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INHIBITION OF POLY(ADP-RIBOSE)POLYMERASE ACTIVITY BY NUCLEOSIDE ANALOGS OF THYMIDINE

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Abstract—The poly ADP-ribosylation of proteins catalyzed by poly(ADP-ribose)polymerase (PARP) is involved in a number of important cellular metabolic activities. We evaluated various analogs of deoxythymidine and deoxyuridine as inhibitors of PARP. Most of these compounds have antiviral and/or anticancer activities. The structural requirements for these nucleoside analogs to be inhibitors of PARP were determined. The compounds evaluated had various substitutions on the 2-, 4- and/or 5-position of the pyrimidine ring, as well as on the 2'-, 3'- and/or 5'-position of the pentose moiety. Inhibition of PARP was strongly dependent on the size of the alkyl or halogen substituent on the 5-position of the pyrimidine ring. Whereas the 5-position of the pyrimidine ring could be varied, alteration of the 2- or 4-position drastically decreased the inhibition of PARP. Kinetic analysis was performed with concentrations of 1–10 μM NAD^+. The K_i values for many compounds were five to seven times lower than the K_i for 3-aminobenzamide, a previously described potent inhibitor of PARP. Compounds with combined substituents at both the 5-position of the pyrimidine ring and the 3'- or 5'-position of deoxyribose generally were potent inhibitors of PARP, as for example 3'-amino-2', 3'-dideoxy-(*E*)-5-(2-bromovinyl)uridine ($K_i = 0.7\ \mu$M), or 5'-azido-2',5'-dideoxy-5-ethyluridine ($K_i = 0.8\ \mu$M). The 5-halogenated analogs had K_i values of 18, 35, 110 and >1000 μM for 5-iodo-2'-deoxyuridine, 5-bromo-2'-deoxyuridine, 5-chloro-2'-deoxyuridine, and 5-fluoro-2'-deoxyuridine, respectively, and the 5-alkyl analogs had K_i values of 45, 2.2, 7, 16 and 180 μM for 5-methyl-2'-deoxyuridine, 5-ethyl-2'-deoxyuridine, 5-propyl-2'-deoxyuridine, 5-butyl-2'-deoxyuridine and 5-pentyl-2'-deoxyuridine, respectively. Two other compounds with substituents in the 5-position of the pyrimidine moiety also had potent activities: (E)-5-(2-bromovinyl)-2'-deoxyuridine ($K_i = 6\ \mu$M) and 5-trifluoromethyl-2'-deoxyuridine ($K_i = 1.6\ \mu$M). Compounds substituted in the 2'-, 3'- and/or 5'-position of the deoxyribose moiety were investigated and 5'-azido-5'-deoxythymidine, 5'-amino-5'-deoxythymidine, 3'-azido-3'-deoxythymidine and 3'-deoxythymidine (d2T) had K_i values of 12, 16, 18 and 30 μM, respectively.

Poly-ADP-ribosylation of proteins is involved in major eucaryotic cellular activities such as DNA repair [for review see Ref. 1], cell transformation [2–4], differentiation [5–9], DNA replication [10] and ligation [11]. ADP-ribosylation of proteins may also affect viral production and infectivity [12, 13] as, for example, ADP-ribosylation of adenovirus core proteins has been reported to have a possible role in viral decapsidation [13]. Poly(ADP-ribose)polymerase (PARP‡), an enzyme located in the nuclei, catalyzes the transfer of the ADP-ribose moiety from NDA^+ to target proteins, thereby forming a poly(ADP-ribose) chain covalently attached to the proteins. PARP requires for its activity either single-stranded or double-stranded "nicked" DNA, and the enzyme can modify a wide range of nuclear proteins, such as histone [14, 15], DNA-polymerases and DNA-ligase [11], Ca^{2+},Mg^{2+}-dependent endonuclease [16] and other proteins [14]. PARP can also automodify itself with subsequent inactivation [17].

