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HUMAN MELANOMA CELLS SENSITIVE TO DEOXYADENOSINE AND DEOXYINOSINE

PETER G. PARSONS* and IAN P. HAYWARD
Queensland Institute of Medical Research, Herston, Queensland, Australia 4006

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Abstract—In an *in vitro* study conducted without the use of adenosine/deoxyadenosine deaminase inhibitors, two human melanoma cell lines, MM96L and MM127, were found to be highly sensitive to killing by continuous treatment with deoxyadenosine (dAdo) (D_{37} 47 μM and 68 μM respectively) compared with fibroblasts (D_{37} 440 μM), Hela cells (D_{37} 1.1 mM) and other melanoma cell lines (D_{37} 0.8 to 2.5 mM). Cross-sensitivity was found to deoxyinosine (dIno) and in part to adenosine but not to related metabolites such as inosine or hypoxanthine. Hypersensitivity to dAdo was associated with deficiency in cell membrane 5′-deoxynucleotidase but not in deaminase activity. dAdo toxicity could be prevented in MM96L by addition of the other three deoxynucleosides together but not by removing dAdo after a brief (2 hr) treatment. Resistant melanoma cells, however, required more than 24 hr dAdo treatment to produce toxicity. DNA synthesis in MM96L cells was reversibly inhibited, and cells tended to accumulate in G1/S. No DNA strand breaks were detected. These results showed that in contrast to the resistant cell line, asynchronous MM96L cells are highly sensitivity to brief treatment, toxicity resulting from an effect associated with inhibition of DNA synthesis. dAdo and dIno, either combined with a deaminase inhibitor or as deaminase-resistant derivatives, may have a favourable therapeutic index for some melanomas *in vivo*.

The association of defective purine metabolism with certain immunodeficiencies and the possibility of exploiting such effects for specific treatment of T cell malignancy, has stimulated many studies of the mechanism of dAdo† toxicity in lymphoid cells. Sensitivity to dAdo has been associated with deficiencies in deoxyadenosine deaminase, purine nucleoside phosphorylase, or 5′-nucleotidase [1–8]. The consequences of such defects include pyrimidine starvation [9, 10], feed-back inhibition of ribonucleotide reductase by elevation of dATP and hence inhibition of DNA synthesis [1, 11, 12], or inhibition of *S*-adenosylhomocysteine hydrolase [13]. Resistance, on the other hand, arose from inhibited transport of the drug across the cell membrane [3, 5] or from lack of kinase activity required to form dATP [14]. Of the many cell types studied in culture, only normal and transformed T lymphocytes have proved to be exceptionally sensitive to dAdo.

During attempts to label purine bases in human melanoma DNA we found that several cell lines were very sensitive to killing by Ado [15] and, in the present work, to dAdo and dIno. Most recent studies have used dAdo deaminase inhibitors in order to avoid the complication of high levels of dIno and hypoxanthine being produced. Such inhibitors, however, may have other effects on purine metabolism and do not suppress deaminase activity equally in all tissues [16]. Since the aim of the present study was to compare dAdo toxicity in a library of human cell lines derived from various tissues, no deaminase inhibitors were used. Attention was focused on aspects of differential cytoxicity relevant to *in vivo* use as well as on the biochemical mechanism.

* Please address all correspondence to: Dr P. G. Parsons, Queensland Institute of Medical Research, Herston, Queensland, Australia 4006.

† Abbreviations used: Ado, adenosine; dAdo, deoxyadenosine; dIno, deoxyinosine; AMP(CH_2)P, adenosine 5′-(α,β-methylene)diphosphate.

MATERIALS AND METHODS

The origins of the human fibroblast strain (PGP) and human melanoma cell lines (MM96–MM253c1) have been described [15, 17, 18]. MM253c1a was a recloned subline of MM253c1. The MM418 melanoma line was established from a primary melanoma tumour of a female. The CCRF-CEM T cell leukemia line [19] and the B cell line (CW), derived by transformation with Epstein–Barr virus of peripheral lymphocytes of a nontumour donor, were obtained from Dr D. J. Moss of this Institute. Cells were cultured in 5% CO_2/air at 37° using Roswell Park Memorial Institute medium 1640 supplemented with 10% (v/v) foetal calf serum, HEPES (3 mM), penicillin (100 I.U./ml) and streptomycin (100 μg/ml). Routine tests for *Mycoplasma* [18] were negative.

