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# *In vitro* Activity of 2-Chlorodeoxyadenosine (CdA) in Primary Cultures of Human Haematological and Solid Tumours

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**2-Chlorodeoxyadenosine (CdA) is a deaminase-resistant purine analogue which has shown clinical activity against various haematological tumours, and is currently undergoing phase II trials. In the present study, the semiautomated fluorometric microculture cytotoxicity assay (FMCA) was used for *in vitro* evaluation of CdA activity in cell suspensions from both haematological and solid tumours. A total of 133 samples from various diagnoses were successfully tested with continuous drug exposure. CdA showed high *in vitro* activity against samples from chronic and acute lymphocytic leukaemia and acute myelocytic leukaemia, but little or no response was observed in the solid tumour groups. Cross-resistance analysis with standard drugs revealed the following rank order of correlation coefficients: cytosine arabinoside (AraC) > daunorubicin > doxorubicin > vincristine > prednisolone > 4-hydroperoxycyclophosphamide > etoposide > cisplatin. The high correlation between CdA and AraC was maintained even if the analysis was based only on the haematological tumours. The results indicate that CdA is differentially active against haematological tumours with little or no activity against solid tumours. CdA also appears highly cross resistant with AraC. If this disease-specific information is substantiated in further clinical trials and extended to other phase I–II drugs, non-clonogenic drug resistance assays such as the FMCA may become useful in new drug evaluation, and in targeting specific diagnoses and patients for phase II trials.**

**Key words: 2-chlorodeoxyadenosine, drug resistance, drug screening**
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## INTRODUCTION

2-CHLORODEOXYADENOSINE (CdA) IS a novel adenosine deaminase-resistant purine analogue of deoxyadenosine [1, 2]. It has recently emerged as the drug of choice for the treatment of hairy cell leukaemia [3–5], and has promising activity in chronic lymphocytic leukaemia [6, 7] and low grade non-Hodgkin's lymphoma [8]. CdA is taken up by cells and converted by deoxycytidine kinase (dCK) to monophosphate. The triphosphate accumulates in cells with high dCK levels and low dephosphorylating activity of 5'-nucleotidase (5NT) [2]. The mechanism by which the phosphorylated congeners of CdA exert their cytotoxic effects is not fully understood, although apoptosis and/or alteration of DNA repair, secondary to pertuberation of the deoxynucleotide pool with subsequent NAD depletion, have been discussed [2, 9, 10].

Because of the high levels of dCK and low levels of 5NT observed in lymphocytes, CdA was originally conceived to be relatively lymphocyte-specific [1, 2]. However, recent studies have shown that CdA also inhibits growth of myeloid progenitors [11], and the drug also appears active in acute myelocytic leukaemia [12]. The major toxicity encountered in the clinical situation is in fact myelosuppression [8, 12]. This may indicate

a broader spectrum of activity than would otherwise be predicted from dCK levels in different tumours, and other potential non-lymphocytic targets for CdA therapy should therefore be considered. In the present study, we investigated the effect of CdA on fresh tumour cells from both haemtological and solid tumours, and compared the effect with that of some standard drugs.

## MATERIALS AND METHODS

*Tumour samples*

A total of 133 successfully analysed tumour cell samples were obtained from patients with acute lymphocytic leukaemia (ALL), acute myelocytic leukaemia (AML), chronic lymphocytic leukaemic (CLL) and solid tumours undergoing routine surgery, diagnostic biopsy or bone marrow/peripheral blood sampling. Tumour tissue from solid tumours was minced to 1 $mm^3$ size, and tumour cells were isolated by collagenase dispersion and Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation as described previously [13]. Leukaemic cells were obtained from bone marrow or peripheral blood by 1.077 g/ml Ficoll-Isopaque (Pharmacia) density gradient centrifugation [14]. Normal mononuclear cells from healthy donors were used in some experiments, and these cells were collected and processed in the same way as the leukaemic samples. Viability was determined by the trypan blue exclusion test, and the proportion of tumour cells was judged by inspection of May-Grünwald-Giemsa stained cytocentrifugate preparations by a trained cytopathologist. Culture medium RPMI 1640 (Flow, Herts, U.K.) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin was used throughout. Cells were cryopreserved in culture medium containing 10% dimethylsulphoxide (DMSO; Sigma, St Louis, Missouri, U.S.A.) and 50% FCS by initial freezing for 24 h at −70°C, followed by storage in liquid nitrogen. Both fresh and cryopreserved samples were used in this study.