Most of the suggested biological functions of PARP are related to repair of DNA damage such as that caused by radiation or alkylating agents [18–20]. Many inhibitors of PARP have been described, but only few have potent inhibitory activity [21–24]. For example, 2'-5'-oligoadenylates, which are synthesized in the cell after interferon treatment, have potent inhibitory activity ($K_i = 4\ \mu$M), and 3-aminobenzamide is also a potent inhibitor of this enzyme having a K_i value of about 3–5 μM [21, 23].

The primary biological function of the ADP-ribosylation reaction is uncertain, in part due to the absence of selective inhibitors of PARP. This lack of selectivity prevents the establishment of a direct linkage of poly ADP-ribosylation of specific proteins to the indicated biological process.

It has been reported that thymidine and some thymidine analogs are inhibitors of PARP [21, 22], and many have good antiviral or anticancer activity [25, 26]. A few have good potential for the therapy of AIDS [for review see Refs. 27 and 28]. Knowledge of the biochemistry of these compounds, as well as the enzymes involved, will be useful for the development of new drugs, as well as for understanding the possible mechanism of antiviral or anticancer activity.

In the present study we have investigated the inhibition of PARP by various deoxyuridine and

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‡ Abbreviations: PARP, poly(ADP-ribose)polymerase; PMSF, phenylmethylsulfonyl fluoride; and DTT, dithiothreitol.

deoxythymidine analogs and present a structure–activity analysis of the inhibition.

MATERIALS AND METHODS

Cells. Murine leukemia L1210 cells were grown as previously described [29]. Asynchronous exponentially growing cells were collected from five roller bottles and used as the enzyme source. Cell counts were made with a model ZB1 Coulter Counter/mode 256 Channelyzer (Coulter Electronics, Hialeah, FL).

3-Aminobenzamide Sepharose 4B affinity column preparation. 4-((3-(Aminocarbonyl)phenyl)amino)-4-oxobutanoic acid was synthesized and coupled to AH-Sepharose 4B by the method of Burtscher *et al.* [30]. The degree of benzamide substitution was based at least 50% on the known amino group content of the AH Sepharose 4B and the absorbance at 250 nm of the gel, yielding a minimal benzamide content of 5 μM/mL of the hydrated gel.

PARP purification. PARP was partially purified from L1210 cells by a method described previously [31], and then purified by affinity column chromatography according to Buki *et al.* [32]. Nuclei were prepared from 1–2 × 10^9 L1210 cells and immediately homogenized using a Dounce homogenizer (25 strokes) in 50 mL of buffer, containing 50 mM Tris–HCl, pH 7.5, 300 mM KCl, 2 mM EDTA, 1 mM phenhylmethylsulfonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), 0.2 M Na_2SO_3, pH 7.5, and 10% glycerol. The homogenate was centrifuged at 20,000 *g* for 30 min and enzymatic activity was recovered in the supernatant. This crude enzyme preparation (up to 150 mg protein) was passed through a 9-mL bed of 3-aminobenzamide Sepharose 4B equilibrated with buffer A containing 50 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 2 mM DTT and 10% glycerol. The column was then washed with 150 mL of buffer A (flow rate 7 mL/cm^2/hr), and the enzyme was quantitatively eluted from the column with buffer A containing 2 mM 3-methoxybenzamide and recovered in a fraction collector. The fractions containing the enzyme (the initial ten column volumes) were pooled and concentrated to 2% of the original volume with Centriprep 30 and Centricon 30 concentrator (Amicon). Residual 3-methoxybenzamide was removed by washing five times with buffer A in the same concentrator systems. Protein concentration was determined according to Bradford [33] using a Bio-Rad protein assay kit. The final enzyme solution was stored at −70° without loss of enzyme activity for at least 1 year.