Clonogenic cell survival was routinely determined as previously described [18] by treating sparse cultures seeded 24 hr previously (3×10^3 cells/16 mm dia. well). After 6 days of continuous treatment, the cultures were labelled for 4 hr with [*methyl*-^{3}H] thymidine (2 μCi/ml, 40 Ci/mmole; Radiochemical Centre, Amersham, U.K.), the colonies dislodged with trypsin (0.2 mg/ml in phosphate-buffered saline) and washed with water onto glass fibre discs for liquid scintillation counting. Cell survival was expressed as a percentage of the control. Unless otherwise stated, all drug treatments were continu-

ous. Stock solutions of drugs were prepared in 0.15 M NaCl just before use.

Incorporation of labelled deoxynucleosides was determined by washing cultures (5×10^4 cells/16-mm well) once with fresh medium and pulsing for 60 min followed by detachment with trypsin and harvesting onto glass fibre discs with water. The samples were solubilised with Soluene 350 (Packard Instruments, Zurich, Switzerland) for determination of dpm by liquid scintillation counting. The nucleosides were [*methyl*-^{14}C] thymidine (0.04 μCi/ml, 54 Ci/mole) mixed either with [G-^{3}H] deoxyadenosine (2 μCi/ml, 30 Ci/mmole) or with [5-^{3}H] deoxycytidine (5 μCi/ml, 27 Ci/mmole).

Flow cytometry was carried out using cells (5×10^5/60-mm plate) treated for various times and then detached, fixed in 50% v/v ethanol, incubated with 50 μg/ml propidium iodide and 100 μg/ml RNase in phosphate-buffered saline at 37° for 30 min. Analysis was performed on a FACS IV instrument (Becton-Dickinson, FACS Systems, CA).

Adenosine and deoxyadenosine deaminase activities were determined by suspending 10^5 cells in 40 μl 20 mM Tris–0.15 M NaCl (pH 7.2), lysis with 0.2% Triton X-100, dilution to 1 ml with 50 μM Ado or dAdo in the same buffer and the absorbance decrease monitored at 265 nm at 25°. Phosphate buffer was avoided because of its inhibiting effect on the enzyme [20].

Intact cell 5′-nucleotidase activity was determined as described by Carson *et al.* [1] using [U-^{14}C] dAMP (Radiochemical Centre) as the substrate. The reaction was followed by paper chromatography of 20 μl aliquots developed in ethanol: 1 M ammonium formate (5:2).

RESULTS

Toxicity of nucleosides in human cell lines and relationship to adenosine deaminase activities

Cell survival was determined by a modification of the clonogenic survival assay involving labelling colonies with ^{3}H-thymidine 6 days after a continuous drug treatment. This assay gives dose–response survival curves similar to visual counting of colonies [18] for DNA-damaging agents; and was confirmed in the present work for dAdo and dIno. The human melanoma cell lines MM96L and MM127 previously identified as being sensitive to Ado [15] were found to be susceptible also to killing by dAdo compared with the MM253c1a line (Fig. 1). The dAdo-sensitive CCRF-CEM T cell line (Fig. 1B) exhibited a survival curve similar to those of MM96L and MM127 whereas a B cell line (CW) resembled the dAdo-resistant MM253c1a. Compared on a concentration basis, dAdo was 5–10-fold more toxic than Ado in MM96L and MM127 but only twice as toxic as Ado in MM253c1a. This was also the case for other resistant cells including 5 other melanoma lines, HeLa cells and a fibroblast strain (Fig. 1 and Table 1).

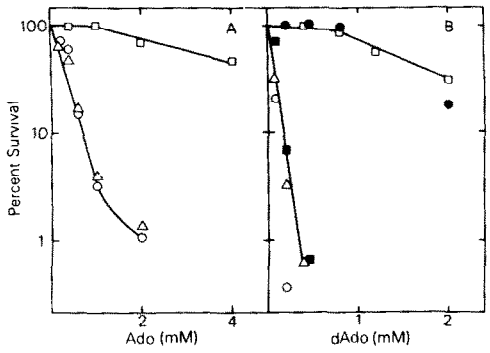

Fig. 1. Survival curves of human cell lines treated with Ado and dAdo. (A) Ado: ○, MM96L; △, MM127; □, MM253c1a. (B) dAdo: ○, MM96L; △, MM127; □, MM253c1a; ■, CCRF-CEM; ●, CW. Points are means of duplicates.

