Biochemical Pharmacology, Vol. 35, No. 22, pp. 4025–4029, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

SELECTIVE TOXICITY OF DEOXYADENOSINE ANALOGUES IN HUMAN MELANOMA CELL LINES

P. G. PARSONS,* E. P. W. BOWMAN* and R. L. BLAKLEY†

* Queensland Institute of Medical Research, Herston, Queensland, Australia 4006; † Division of Biochemical and Clinical Pharmacology, St. Jude Childrens Research Hospital, Memphis, TN 38101, U.S.A.

(*Received* 31 *January* 1986; *accepted* 13 *June* 1986)

Abstract—The *in vitro* toxicities of 19 analogues of deoxyadenosine were tested using a panel of human melanoma cell lines including two lines sensitive to deoxyadenosine and deoxyinosine. The 2-fluoro-, 2-chloro-, 2-bromo- and 2-amino-8-aza derivatives were the most toxic and showed selectivity against deoxyadenosine-sensitive cells. 2-Bromodeoxyadenosine (BrdAdo) and its 5′-phosphate were less potent than the chloro compound but showed the greatest selectivity. In further studies of BrdAdo a third sensitive melanoma line was identified of the eight tested. A treatment time of 24 hr or more was required to develop toxicity to BrAdo; this could be prevented by deoxycytidine or cytidine added to the medium but not by other nucleosides. Flow cytometry showed that BrdAdo blocked cells in the G1 and S phases of the cell cycle. DNA synthesis as judged by thymidine incorporation was rapidly inhibited by BrdAdo to an extent which reflected the sensitivity of the particular cell line; RNA synthesis was less affected. Exposure to BrdAdo for 48 hr induced breaks in the preformed DNA of sensitive but not resistant cells. The results suggest that the toxicity of BrdAdo is associated with prolonged inhibition of DNA synthesis and subsequent DNA fragmentation.

The exceptional sensitivity of normal and transformed T lymphocytes to dAdo‡ has prompted attempts to derive specific treatments for T cell malignancies. The high levels of intracellular and extracellular deaminase activity necessitate the use of deaminase inhibitors [1] for dAdo to have significant toxicity *in vivo*. An alternative approach is the use of analogues such as 2-halogenodeoxyadenosines, which are not substrates for deoxyadenosine deaminase but are phosphorylated and incorporated into DNA and have significant toxicity and T-cell selectivity *in vivo* and *in vitro* [2–6]. Of particular interest is the finding that 2-chlorodeoxyadenosine is toxic to resting T cells [4].

Compared with T cells, a human fibroblast strain and seven cell lines established from solid tumours were found to be insensitive to 2-chlorodeoxyadenosine [3, 4]. We recently identified two human melanoma lines sensitive to killing by adenosine, dAdo and dIno [7, 8]. The toxicity of a range of dAdo analogues, available through the transdeoxyribosylation reaction [3, 5], has therefore been tested using these and other human cell lines to identify structure/activity relationships and to determine whether the 2-halogeno compounds act in a similar fashion to dAdo itself.

MATERIALS AND METHODS

The origins of HeLa-S_3 and the human melanoma cell lines MM96-MM473 have been described [8, 9]. NFF and HFM were strains of human fibroblasts established from neonatal foreskin and foetal muscle respectively. The CCRF-CEM T cell leukaemia line [10] was obtained from Dr D. Hedley, Ludwig Institute for Cancer Research, Sydney, Australia.

Cell cultures were maintained at 37° in 5% CO_2/air in Rosewell Park Memorial Institute Tissue Culture Medium 1640 (Commonwealth Serum Laboratories, Melbourne, Australia) supplemented with 10% (v/v) foetal calf serum, 100 μg streptomycin per ml, 100 IU penicillin per ml and 3 mM 4-2(hydroxyethyl)-1-piperazine-ethanesulphonic acid. Unless otherwise stated, cell survival was determined by a colony-type assay [9], as follows. The drug was added to cells seeded 24 hr previously (2×10^3 cells/16 mM well) and after 5–7 days the cultures were labelled with [^{3}H]thymidine (2 μCi/ml) for 2 hr, detached with trypsin and washed onto glassfibre discs for liquid scintillation counting. Survival was calculated as percentage of control cpm and curves were plotted using five different drug concentrations. In some experiments, visual counting of colonies was employed; cultures (1000 cells/60 mm plate) were treated and after 7 days the colonies (>50 cells) were stained with Giemsa and counted.