PARP assay. PARP assay was performed in a 100 μL volume of buffer containing 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 20% glycerol, PARP (0.05 U), 1–10 μM NAD^+ (containing 20,000 cpm [^{14}C]NAD^+), calf thymus DNA (80 μg/mL), histones (40 μg/mL) and various amounts of the nucleoside analogs. The incubation was carried out for 1 hr at room temperature. The reaction was stopped by the addition of cold 20% trichloroacetic acid–2% $Na_4P_2O_7$, filtered through a Whatman GF/C filter disc, washed three times with 5% cold trichloroacetic acid–0.5% $Na_4P_2O_7$, once with 95% cold ethanol, and counted in 5 mL Optifluor-O (Packard Instrument Co., Downers Grove, IL) by liquid scintillation spectroscopy. One unit of enzyme activity was defined as being equivalent to 1 nmol of ADP-ribose incorporated into acid-insoluble material/min at 25°.

RESULTS

Purification of PARP from mouse L1210 cells. The enzyme was partially purified by affinity chromatography, and a summary of the purification achieved is shown in Table 1. The enzyme was evaluated for dependence of its activity on dsDNA, histone and NAD^+, and Fig. 1 shows typical data obtained. The enzyme was free from glycohydrolase contamination.

Inhibition kinetics of PARP by nucleoside analogs of deoxyuridine and thymidine. Various concentrations of the nucleoside analogs were competed against several levels of NAD^+ (1–10 μM). Figure 2 depicts the type of inhibition of PARP by two nucleoside analogs in the terms of Dixon plots, and these data indicate that the inhibition may be noncompetitive, at least in the tested range of the NAD^+. Similar inhibition kinetics were obtained with the other nucleoside analogs. The data from two independent experiments were combined, and K_i values were calculated (Table 2). The variation was less than 20%. In addition, the K_i relative to that for 3-aminobenzamide was calculated by dividing the K_i of the nucleoside inhibitors by the K_i of 3-aminobenzamide.

Effect of substitution on the pyrimidine ring on the inhibition of PARP. Various 5-substituted analogs of deoxyuridine were evaluated as inhibitors of

Table 1. Summary of partial purification of poly (ADP-ribose)polymerase from L1210 cells

Purification stage	Volume (mL)	Total protein (mg)	Specific activity (U/mg*)	Total activity (U)	Purification (fold)	Yield (%)
Crude extract from nuclei	32	147	1.03	152	1.0	100
Pooled fractions after affinity chromatography	2.13	3.19	33.0	105	32	69

* One unit is the transfer of 1 nmol of NAD^+/min to protein.

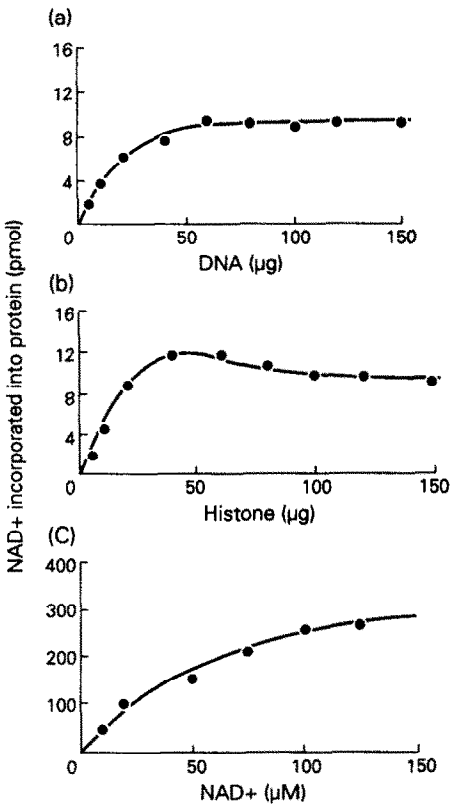


Fig. 1. Poly(ADP-ribose)polymerase activity as a function of "nicked" dsDNA (A), histone (B) and NAD^+ (C) concentrations. The assays were performed as described in Materials and Methods.