*Reagents and drugs*

Fluorescein diacetate (FDA; Sigma) was dissolved in DMSO and kept frozen (−20°C) as a stock solution (10 mg/ml) protected from light. CdA was obtained from Dr Zygmunt Kazimierczuk, The Foundation for Diagnostics and Therapy, Warzaw, Poland. Vincristine (Vcr), cytosine arabinoside (AraC), prednisolone (Pred), etoposide (VP16), cisplatin (Cisp), daunorubicin (Dnr) and doxorubicin (Dox) were obtained from commercial sources. 4-Hydroperoxycyclophosphamide (4HC), an active metabolite of cyclophosphamide, was a kind gift from Asta Pharma. CdA was generally tested at five concentrations, whereas the drugs for comparison were tested at empirically derived cut-off concentrations (EDCC) as previously described [14]. Experimental plates were prepared with 20 μl/well of drug solution at 10 times the desired final concentration with the aid of a programmable pipetting robot (PROPETTE; Perkin Elmer, Norwalk, Connecticut, U.S.A.) as described [14]. The plates were stored frozen at −70°C for further use. Drug stability during storage conditions was estimated by repeated testing of sensitive cell lines (unpublished data). CdA could be stored in this way for at least 1–2 months. The experiments were performed with continuous drug exposure. No apparent difference between fresh and cryopreserved samples was observed.

*Equipment*

The 96-well scanning fluorometer (Fluoroscan 2; Flow) is equipped with a xenon lamp and broadband interference filters exciting fluorescence at 485 nm for FDA. The emitted light from a vertical light path of each well was sequentially read at 538 nm. One plate was read in approximately 1 min. In most experiments, cells, media and drugs were added to the wells by the pipetting robot, PROPETTE. Addition of buffer and fluorochrome was performed with the aid of an automated 96 well dispenser, Multidrop (Flow).

*Cytotoxicity assay procedure*

The principal steps of the fluorometric microculture cytotoxicity assay (FMCA) procedure have been described previously [13, 14]. On day 1 180 μl of the tumour cell preparation (0.5–5 × $10^5$ cells/ml culture medium) were seeded into the wells of V-shaped 96-well experimental microtitre plates (Nunc, Roskilde, Denmark) prepared as described above. Six blank wells received only culture medium, and six wells with cells but without drugs served as controls. The culture plates were then incubated at 37°C in humidified atmosphere containing 95% air and 5% $CO_2$. At the end of the incubation period (72 h), the plates were centrifuged (200 *g*, 7 min) and the medium removed by flicking the plate. After one wash with phosphate buffered saline (PBS), 200 μl of PBS containing FDA (10 μg/ml) was added to all wells. Subsequently, the plates were incubated for 1 h before reading the fluorescence in the Fluoroscan 2. The fluorometer was blanked against wells containg PBS including the dye but without cells. Quality criteria for a successful assay included a fluorescence signal in control cultures of >5 × mean blank values, mean coefficient of variation (CV) in control cultures of < 30% and > 70% of tumour cells prior to incubation. The success rate was > 80% and the most common cause of assay failure was a low fluorescence signal in controls and/or too low a proportion of tumour cells.

*Quantification of FMCA results*

The results obtained by the indicator FDA are presented as survival index (SI) defined as fluorescence of experimental as a percentage of control cultures with blank values subtracted. A ≥ 50% decrease in SI was arbitrarily defined as *in vitro* response.

