nature. During the course of this work, we have examined several benzamides substituted in the 3-position for their ability to inhibit the enzyme. Table 1 shows the effect of these compounds at 50μM (equimolar with substrate) on the activity of poly(ADP-ribose) synthetase activity *in vitro*. All the benzamides were more potent inhibitors than either nicotinamide or thymidine in this system. With the exception of 3-nitrobenzamide, inhibition was greater than 90%. 3-Aminobenzamide and 3-methoxybenzamide were both found to be competitive inhibitors, with K_i values (means ± S.E.M. of four experiments) of 1.8 ± 0.2μM and 1.5 ± 0.3μM respectively (results not shown). The greater degree of inhibition by the benzamides than by nicotinamide is not readily explained on the basis of simple structural or electron properties of the molecules.

3-Aminobenzamide was chosen to determine the effect of altering the carboxamide moiety on the ability of such compounds to inhibit the synthetase. It was surprising to find that both 3-aminobenzoic acid and 3-aminoacetophenone were inhibitory, although the inhibition was much less than with any of the benzamides (Tables 1 and 2). The corresponding nicotinamide analogues, nicotinic acid and 3-acetylpyridine, had no effect on enzyme activity (Table 2, and Preiss *et al.*, 1971). In contrast, alkylation of the amide group in both nicotinamide (Preiss *et al.*, 1971) and 3-aminobenzamide (to give 3-amino-*N*-methylbenzamide; Table 2) abolished inhibition. At present the inhibition by 3-aminobenzoic acid cannot be explained. It appears to be specific for 3-aminobenzoic acid; 3-nitro-, 3-hydroxy-, 2-amino- and 4-amino-benzoic acids all have no effect on enzyme activity. 3-Aminobenzoic acid has been shown to inhibit NAD^{+} glycohydrolase (EC 3.2.2.5) by formation of an adduct with the NAD^{+} substrate (Guardiola *et al.*, 1957). Such a mechanism cannot account for the inhibition of poly(ADP-ribose) synthetase, because neither 2-amino- nor 4-amino-benzoic acid (equally potent inhibitors of NAD^{+} glycohydrolase) has any affect on synthetase activity. Furthermore, the lack of inhibition by the 2- and 4-isomers also precludes the possibility that inhibition by 3-aminobenzoic acid is by nucleophilic removal of ADP-ribose residues from the protein. Inhibition by 3-aminoacetophen-

Table 1. *Effect of various benzamides on poly(ADP-ribose) synthetase activity*
Enzyme activity was assayed as described in the Materials and Methods section. Inhibitors were added to a final concentration of 50μM (equimolar with substrate). Each value is the mean of at least four separate determinations.

Compound	NAD^{+} incorporated into acid-insoluble material (nmol/min per mg of protein)	Inhibition (%)
None	2.40	—
Benzamide	0.09	96
3-Aminobenzamide	0.23	90
3-Bromobenzamide	0.12	95
3-Hydroxybenzamide	0.09	96
3-Methoxybenzamide	0.06	98
3-Nitrobenzamide	0.67	71
Nicotinamide	0.88	63

Table 2. *Effect of analogues of 3-aminobenzamide on poly(ADP-ribose) synthetase activity*
Enzyme activity was assayed as described in the Materials and Methods section. Inhibitors were added to a final concentration of 50 μM (equimolar with substrate). Each value is the mean of at least four separate determinations.

Compound	NAD^+ incorporated into acid-insoluble material (nmol/min per mg of protein)	Inhibition (%)
None	2.40	—
3-Aminobenzoic acid	2.16	10
Nicotinic acid	2.43	0
3-Hydroxybenzoic acid	2.38	0
3-Nitrobenzoic acid	2.45	0
2-Aminobenzoic acid	2.59	0
4-Aminobenzoic acid	2.47	0
3-Aminoacetophenone	1.66	31
3-Acetylpyridine	2.47	0
Acetophenone	1.53	36
3-Amino-*N*-methylbenzamide	2.38	0
3-Acetamidobenzamide	0.04	98
3-Succinylaminobenzamide	0.22	>91

one is less specific, as shown by the finding that acetophenone also inhibits (for a more detailed study on inhibition by acetophenones, see Purnell & Whish, 1980).

It was noteworthy that inhibition was retained when the amino group was acylated, as in 3-acetamidobenzamide and 3-succinylaminobenzamide. An accurate value could not be obtained for the latter compound, owing to its extremely low solubility. In addition, because detoxification of compounds containing aromatic amino groups often occurs by acetylation, 3-aminobenzamide detoxification will not lead to loss of inhibitor potency. Finally, coupling via the amino group should provide a very useful means by which an affinity medium for purification of the enzyme can be obtained.

In conclusion, the inhibitors described above will be useful for studies *in vitro* and, more importantly, since two, 3-aminobenzamide and 3-methoxybenzamide, are physiologically specific (M. R. Purnell & W. J. D. Whish, unpublished work), they will be extremely valuable as probes for use as inhibitors of poly(ADP-ribose) synthetase *in vivo*.

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References

Clark, J. B., Ferris, G. M. & Pinder, S. (1971) *Biochim. Biophys. Acta* **238**, 82–85
Finan, P. A. & Fothergill, G. A. (1961) *J. Chem. Soc.* 2824–2825
Guardiola, A. L., Paretsky, D. & McEwan, W. (1957) *J. Am. Chem. Soc.* **80**, 418–421
Hayaishi, O. & Ueda, K. (1977) *Annu. Rev. Biochem.* **46**, 95–116
Hilz, H. & Stone, P. R. (1976) *Rev. Physiol. Biochem. Pharmacol.* **76**, 1–58
Khan, M. G. & Shall, S. (1976) *Biochem. Soc. Trans.* **4**, 778–781
Leiber, U., Kittler, M. K. & Hilz, H. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 1347–1350
Levi, V., Jacobson, E. L. & Jacobson, M. K. (1978) *FEBS Lett.* **88**, 144–146
Meuth, M., Aufreite, E. & Reichard, P. (1976) *Eur. J. Biochem.* **71**, 39–43
Preiss, J., Schlaeger, R. & Hilz, H. (1971) *FEBS Lett.* **19**, 244–246
Purnell, M. R. & Whish, W. J. D. (1980) *Biochem. Soc. Trans.* **8**, 175–176
Purnell, M. R., Stone, P. R. & Whish, W. J. D. (1980) *Biochem. Soc. Trans.* **8**, 215–227
Radziszewski, B. (1885) *Ber. Dtsch. Chem. Ges.* **18**, 355–356
Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544–552
Shall, S. (1975) *J. Biochem. (Tokyo)* **77**, 2p