The ability of cell lysates to deaminate Ado and dAdo to inosine and dIno respectively was determined as a possible reason for the range of nucleoside sensitivities found. The results showed that allowing for differences in cell volume, the deaminase activities in MM96L and MM127 were similar to those in relatively resistant cells such as MM253c1a, HeLa and PGP (Table 1).

The variety of possible dAdo metabolites and proposed mechanisms of toxicity prompted a survey of the toxicity of a wide range of related compounds. Of the known metabolites, including deoxyribose-1-phosphate formed by phosphorylase cleavage of adenine from the sugar moiety, only dIno showed

Table 1. Comparison of cell survival and deaminase activities in cultured human cells

Cell	Cell volume (μm^3)	D_{37} (mM)*		Deaminase (nmole/min/10^6 cells)	
		Ado	dAdo	Ado	dAdo
Melanoma					
MM96L	1360	0.45	0.048	0.77	0.45
MM127	2100	0.25	0.068	0.63	0.39
MM138	1360	1.7	0.81	0.95	0.62
MM170	6500	3.8	2.5	2.4	1.5
MM418	5100	1.6	0.96	3.2	2.1
MM253c1a	6600	3.1	2.0	4.7	2.8
MM253-3D	6800	3.4	1.7	4.8	2.6
Other					
HeLa-S_3	1300	2.1	1.12	1.1	0.6
PGP fibroblasts	6400	1.3	0.44	1.1	0.62

* Dose required to reduce survival to 37%.

Table 2. Comparison of the toxicity of dAdo metabolites and inhibitors in human melanoma cells

Drug	D_{37} (mM)		
	MM96L	MM127	MM253c1a
dAdo	0.048	0.068	2.0
Ado	0.45	0.25	3.1
Adenine	1.7	0.59	1.33
Hypoxanthine	2.6	2.2	2.2
dIno	0.14	0.084	3.7
D-Deoxyribose-1-phosphate	1.1	1.0	0.9
$AMP(CH_2)P$	>0.5	>0.5	>0.5
Inosine	4.1	5.0	6.1
Deoxyguanosine	0.040	0.048	0.064
Homocysteine	2.7	0.64	3.0
S-Adenosylhomocysteine	>0.5	>0.5	>0.5
5-Azacytidine*	2.5	4.0	4.4
Hydroxyurea	0.62	0.45	0.18
3-Aminobenzamide	6.0	4.9	12
Dipyridamole*	0.12	0.22	0.30

* μM

selectivity for MM96L and MM127 (Table 2 and Fig. 2A). The 5′-nucleotidase inhibitor $AMP(CH_2)P$ had little toxicity in any of the lines tested. dIno, which was also highly selective against CCRF-CEM cells (Fig. 2B), was almost as toxic as dAdo itself.

The possibility that dAdo toxicity resulted from inhibition of DNA methylation by feed-back inhibition of *S*-adenosylhomocysteine hydrolase by *S*-deoxyadenosylhomocysteine [13] was tested in two ways. Firstly, exogenous homocysteine or *S*-adenosylhomocysteine had little selectivity for MM96L compared with the resistant MM253c1a line (Table 2). Secondly, the DNA methylation inhibitor 5-azacytidine was also found to have similar toxicity in both cell lines.

It is known that dAdo is phosphorylated intracellularly to dATP which inhibits ribonucleotide reductase and thus lowers the dCTP and dGTP pool sizes [5, 21]. However, neither hydroxyurea, which inhibits ribonucleotide reductase [22], nor 3-aminobenzamide, which lowers deoxynucleotide pool sizes [23] had any significant selectivity for the dAdo-sensitive cell lines. Dipyramidole inhibits both influx and efflux of nucleosides in mammalian cells [24] and may therefore decrease or increase [25] the toxicity of dAdo depending upon the particular system. Used either alone or in combination with dAdo, this agent exerted a similar toxicity in the three cell lines studied.

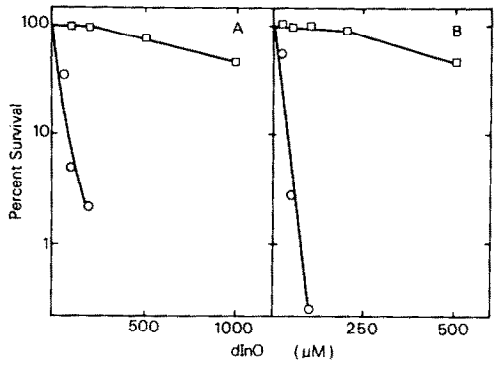

Fig. 2. Survival curves of cells treated with dIno. (A) ○, MM96L; □, MM253c1a. (B) ○, CCRF-CEM; □, CW. Points are means of duplicates.