*Statistical analysis*

SI values at different concentrations for the various diagnoses were compared using the Student's *t*-test. Pearson's correlation coefficient was calculated to compare the activity of CdA with standard chemotherapeutic drugs.

## RESULTS

A total 133 samples were successfully analysed and the histological types of these samples are summarised in Table 1. The effect of CdA on SI for ALL, AML, CLL and solid tumours is shown in Figure 1. A concentration-dependent decrease in SI was observed for normal mononuclear cells and for all the haematological tumour types whereas solid tumours were unre-

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*Table 1. Tumour types successfully* analysed for CdA activity*

| Tumour type | Number |
|---|---|
| CLL | 17 |
| ALL | 17 |
| AML | 34 |
| Renal | 8 |
| ACC | 5 |
| Sarcoma | 11 |
| Ovarian | 15 |
| Lung† | 10 |
| Breast | 5 |
| Other‡ | 11 |
| Total | 133 |

*Successful analysis = signal/noise > 5, coefficient of variation in control cultures < 30% and a proportion of tumour cells > 70%. Overall success rate = 85%. ALL, acute lymphocytic leukaemia; AML, acute myelocytic leukaemia; CLL, chronic lymphocytic leukaemia; ACC, adrenocortical carcinoma. †This group consists of five small cell lung cancer and five non-small cell lung cancer samples. ‡Other tumours include: cancer of the thyroid (2), Wilm's tumour (1), schwannoma (1), carcinoid (1), oesophageal cancer (1), melanoma (1), cancer of the cervix (1), mesothelioma (1), bladder cancer (1), unknown primary (1).

sponsive. The dose response curve was most left-shifted for the normal mononuclear cell samples, closely followed by the CLL group. The differences between the solid tumour group and the haematological tumours were highly significant ($P < 0.001$) at all concentrations tested.

When the frequency of samples showing a ≥ 50% decrease in SI was determined, a similar pattern was apparent (Figure 2). Haematological tumours showed a high and increasing frequency of responders with increasing concentrations of CdA, again with a tendency for CLL to be the most sensitive tumour type. At the midconcentration tested (0.2 μg/ml), the 95% confidence limits for the *in vitro* response rate (%) were 56–96, 49–93, 52–84 and 0–5% for CLL, ALL, AML and solid tumours, respectively. At the highest concentration of CdA, four solid tumour samples showed ≥ 50% decrease in SI (1/15 ovarian, 1/11 sarcoma, 1/5 non-small cell lung cancer, 1/1 bladder cancer).

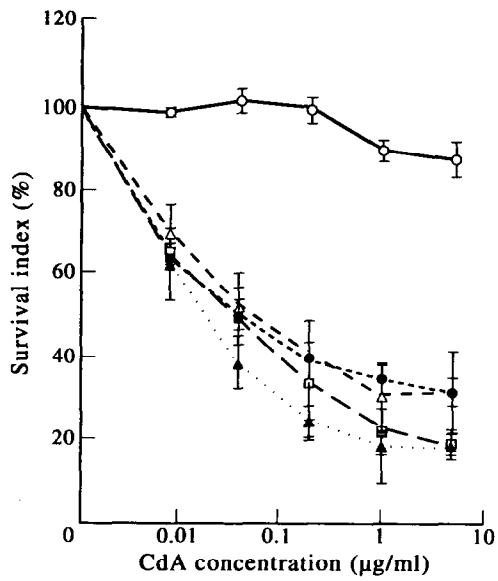


**Figure 1. Effect of increasing concentrations of CdA on SI in normal mononuclear cells (▲ $n = 5$), CLL (□ $n = 17$), ALL (△ $n = 17$), AML (● $n = 34$) and various solid tumours (○ $n = 52$). The results are expressed as SI values and presented as mean values + S.E.**