For flow cytometry, cells (2×10^5/60 mm plate) were detached with trypsin, and fixed in 50% (v/v) ethanol–phosphate buffered saline. After addition to the cell pellet of 250 μl phosphate buffer containing RNase (0.5 mg/ml), Triton X-100 (0.2%) and propidium iodide (50 μg/ml), the DNA content was determined using a FACS IV cell sorter (Becton-Dickinson FACS Systems, Sunnyvale, CA, U.S.A.). Chicken erythrocytes and human fibroblasts were used as internal and external standards respectively.

DNA synthesis was determined using cells seeded 24 hr previously (5×10^4/16 mm well). At various times after commencement of BrdAdo treatment, [^{3}H]thymidine (1 μCi/ml) was added. After 30 min incubation, cells were harvested for liquid scin-

‡ Abbreviations: dAdo, deoxyadenosine; BrdAdo, 2-bromodeoxyadenosine; dIno, deoxyinosine.

tillation counting as described above. For simultaneous determination of RNA synthesis, a mixture of [^{3}H]thymidine (1 μCi/ml) and [^{14}C]uridine (0.05 μCi/ml) was used and the harvested cells were solubilised with Soluene 350 (Packard Instruments, Zurich, Switzerland) for calculation of dpm.

For determination of DNA strand breaks, cells (3×10^5/60mm plate) were labelled with 0.05 μCi/ml [^{14}C]thymidine for 24 hr, washed and exposed to the drug for 48 hr. The cells were harvested, mixed with [^{3}H]-labelled controls (0.1 μCi/ml [^{3}H]thymidine) and co-eluted from Millipore BS-2 filters with tetraethylammonium hydroxide (pH 12.6) as previously described [11].

The deoxyadenosine analogues were synthesized as previously described [3, 5] and were dissolved in medium. dAdo and dIno were dissolved in saline prior to dilution into medium.

RESULTS

Cell survival

The *in vitro* toxicity of continuous exposure to dAdo, dIno and 19 analogues of dAdo was compared using two human melanoma cell lines (MM96L, MM127) known to be sensitive to the fomer agents [8], and a line (MM253c1) known to be resistant. The 2-fluoro-, 2-chloro-, 2-bromo-, 2-bromo-5′-phosphate and 8-aza-2-amino derivatives of dAdo gave D_{37} values of <20 μM (Table 1). Similar results were obtained using HeLa cells (not shown). The following compounds had no significant toxicity at <20 μM: 8-bromo-, 8-chloro-, 2-methylthio-, 2-trifluoromethyl-, 8-aza, 8-aza-7-deaza- and 2-aminodeoxyadenosine; 6-methylamino-, 6-benzylamino and 6-dimethylamino-9-(2′-deoxyribosyl)purine; 8-bromo- and 8-trifluoromethyl-3-(2′-deoxyribosyl)-adenine; 2-bromoadenosine and 2′-deoxyribosyl isoguanine. Except for dAdo and dIno, survival was not determined at drug levels >20 μM because of poor solubility of some of the analogues. 2-Chlorodeoxyadenosine was approximately 10-fold more potent than the other 2-halogeno derivatives but comparing the MM96L/MM253c1 D_{37} ratios, BrdAdo was three-fold more selective against the sensitive cell lines. Study was therefore focused on the latter compound.

The survival curves of resistant cells exhibited shoulders whereas sensitive cells gave a linear response (Fig. 1); cell survival determined by [^{3}H]thymidine labelling of cultures or by visual counting of colonies gave similar results (Fig. 1A). The melanoma line MM127 and the T cell leukaemia line CCRF-CEM, known to be sensitive to dAdo, were also very sensitive to BrdAdo (Fig. 1B). A survey of other lines (Table 2) revealed sensitivity to