Modification of dAdo toxicity

With the exception of a small protective effect of uridine and deoxycytidine (Table 3), none of the compounds listed in Table 2 affected dAdo toxicity when administered 1 hr beforehand and left on the cells for the treatment period (results not shown). As previously found in other mammalian cells [5], the combination of three deoxynucleosides prevented dAdo toxicity in MM96L and MM253c1a cells; the three ribonucleosides had no effect (Table 3).

The cross-sensitivity of cells to Ado and dAdo, the possibility of Ado acting as a hormone [26] and the neural crest origin of melanoma cells prompted the use of agents such as caffeine and adrenalin known to modify the biological response to Ado [26]. Neither of these compounds had any effect on dAdo toxicity (Table 3).

Temporal responses of dAdo toxicity

The temporal dependence of Ado toxicity was studied using single, approximately equitoxic doses of dAdo for MM96L and MM253c1a. In the first set of experiments the drug was removed at various times after initiating treatment. The results showed that MM96L cells required only brief (2–4 hr) exposure to dAdo to obtain maximum kill whereas MM253c1 cells required > 24 hr treatment (Fig. 3A). A second set of experiments addressed the question of whether toxicity was due to dAdo itself or to a metabolite resulting from deamination and possibly further metabolism by enzymes either in the foetal calf serum or in the cells. The medium was removed from treated cultures at various times and placed on fresh cells for determination of survival. During the

Table 3. Modification of dAdo toxicity

Drug	Concentration*	Cell survival (% control) MM96L plus 0.2 mM dAdo	MM253c1a plus 4 mM dAdo
Control		8.5 ± 1.8†	7.6 ± 2.2
Deoxycytidine	200 μM	16 ± 2.1	12 ± 2.3
Deoxycytidine + thymidine + deoxyguanosine	20 μM each	46 ± 8	81 ± 5
Uridine	200 μM	12 ± 2.2	10 ± 1.5
Uridine + cytidine + guanosine	200 μM each	11 ± 3.1	11 ± 2.2
Caffeine	1 mM	7.1 ± 1.2	5.9 ± 1.8
Adrenalin	15 μM	7.5 ± 2.3	8.1 ± 2.6

* Cell survival using the drug alone was >90%.
† Mean and SD of duplicates.

24-hr period studied, toxicity slowly declined to a level similar to that expected for an equivalent concentration of dIno (Fig. 3B).

5′-Nucleotidase activity

Three melanoma lines were assayed as intact cells in isotonic buffer for the presence of membrane 5′-nucleotidase using ^{14}C-dAMP as the substrate. The MM96L and MM127 lines had very low activity compared with MM253c1a (Table 4); the specificity of the assay was confirmed using the 5′-nucleotidase inhibitor AMP(CH_2)P. CCRF-CEM cells used as a control had little activity, as expected from previous reports [1, 4, 8].

Effect of dAdo on RNA and DNA synthesis

Cells were labelled with ^{3}H-dAdo to determine whether transport was inhibited in the resistant MM253c1a line and, because most of the label is incorporated into RNA, to determine the effect of dAdo on RNA synthesis. The dose response for a 1 hr treatment showed little inhibition of RNA synthesis in MM96L cells until supratoxic doses were used (Fig. 4A); the effect in MM253c1a cells was even less. Both cell lines incorporated the same amount of label in controls (high specific activity ^{3}H-dAdo) and in dAdo-treated cells (low specific activity) suggesting that uptake and incorporation of label is rapid and does not reach saturation.

DNA synthesis, as judged by incorporation of ^{14}C-thymidine, was inhibited to 30% of controls in MM96L by dAdo doses of minimal toxicity (Fig. 4A), but did not fall below 20% even at the highest levels used. This plateau effect was also shown by MM253c1a except that the degree of inhibition was much less. ^{3}H-Deoxycytidine, like thymidine, is incorporated into DNA in these cells [27] and following dAdo treatment gave a similar pattern of incorporation into MM96L cells as thymidine (results not shown). Incorporation of ^{3}H-deoxycytidine by MM253c1a was inhibited more than that of ^{3}H-thymidine.

The temporal response of nucleoside incorporation was determined following a 1 hr treatment with 0.4 mM dAdo (Fig. 4B). RNA synthesis decreased to 60% of controls by 4 hr in MM253c1a, with some recovery after 24 hr, whereas MM96L cells showed little change. DNA synthesis, on the other hand, was inhibited immediately in MM96L and recovered after 4 hr. In MM253c1a cells, DNA synthesis followed the pattern of RNA synthesis.