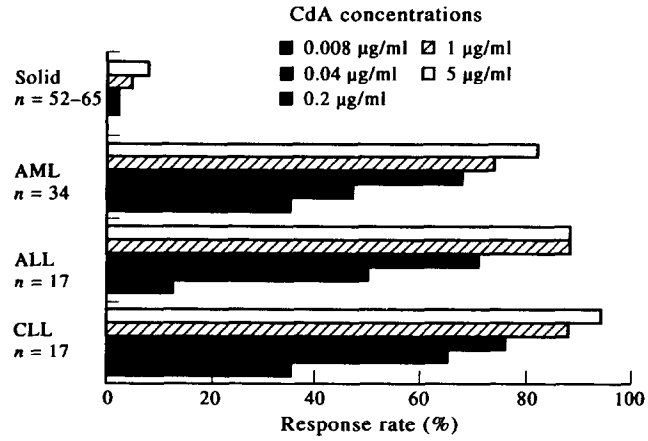


**Figure 2. Effect of indicated concentrations of CdA on the percentage of samples showing a > 50% decrease in SI (*in vitro* response rate) for CLL, ALL, AML and solid tumour samples. The numbers of samples are indicated.**

The relationships between SI values at EDCC for CdA and standard drugs are shown in Figure 3. SI values for CdA and AraC were strongly correlated (a; $r = 0.83$) whereas a much weaker relationship was observed between CdA and Cisp (b; $r = 0.35$). The high correlation between CdA and AraC was maintained even if the CdA-resistant solid tumours were excluded from the analysis (Figure 3c). Relatively high correlations for all tumours were also obtained between CdA and the anthracyclines ($r = 0.62$–$0.72$), whereas Vcr and CdA showed intermediate correlation ($r = 0.51$). Pred, 4HC and VP16 showed generally lower correlation coefficients ($r < 0.5$). When the analysis was based only on haematological tumours, the correlations for most drugs became weaker to a variable extent, most evident for Pred (Figure 3c). In contrast, the correlation coefficient of VP16 became higher when the solid tumour samples were excluded.

Information on previous clinical treatment status was available for 47 of the haematological samples. Although the non-treated group ($n = 15$) showed lower mean SI values compared to the previously treated group ($n = 31$–$32$) at 0.04 and 0.2 μg/ml CdA, these differences were not statistically significant (not shown). Solid tumours were essentially non-responsive, irrespective of previous treatment status.

## DISCUSSION

The clinical approach for identification of cancer drug efficacy in different tumour types has, during the past decade, been the empiric phase II trial [15]. Such phase II trials have a number of problems: a limited number of tumour types are tested, the process is expensive, it takes many years to complete, and patients incur significant morbidity with low probability of benefit. Given the poor outcome for many patients in phase II clinical trials [15–19], there is obviously a need for laboratory tests that could provide accurate information on the probability of response in different tumour types, and which could assist in directing the efforts of phase II trials.