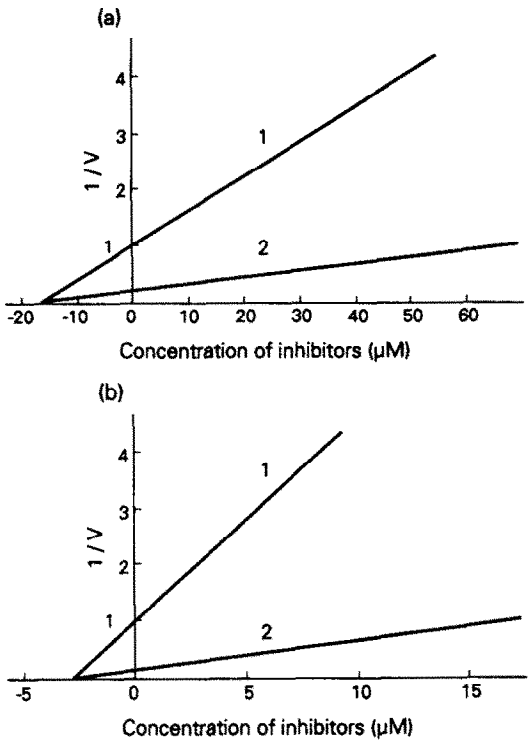


Fig. 2. Dixon plots of poly (ADP-ribose)polymerase inhibition by 5'-amino-5'-deoxythymidine (A) and 5'-azido-2',5'-dideoxy-(*E*)-5-(2-bromovinyl)uridine (B). The Assays were performed as described in Materials and Methods. The concentrations of NAD^+ were 1 μM (1) and 10 μM (2).

PARP; these included 5-alkyl, 5-halogen and 5-haloalkyl substituents, as well as deoxyuridine analogs substituted in the 2- and 4-position of the pyrimidine ring.

Data in Table 2 show that the K_i for 5-alkyl-substituted deoxyuridine analogs was dependent on the length of the alkyl substituent, and the optimal substituents were ethyl and propyl moieties. The greater inhibition was produced by 5-ethyl-2'-deoxyuridine ($K_i = 2.2\,\mu M$), whereas the K_i for 5-propyl-2'-deoxyuridine was 7 μM. Relative to the K_i of 5-ethyl-2'-deoxyuridine, the K_i value increased as the size of the alkyl substituent in the 5-position either decreased or increased. Thus, the K_i values for 5-methyl-2'-deoxyuridine and 2'-deoxyuridine were 45 and >1000 μM, respectively, and those for 5-propyl-, 5-butyl-, 5-pentyl- and 5-hexyl-2'-deoxyuridine were 7, 16, 180 and >1000 μM, respectively. Data in Table 2 also show that the K_i value of the halogen substitution in the 5-position of the deoxyuridine was dependent on the size of halogen, and the most potent inhibitor was 5-iodo-2'deoxyuridine with a K_i value of 18 μM.

Two other halogenated compounds, (*E*)-5-bromovinyl-2'-deoxyuridine and 5-trifluoromethyl-2'-deoxyuridine, were potent inhibitors of PARP with K_i values of 6 and 1.6 μM, respectively. 5-Methylamino-2'-deoxyuridine did not inhibit PARP even at a 1 mM concentration (Table 2). Compounds substituted in the 4-position of 2'-deoxyuridine (4-*N*-oxy-2'-deoxyuridine, 4-*N*-oxy-5-methyl-2'-deoxyuridine and 4-*N*-oxy-5-ethyl-2'-deoxyuridine) showed no inhibitory effect on PARP activity (Table 2).

Effect of substitution in the pentose moiety on the inhibition of PARP. Table 2 presents the data of the inhibition of PARP by various 2'-, 3'- and 5'-modified analogs of thymidine. These data indicate that amino and azido modifications of the 3'- or 5'-position of the pentose moiety resulted in an increase in the inhibition of PARP (decrease in K_i) relative to thymidine. However, modification of both the 3'- and 5'-positions by amino groups led to an increase of the K_i value. The modification by phosphate groups at the 5'-position drastically increased the K_i values to >1 mM. The PARP inhibition constant was decreased slightly to 30 μM from that of thymidine in the case of 3'-deoxythymidine (d2T), but was increased almost six times by dehydrogenation of d2T producing a 2', 3'-unsaturated bond (d4T). Arabinose nucleoside analogs (5'-amino-5'-deoxyarabinofuranosylthymine, (*E*)-5-(2-bromovinyl)-1-arabinofuranosyluracil) were less inhibitory compared to the corresponding deoxy-