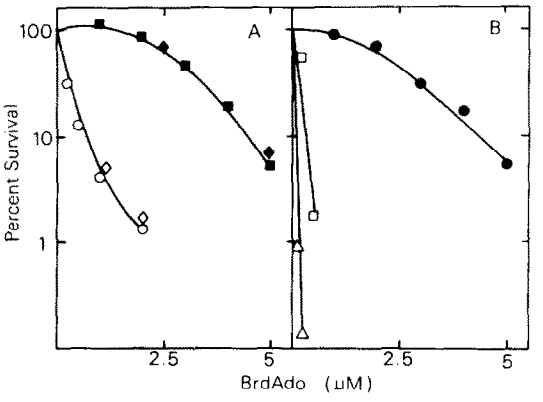

Fig. 1. Survival of cell lines treated with BrdAdo. (A) ■ MM253c1; ◆ MM253c1, determined by colony counting; □ MM96L; ◇ MM96L determined by colony counting. (B) ● HeLa; □ MM127; △ CCRF-CEM. Points are means of duplicates.

Table 2. Toxicity of BrdAdo in human cells

Cell	D_{37} (μM)
CCRF-CEM	0.03
HeLA	2.8
HFM fibroblasts	1.5
NFF fibroblasts	1.9
MM96L	0.18
MM127	0.21
MM170	2.1
MM229	0.33
MM253c1	3.2
MM386	2.2
MM418	2.3
MM473	1.8

Table 1. Toxicity of dAdo and dAdo analogues in human cell lines

Compound	D_{37}^{*} (μM)		
	MM96L	MM127	MM253c1
dAdo	95	61	2100
dIno	120	79	3500
2-Fluorodeoxyadenosine	0.11	0.12	0.35
2-Chlorodeoxyadenosine	0.022	0.012	0.14
BrdAdo	0.18	0.21	3.2
2-Bromodeoxyadenosine-5′-phosphate	0.51	0.45	70
8-Aza-2-aminodeoxyadenosine	0.30	0.65	5.2

* Dose required to reduce survival to 37%.

Table 3. Modification of BrdAdo toxicity by other nucleosides

Nucleoside	Concentration* (μM)	Cell survival (% control) MM96L plus 0.5 mM BrAdo	HeLa plus 5 mM BrAdo
Control		12 ± 2.1†	8.5 ± 2.3
Deoxycytidine (1)	100	91 ± 5.7	93 ± 7.1
dAdo (2)	10	9.1 ± 1.8	9.2 ± 2.5
Deoxyguanisine (3)	10	10 ± 2.3	8.9 ± 1.6
Thymidine (4)	10	9.5 ± 1.9	7.9 ± 2.9
(1) + (2) + (3) + (4)		93 ± 3.5	97 ± 3.1
Cytidine (5)	50	63 ± 4.5	41 ± 5.2
Adenosine (6)	10	10 ± 3.4	8.9 ± 3.1
Guanosine (7)	10	11 ± 2.7	6.8 ± 3.1
Uridine (8)	50	14 ± 2.2	9.2 ± 1.7
(5) + (6) + (7) + (8)		67 ± 5.8	40 ± 6.2

* Survival was >90% when used alone.
† Mean ± SD (N = 2).

BrdAdo in three human melanoma lines compared with Hela, two fibroblast strains and five other melanoma lines. The D_{37} for dAdo in the BrdAdo-sensitive MM229 line was 105 μM.

The effect of the natural nucleosides on BrdAdo toxicity was compared using MM96L and Hela (Table 3). Deoxycytidine and to a lesser extent, cytidine, almost completely inhibited the toxicity of BrdAdo. Various combinations of nucleosides had no effect unless cytidine or deoxycytidine were present (results not shown).

Effect of BrdAdo on cell cycle progression

The effect of toxic levels of BrdAdo on cell cycle progression was determined by DNA flow cytometry. Cell counts showed that <10% of cells were lost during these experiments. Colcemid, used to demonstrate that the controls (Figs 2A and 3A) were progressing through the cycle, caused cells to accumulate in G2/M (Figs 2B and 3B). The effect was more pronounced in HeLa than in MM96L. Cells exposed to BrdAdo for 24 hr showed progression of G2/M cells into a G1/S block (Figs 2C and 3C). The fact that colcemid scarcely increased the proportion of G2/M cells in the presence of BrdAdo (Figs 2D and 3D) indicates that most of the cells already in S remained in that phase. No difference between MM96L and HeLa was observed at this BrdAdo level (5 μM). When 1 μM BrdAdo was used, MM96L cells were again blocked in G1/S whereas HeLa were not affected (results not shown).