Recently, some preliminary evidence was published suggest-

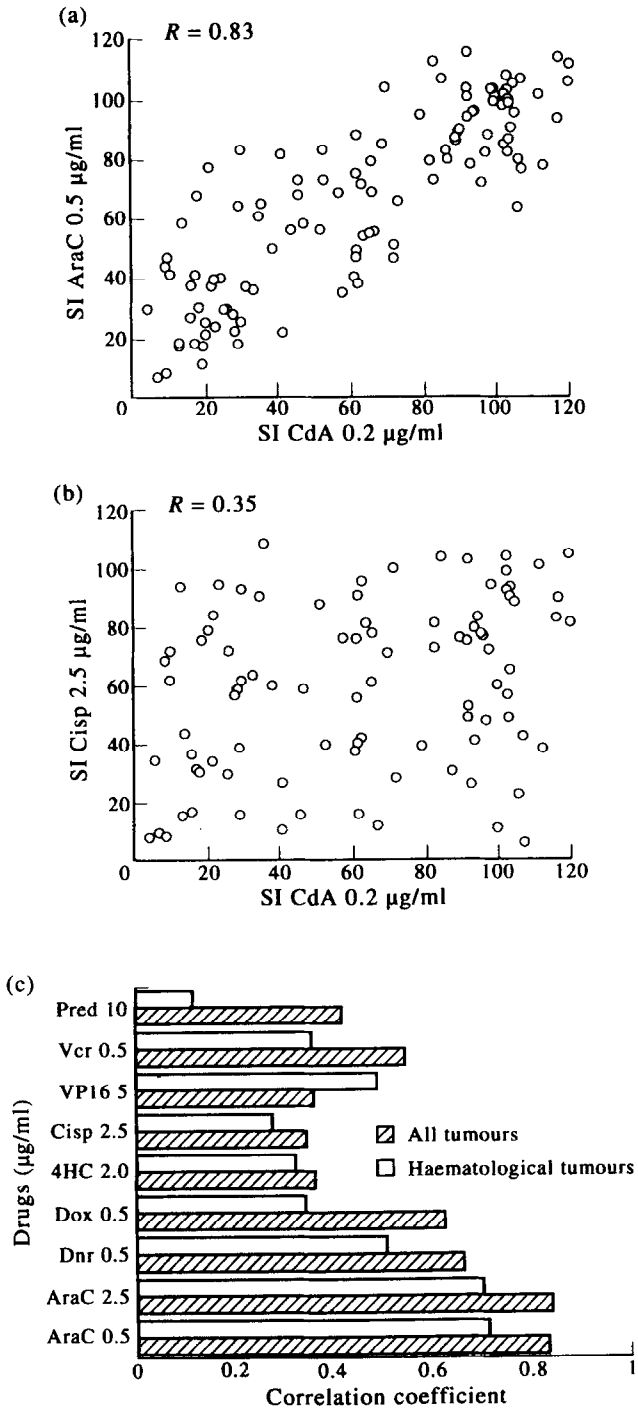


**Figure 3. Relationship between SI values for CdA and AraC (a), and CdA and Cisp (b) at indicated concentrations for samples where parallel data were available ($R$ = 0.83, $n$ = 121 in a, $R$ = 0.35, $n$ = 113 in b). In c, the correlations between CdA and some standard drugs are shown for all tumour types (hatched bars, $n$ = 107–123) and for haematological tumours only (unfilled bars, $n$ = 50–65).**

ing that disease-specific activity could be accurately detected in accordance with clinical experience by non-clonogenic drug resistance assays based on measurement of cell kill in the whole tumour cell population [20, 21]. Corrobating these findings, we have recently found that the FMCA can detect disease-specific drug activity for a series of standard drugs in a retrospective study [22, 23]. The present study was undertaken to prospectively evaluate the ability of the FMCA to identify disease-specific activity for a panel of early phase I–II drugs of which CdA was the first to be completed.

The present results demonstrate that CdA show *in vitro* activity against haematological tumours, including AML, compatible with recent clinical experience [7, 12]. In the phase II study on AML showing complete remissions in 8 out of 17 pediatric patients [12], 7 ALL patients were also included. Although oncolytic responses were observed in the majority of these patients, only 1/7 achieved remission. When considering these data, it is interesting to note the lower *in vitro* response rate obtained for ALL compared to AML and CLL samples at clinically achievable steady state concentrations (0.008 µg/ml, see below). However, clinical experience of CdA in ALL is still very limited, and the true clinical activity remains to be established. In addition, for AML, the clinical data on CdA efficacy need to be confirmed in adult patient populations.