Table 2. Inhibition constant of various nucleoside analogs for inhibition of poly(ADP-ribose)polymerase

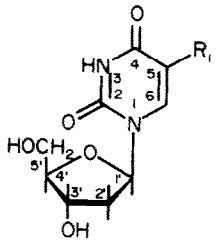
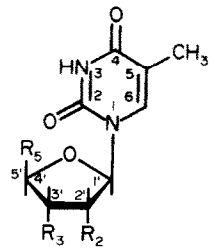
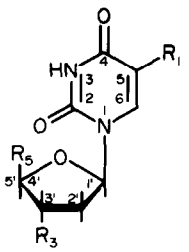
	K_i (μM)	K_i nucleoside/K_i aminobenzamide
Compounds substituted in the 5-position of the pyrimidine moiety (R_1)		
		
2′-Deoxyuridine	>1000	
5-Methyl-2′-deoxyuridine	45	10.8
5-Ethyl-2′-deoxyuridine	2.2	0.47
5-Propyl-2′-deoxyuridine	7	1.52
5-Butyl-2′-deoxyuridine	16	3.47
5-Pentyl-2′-deoxyuridine	180	39.1
5-Hexyl-2′-deoxyuridine	>1000	
5-Methylamino-2′-deoxyuridine	>1000	
5-Fluoro-2′-deoxyuridine	>1000	
5-Chloro-2′-deoxyuridine	110	23.9
5-Bromo-2′-deoxyuridine	35	7.6
5-Iodo-2′-deoxyuridine	18	3.9
5-Trifluoromethyl-2′-deoxyuridine	1.6	0.35
(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine	6	1.30
Compounds substituted in the 4-position of the pyrimidine moiety		
4-*N*-oxy-2′-Deoxyuridine	>1000	
4-*N*-oxy-5-Methyl-2′-deoxyuridine	>1000	
4-*N*-oxy-5-Ethyl-2′-deoxyuridine	>1000	
Compounds substituted in the 2′-, 3′- or 5′-position of the pentose moiety of thymidine (R_2, R_3, R_5)		
		
3′-Azido-3′-deoxythymidine	18	3.9
3′-Amino-3′-deoxythymidine	50	10.7
5′-Amino-5′-deoxythymidine	16	3.48
5′-Azido-5′-deoxythymidine	12	2.61
3′,5′-Diamino-3′,5′-didehydrothymidine	80	17.4
3′-Deoxythymidine (d2T)	30	6.52
3′-Deoxy-2′,3′-didehydrothymidine (d4T)	180	39.1
5′-Amino-5′-deoxyarabinofuranosylthymine	100	21.7
dTMP	>1000	
dTDP	>1000	
dTTP	>1000	

Table 2 (*continued*)

	K_i (μM)	K_i nucleoside/K_i aminobenzamide
Compounds substituted in both the pyrimidine and pentose moieties (R_1, R_3, R_5)		
		
3′-Azido-2′,3′-dideoxy-5-bromouridine	11	2.39
5′-Azido-2′,5′-dideoxy-(*E*)-5-(2-bromovinyl)uridine	2.8	0.61
3′-Azido-2′,3′-dideoxy-(*E*)-5-(2-bromovinyl)uridine	0.8	0.17
3′-Amino-2′,3′-dideoxy-(*E*)-5-(2-bromovinyl)uridine	0.7	0.15
5′-Amino-2′,5′-dideoxy-5-iodouridine	2.8	2.39
5′-Azido-2′,5′-dideoxy-5-iodouridine	3.0	0.65
3′-Azido-2′,3′-dideoxy-5-iodouridine	1.4	1.15
5′-Azido-2′,5′-dideoxy-5-ethyluridine	0.8	0.17
3′-Azido-2′,5′-dideoxy-5-ethyluridine	1.0	0.22
2-5′-Anhydro-3′-azido-2′,3′-dideoxy-5-iodouridine	>100	
2-5′-Anhydro-3′-azido-3′-deoxythymidine	>100	
2-5′-Anhydro-3′-deoxythymidine	>1000	
(*E*)-5-(2-Bromovinyl)-1-arabinofuranosyluracil	50	10.9
1-(2′-Deoxy-2′-fluoro-arabinofuranosyl)-5-methyluracil	>100	
1-(2′-Deoxy-2′-fluoro-arabinofuranosyl)-5-ethyluracil	24	5.22
Aminobenzamide	4.6	