Effect of BrdAdo on DNA and RNA synthesis

Using a 1 hr treatment period and concentrations of minimal toxicity, BrdAdo strongly inhibited DNA synthesis (determined as incorporation of [^{3}H]thymidine) in MM96L compared with HeLa (Fig. 4). Supratoxic levels inhibited DNA synthesis in both cell lines, in agreement with the observed cell cycle block in G1/S.

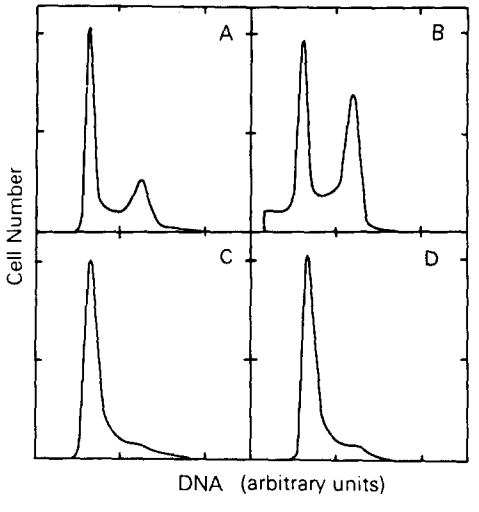

Fig. 2. Effect of BrdAdo (24 hr treatment) on the cell cycle progression of MM96L, as judged by flow cytometry. (A) Untreated cells. (B) Cells treated with 1 μg/ml colcemid for 24 hr. (C) Cells treated with 5 μM BrdAdo for 24 hr. (D) Cells treated simultaneously with 1 μg/ml colcemid and 5 μM BrdAdo for 24 hr.

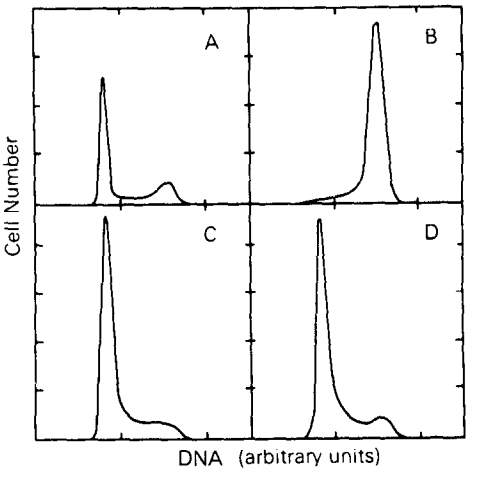

Fig. 3. Effect of 5 μM BrdAdo (24 hr treatment) on the cell cycle progression of HeLa. Same legend as Fig. 2.

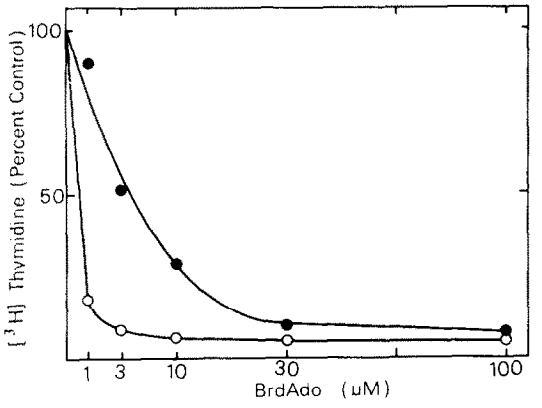

Fig. 4. BrdAdo dose response for inhibition of DNA synthesis in MM96L (○) and Hela (●) after a 1 hr treatment. Points are means of duplicates.

A study of the treatment time response showed that inhibition of DNA synthesis by 10 μM BrdAdo was rapid in both cell lines, reaching a minimum within the first hour (Fig. 5). The inhibition was less for HeLa than for MM96L. Inhibition of RNA synthesis in MM96L occurred more slowly and to a lesser extent compared with DNA synthesis. In contrast RNA synthesis was inhibited rapidly in HeLa but reached the same level as in MM96L. Parallel cultures seeded at lower cell densities were used to determine the effect of treatment time on long-term cell survival. The results (Fig. 5) showed that significant toxicity was only generated when cells were treated for at least 24 hr.