Dose and time responses of RNA synthesis to dAdo treatment were also determined using [2-^{14}C] uridine and [8-^{3}H]guanosine; the results (not shown) were similar to those obtained using ^{3}H-dAdo.

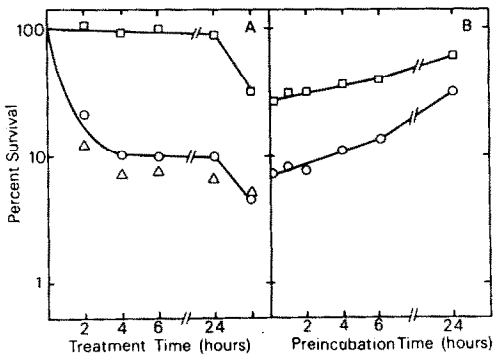

Fig. 3. Temporal relationships between dAdo toxicity, treatment time, and preincubation in culture medium. (A) Cell treatment time: ○, MM96L (0.2 mM dAdo); △, MM127 (0.2 mM dAdo); □, MM253c1a (2 mM dAdo). (B) dAdo preincubated for various times in culture medium and then used for continuous treatment of cells: ○, MM96L (0.2 mM dAdo); □, MM253c1a (2 mM dAdo). Points are means of duplicates.

Table 4. 5′-Nucleotidase activity in human cell lines

Cell line	5′-Nucleotidase activity* Control	Plus 2.5 mM AMP(CH_2)P
CCRF-CEM	2.5 ± 0.3†	2.7 ± 0.4
MM96L	4.2 ± 0.5	4.9 ± 0.4
MM127	5.4 ± 0.4	5.1 ± 0.3
MM253c1a	151 ± 9	22 ± 3

* pmol of substrate ([U-^{14}C]-dAMP) hydrolysed per min per 10^6 cells.
† Mean and S.D. of duplicates.

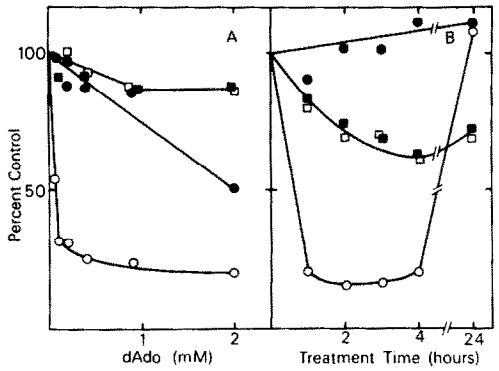

Fig. 4. Effect of dAdo on DNA and RNA synthesis. (A) Dose response of cells treated with dAdo for 1 hr and then labelled with nucleoside for 1 hr. Incorporation by controls shown in parentheses. DNA synthesis: ○, incorporation of ^{14}C-thymidine by MM96L (970 dpm); □, incorporation of ^{14}C-thymidine by MM253c1a (2950 dpm). RNA synthesis: ●, incorporation of ^{3}H-deoxyadenosine by MM96L (1130 dpm); ■, incorporation of ^{3}H-deoxyadenosine by MM253c1a (3400 dpm). (B) Temporal response of cells treated with 0.4 mM dAdo for various times and then labelled with nucleoside for 1 hr. DNA synthesis (incorporation of ^{14}C-thymidine): ○, MM96L; □, MM253c1a. RNA synthesis (incorporation of ^{3}H-deoxyadenosine): ●, MM96L; ■, MM253c1a. Points are means of triplicates. All S.D. < 10%.

Effect of dAdo on the cell cycle

Cells were treated with various doses of dAdo for one doubling time (24 hr) to determine the cell cycle specificity of growth arrest. The results (Table 5) showed that toxic doses depleted the proportion of cells in S and G2 with consequent accumulation in G1. However, even at doses causing overt fragmentation and treament times of 48 hr (results not shown), a substantial proportion of cells was found in the S and G2 phases.