riboside (5′-amino-5′-deoxythymidine and (*E*)-5-bromovinyl-2′-deoxyuridine).

Effect of substitution in the pentose and pyrimidine moieties on the inhibition of PARP. Combinations of different substituents at the 5-position of the pyrimidine ring, and the 3′- or 5′-position of the pentose moiety were evaluated. Table 2 shows inhibition kinetics and the K_i values for several double-substituted nucleoside analogs. These data show a significant decrease in the K_i relative to the parent compounds. For several analogs the K_i was less than 1 μM, which is five to seven times lower than the K_i value determined for 3-aminobenzamide (4.6 μM), a compound considered to be a very potent inhibitor of PARP.

DISCUSSION

ADP-ribosylation of proteins has been reported to play a role in many aspects of cellular metabolism, such as DNA repair [1], cell transformation [2–4] and differentiation [5–9], but the precise functional role of ADP-ribosylation of proteins is yet to be elucidated. In the absence of a molecular genetic approach to obtain an understanding of the physiological role of ADP-ribosylation of proteins, the use of PARP inhibitors may be a useful alternative strategy. Until recently, only two highly active inhibitors of PARP, 3-aminobenzamide [21, 23] and oligoisoadenylates (pppA2′p5′A2′-p5′pA) [24], were available. Both of these compounds have side-effects, especially oligoisoadenylates, which alter other enzymatic activities too [for review see Refs. 34 and 35]. Suto *et al.* [36] have reported recently that several 5-substituted dihydro-isoquinolinones are very potent inhibitors of PARP with K_i values of <1.0 μM. 5-Bromo-2′-deoxyuridine and thymidine have been reported to be inhibitors of PARP [21], but the inhibition constants for these two compounds are too high to consider them for potential inhibition of the PARP activity *in vivo*. We have investigated various deoxyuridine and thymidine analogs and found that some are potent inhibitors with inhibition constants (K_i) five to seven times lower than the K_i for 3-aminobenzamide. The value of K_i is strongly dependent on the nature of the 5-substituent of deoxyuridine. Electronegative substituents that are 2.15 Å or larger (iodo, trifluoromethyl, bromovinyl) or neutral (alkyl) substituents in the 5-position of the pyrimidine moiety are the most potent compounds. The substitution of the 3′- or 5′-position of the deoxyribose also affects the K_i value. The physico-chemical basis for the varying activities of the

nucleoside analogs as influenced by the nature of the substituents in the various positions is not understood as yet. Ideally, one would like to be able to examine the enzyme by X-ray crystallography and NMR in the presence and absence of the most potent inhibitor we have found. Such information would afford the opportunity to direct synthetic efforts for the development of inhibitors with high selectivity. The combination of different substitutions has been shown to lead to an increase in inhibition, that is a decrease in the k_i value.

It is interesting to note that practically all of the compounds evaluated have antiviral and/or anticancer activities and some are even in clinical use [25–28]. At present, there is no unequivocal evidence for a connection between the antiviral and antitumor activities of these nucleoside analogs and inhibition of PARP. Nevertheless, many of the most potent inhibitors of PARP are also very potent antiviral agents, suggesting that poly ADP-ribosylation of proteins may play a role in viral reproduction, although presently there is little evidence for such a relationship. The data obtained may be useful for further investigation of the physiological roles of poly ADP-ribosylation of proteins, as well as for developing a new basis for drug selection.

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