Strand breaks in preformed DNA

A variety of BrdAdo doses and treatment times were evaluated in attempts to detect DNA strand breaks by alkaline elution. In this method, breaks are detected by an increased elution rate from filters by alkali. No breaks were detected unless an exposure time of 48 hr was used. Under these conditions, an increased elution rate compared with

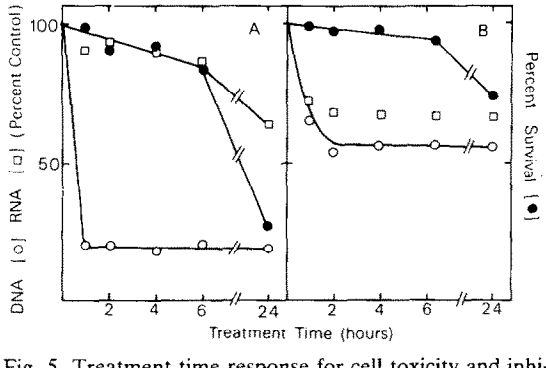

Fig. 5. Treatment time response for cell toxicity and inhibition of DNA and RNA synthesis by 10 μM BrdAdo. (A) MM96L: ● cell survival; ○ DNA synthesis measured by incorporation of [³H]thymidine; □ RNA synthesis measured by incorporation of [¹⁴C]uridine. (B) HeLa: same symbols as in (A). Points are means of duplicates.

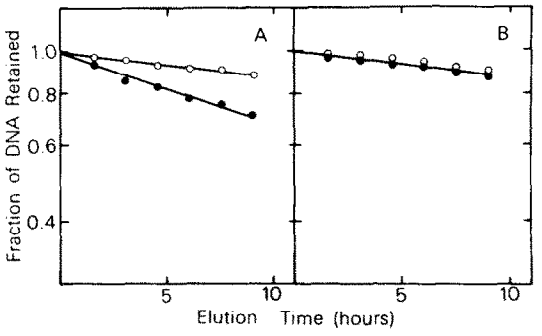

Fig. 6. Induction of breaks in preformed DNA after treatment of cells with 5 μM BrdAdo for 48 hr. (A) MM96L: ○ controls; ● treated. (B) Hela: same symbols as in (A).

controls was found in MM96L but not in HeLa (Fig. 6).

DISCUSSION

This study identified five 2-substituted deoxyadenosine analogues having significant toxicity against human melanoma cells in culture. The toxicity of 2-fluoro-, 2-chloro- and 2-bromodeoxyadenosine to other cell types *in vitro* has been reported [2–6, 12]. It is of interest that the relative toxicities of the halogen analogues depend upon the particular cell line. BrAdo was more toxic than 2-fluorodeoxyadenosine in CCRF-CEM cells [3] whereas the reverse was found in the melanoma lines; and the difference between the toxicities of the fluoro and chloro derivatives was less for melanoma cells than for CCRF-CEM. Within the melanoma group, 2-fluorodeoxyadenosine was much less selective against MM96L and MM127 compared with the other active analogues. The mechanism(s) of toxicity of deoxyadenosine and its analogues are complex and may involve several requirements such as transport, phosphorylation and incorporation into DNA as discussed below. The present findings suggest that 2-substituents of moderate size and polarity meet these requirements. Concerning the latter property, it is of interest that the 2-amino analogue only became potent when combined with an 8-aza substitution. It may therefore be worthwhile to synthesize and test a series of 2-amino derivatives of 8-azadeoxyadenosine.