DISCUSSION

This study identified for the first time nonlymphoid human cells having exceptional sensitivity to killing by dAdo. Since only 10% of the human melanoma cell lines studied so far in this laboratory are dAdo-sensitive, and no immunodeficiencies were reported for the donors [17], it must be assumed that sensitivity was not genetically determined but arose either *in vivo* during neoplastic development or *in vitro* by selection. Concerning the latter possibility, it has been pointed out that culture medium usually contains no added nucleosides and may therefore select cells capable of *de novo* nucleotide synthesis at the expense of cells dependent on salvage pathways [11]. This seems unlikely to be the case for the present cell lines for several reasons. Firstly, the similar incorporation of ^{3}H-dAdo by untreated sensitive and resistant cells suggests that the salvage pathway is active in each. Secondly, the action of dAdo closely resembles that in the T-cell line CCRF-CEM, and enzyme-deficient T cells are susceptible to dAdo *in vivo*. In addition, it is equally possible that an active system for *de novo* synthesis confers a selective advantage in tumour cells *in vivo* where the nutrient supply may be depleted.

Table 5. Effect of 24 hr dAdo treatment of progression through the cell cycle

	MM96L			MM253c1a		
dAdo (mM)	G1	S	G2	G1	S	G2
0	66*	12	27	67	12	21
0.4	70	12	18	—	—	—
0.8	71	10	19	—	—	—
1.2	79	7	14	—	—	—
2	83†	3	14	—	—	—
4	—	—	—	87	7	6
8	—	—	—	88	7	5

* Percent of total cells.

† 37% of total fluorescence attributed to cell fragments (DNA content <50% of G1).

An extensive series of cell survival studies was carried out in order to eliminate toxicity mechanisms involving inhibition of transmethylation [13], uridine starvation [9, 10], accumulation in other purine pools [28, 29] and hormone-like messenger effects [26]. The prevention of toxicity by adding the other three deoxynucleosides, rapid inhibition of DNA synthesis but not RNA synthesis [30–32] and accumulation of cells in G1/S strongly suggest that MM96L cells, like T cells, convert dAdo to dATP which being in large excess, inhibits ribonucleotide reductase. The small protective effect of deoxycytidine is consistent with dAdo being phosphorylated by deoxycytidine kinase. Inhibition of DNA synthesis *per se* did not appear to be the prime cause of toxicity, however, because ^{3}H-thymidine incorporation recovered during the first 24 hr following a toxic treatment and a brief treatment with dAdo was toxic to the majority of cells. In addition, DNA synthesis could not be completely suppressed, and the G1/S block was not complete. Inhibition of ribonucleotide reductase by dAdo was by no means complete in L1210 cells [25]. The reason for the cell cycle independent toxicity of dAdo and dAdo analogues [33, 34] therefore remains unclear.

Few studies have addressed the possibility of dIno being toxic [5, 6, 35]. dIno can be phosphorylated to dIMP [1] but no accumulation of dITP was found in nucleoside phosphorylase-deficient lymphocytes treated with dIno [14]. There is little evidence to suggest that dITP would be formed enzymatically and it seems more likely that dIMP is converted to dAMP [8] and thence to dATP; this pathway would then explain the equivalent toxicities of dIno and dAdo. The possibility of dIno being active by conversion to hypoxanthine [36] or inosine [29] was ruled out in this study. The somewhat lower degree of cross-sensitivity to Ado found also with T cells [35] may result from conversion to dAdo. However, there is evidence that Ado and dAdo act by different mechanisms in human lymphocytes [37]; and exogenous dAdo did not alter the adenine ribonucleotide pool in Novikoff hepatoma cells [38].

The high sensitivity to dAdo shown by CCRF-CEM, MM96L and MM127 cells compared with other cell lines is best explained by accumulation of

nucleotides due to 5′-nucleotidase deficiency rather than other enzymes involved in purine metabolism [2, 26]. Deficiency in purine nucleoside phosphorylase [1, 6] was unlikely because no cross-sensitivity to deoxyguanosine was found. Adenosine deaminase activity was found in all cells studied, and dAdo-sensitive cells were also sensitive to dIno, the deamination product of dAdo. Alterations in kinase activity have not previously correlated with the observed sensitivity to deoxyadenosine [5].

Several aspects of this study are relevant to designing therapeutic approaches applicable to the minority of human tumours which may be dAdo-sensitive. First, it may be feasible to identify such tumours by assay of 5′-nucleotidase and related enzymes. Second, a single brief treatment may enhance the inherent therapeutic index. Third, plasma levels of the other deoxynucleosides (approximately 1 μM) [39] would appear from the present work to be too low to prevent tumour toxicity. This contrasts to *in vitro* studies of dAdo combined with deaminase inhibitors where 1 μM deoxycytidine alone was sufficient to prevent toxicity [12].

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