The present *in vitro* results are also in accordance with a recent study using the differential staining cytotoxicity (DISC) assay in haematological specimens [24]. In addition, the present study showed a marked difference between haematological and solid tumours with respect to CdA sensitivity. The solid tumours showed an extremely low *in vitro* response rate at all concentrations tested. One should note that clinically achievable steady-state concentrations corresponds roughly to the lowest concentration tested (0.008 µg/ml; 30 nM) and 0.04 µg/ml (150 nM) corresponds to the achievable peak concentration [25]. This is in contrast to a previous study using a clonogenic assay, where CdA was reported active against some solid tumours with 40% of samples showing a > 50% decrease in colony formation at 1 µg/ml [26]. In the present study, only 4% of the solid tumour samples showed a > 50% decrease in SI at 1 µg/ml. An explanation for the apparent discrepancy may be related to the different endpoints used, inhibition of cell proliferation versus cell kill, whereas the FMCA provides a more 'robust' endpoint, in that cell kill rather than inhibition of cell proliferation is a prerequisite for successful treatment. The lack of haematological specimens in the study of colony formation [26] also precludes the possibility of a comparison with clinically sensitive tumours even if lower concentrations should be used.

Little is known about the activity of CdA in solid tumours. However, preliminary results from an ongoing phase I dose-finding study of CdA activity in patients with solid non-haematological and non-lymphoreticular tumours was recently reported [27]. In this study, no antitumour activity was observed for the first 14 patients tested. The completion of this and other phase I–II studies in solid tumours will allow evaluation of the accuracy of the present *in vitro* predictions.

When cross-resistance patterns with some standard drugs were analysed we found the highest correlation with AraC. This is not surprising since both CdA and AraC are believed to be activated through phosphorylation to its monophophates by the same key enzyme [2]. However, clinically, cross-resistance does not always occur between agents sharing a common metabolic pathway [26]. Furthermore, in a recent report utilising the non-clonogenic DISC assay in haematological tumours, CdA was reported to show little cross-resistance with AraC estimated by the correlation coefficients obtained [24]. In contrast, a high correlation was found for the alkylating drug nitrogen mustard. The reason for this discrepancy is not entirely clear. However, in the latter study, only 24 observations was used for the cross-resistance analysis. One prerequisite for meaningful correlations is that both agents are used at concentrations sufficient to produce a significant scatter of survival indices. In the present

study SI, median values and percentile distributions for AraC and CdA were comparable at concentrations where high correlation coefficients were obtained. The ability of CdA to kill non-dividing cells is also shared by AraC when used in primary cultures of haematological tumours, including those with very low growth fraction [21]. In addition, the overall *in vitro* activity spectrum for CdA is very similar to that observed for AraC (not shown), indicating a similar mode of action.

Relatively high correlations were also obtained between CdA and the anthracyclines whereas Vcr showed intermediate correlations and 4HC, Pred and VP16 lower correlation coefficients. When the analysis was based only on haematological tumours, the correlation for most drugs became weaker. For at least some of the drugs, this is possibly due to the removal of parallel (but mechanistically unrelated) resistance of the solid tumours, most apparent in the case of Pred which has no *in vitro* activity in solid tumour samples. On the other hand, the correlation coefficient for VP16 was increased when the analysis was based only on the haematological tumours. Since VP16 but not CdA is active *in vitro* in solid tumours, a stronger relationship between these drugs in haematological tumours was probably masked by the inclusion of the solid tumours in the analysis. The cross-resistance analysis may have implications when combination therapy with CdA is designed. Indeed, in 31 samples from patients with AML, a higher frequency of additive and synergistic interactions between CdA and AraC or daunorubicin was obtained than between CdA and VP16 or amsacrine (not shown). The fact that CdA also appears active in samples from patients previously treated with chemotherapy, makes the evalution of the drug in the second line setting an important task. In this context, it is also of interest to note that at least CdA treated CLL patients appear to retain their CdA sensitivity at relapse [28].

In summary, the results indicate that CdA is differentially active against haematological and solid tumours, and the drug appears to be cross-resistant with AraC but not with alkylating drugs. If this disease-specific activity is substantiated in clinical trials and extended to other phase I–II drugs, non-clonogenic assays like FMCA may be useful in new drug evaluation and in targeting specific diagnoses and patients for initial phase II trials.


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