A second finding was that some cell lines derived from human solid tumours (melanoma) were exceptionally sensitive to the 2-halogeno analogues, approaching the sensitivity of the CCRF-CEM T cell leukemia line. We chose BrdAdo for further study not because it was considered to be the most promising analogue for *in vivo* use but because it had slightly better selectivity against the hypersensitive lines than the more potent chloro compound, and because the 5'-monophosphate, a potential pro-drug, was available for comparison. The possibility of using nucleotides as pro-drugs has been studied in other systems [13]. Deoxycytidine and cytidine were much more effective in preventing the toxicity of BrdAdo than in preventing the toxicity of

dAdo [8]. Indicative of a differential effect on the purine/primidine balance or a reduced efficiency of BrdAdo as a substrate for deoxycytidine kinase compared with dAdo, the possibility of such protection occurring *in vivo* may need to be included in the evaluation of dAdo analogues.

Since the doubling times for MM96L and HeLa are approximately 24 hr, the results suggested that BrdAdo toxicity requires inhibition of DNA synthesis for a period of at least one doubling time. Inhibition of DNA synthesis by BrdAdo could result from feed-back inhibition of ribonucleotide reductase as proposed for dAdo itself [14–16], by incorporation of BrdAdo into DNA as found for 2-chlorodeoxyadenosine [4], or by a combination of these effects. The requirement for long exposure times to induce toxicity and DNA fragmentation, found also using 2-chlorodeoxyadenosine [17], could be due to inhibited repair of spontaneous depurination caused by altered nucleotide pools and/or inappropriate incorporation of nucleotides. No explanation can yet be given for the exceptional toxicity of BrdAdo in the MM96L, MM127 and MM229 lines, except to note the association with 5′-nucleotidase deficiency [8], high sensitivity to inhibition of DNA synthesis by BrAdo and formation of strand breaks. In due course it will be of interest to determine the deoxycytidine kinase:5′-nucleotidase ratio, considered to be a critical aspect of the sensitivity of T cells to 2-chlorodeoxyadenosine [4].

Acknowledgement—This work was supported by the National Health and Medical Research Council, Canberra, Australia.

REFERENCES

1. C. A. Koller, B. S. Mitchell, M. R. Grever, E. Mejias, L. Malspeis and E. N. Metz, *Cancer Treat. Rep.* **63**, 1949 (1979).
2. D. A. Carson, D. B. Wasson, J. Kaye, B. Ullman, D. W. Martin, R. K. Robins and J. A. Montgomery, *Proc. natn. Acad. Sci. U.S.A.* **77**, 6865 (1980).
3. M.-C. Huang, K. Hatfield, A. W. Roetker, J. A. Montgomery and R. L. Blakley, *Biochem. Pharmac.* **30**, 2663 (1981).
4. D. A. Carson, D. B. Wasson, R. Taetle and A. Yu, *Blood* **62**, 737 (1983).
5. M.-C. Huang, T. L. Avery, R. L. Blakley, J. A. Secrist III and J. A. Montgomery, *J. med. Chem.* **27**, 800 (1984).
6. D. A. Carson, D. B. Wasson and E. Beutler, *Proc. natn. Acad. Sci. U.S.A.* **81**, 2232 (1984).
7. P. G. Parsons and I. P. Haywood, *Biochem. Pharmac.* **32**, 871 (1983).
8. P. G. Parsons and I. P. Hayward, *Biochem. Pharmac.* **35**, 655 (1986).
9. P. G. Parsons, *Biochem. Pharmac.* **34**, 1801 (1985).
10. G. Foley, H. Lazarus, S. Farber, B. Uzman, B. Boone and R. McCarthy, *Cancer* **18**, 522 (1965).
11. P. G. Parsons, S. G. Smellie, L. W. Morrison and I. P. Haywood, *Cancer Res.* **42**, 1454 (1982).
12. J. A. Montgomery and K. Hewson, *J. med. Chem.* **12**, 498 (1969).
13. H. P. Johnston, P. Hawley, S. E. White, I. Gibson and D. M. Tidd, *Br. J. Cancer* **51**, 505 (1985).
14. I. H. Fox and W. N. Kelley, *A. Rev. Biochem.* **47**, 655 (1978).
15. J. K. Henderson, F. W. Scott and J. K. Lowe, *Pharmac. Ther.* **8**, 573 (1980).
16. J. Kurtzberg and M. S. Hershfield, *Cancer Res.* **45**, 1579 (1985).
17. S. Seto, C. J. Carrera, M. Kubota, D. B. Wasson and D. A. Carson, *J. clin. Invest.* **75**, 377 (1985).