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Short communication

Differential cytotoxicity of Combretastatins A1 and A4 in two daunorubicin-resistant P388 cell lines

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Summary. Combretastatin A4, a novel anti-mitotic agent was effective against two P388 cell lines with acquired resistance to daunorubicin. In contrast, Combretastatin A1, a close structural analogue of A4, showed a high degree of cross-resistance. Combretastatin A1 was also more efficient at increasing intracellular daunorubicin concentrations in both resistant cell lines. Neither agent was capable of altering anthracycline accumulation in the parental (sensitive) cell line. We propose that the cross-resistance to Combretastatin A1 occurs, at least in part, as a result of the increased affinity of the drug-efflux process operative in these resistant cells for Combretastatin A1 vs Combretastatin A4. Hence, Combretastatin A4 may play a role in the treatment of tumours with acquired resistance to the anthracycline antibiotics.

Introduction

Combretastatin A4, a natural product isolated from Combretum caffrum [7], is presently being investigated under the sponsorship of the Cancer Research Campaign Clinical Trials scheme. This agent has been shown to be a potent inhibitor of tubulin assembly [5]. A close structural analogue, Combretastatin A1, has also been shown to alter tubulin dynamics [6]. This mechanism of action is common to the clinically useful vinca alkaloid class of anti-cancer agents. These agents are known to belong to the group of compounds that show extensive cross-resistance in multi-drug-resistant (MDR) cell lines.

This report describes the effect of Combretastatins A1 and A4 on two cell lines with acquired resistance to daunorubicin that exhibit typical MDR characteristics. A role for Combretastatin A4 in the treatment of drug-resistant tumours is proposed.

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Materials and methods

Chemicals. Combretastatins A1 and A4 (NSC 600032 and NSC 817373) were kindly provided by Prof. G. R. Pettit (Dept. of Chemistry, Arizona State University, Tempe, Ariz). Daunorubicin (May and Baker, Dagenham, UK), vincristine, vinblastine, vindesine (Eli Lilly, Basingstoke, UK) and verapamil (Sigma Chemical Co., Poole, Dorset, UK) were used without further purification.

Cell culture. Two P388 cell lines (P388R8/13 and P388R8/22) with acquired resistance to daunorubicin were developed from the parental (P388) line by incremental challenge with the drug in vitro. Both resistant cell lines have been shown to contain amplified gP170 DNA sequences (approx. 50-fold; M. Fox, personal communication) compared with the parental line. All cell lines were grown in RPMI medium supplemented with 10% horse serum (Gibco, UK). All cell lines were mycoplasma-free and replaced from frozen stock at 3-month intervals. Cell viability was determined immediately before all experiments and in all cases was >95% as assayed by trypan blue exclusion.

Cell-survival studies were carried out following drug treatment for one cell-cycle period on cells in exponential growth either by back extrapolation of growth curves for the 10 days following drug treatment or by a clonogenic assay of colonies on noble agar (0.5%, Difco, Detroit, USA; 200 cells/plate). Colonies were counted 10–14 days after drug treatment. All survival experiments were carried out in triplicate.

Drug accumulation studies. The effect of Combretastatins A1 and A4 on the accumulation of daunorubicin (^{14}C; 52 mCi/mmol, 92 μCi/mg, Amersham, UK) was measured by co-incubation of the drugs as described by Capranico et al. [1]. Briefly, incubations were carried out on each cell line ($10^6 ml^{-1}$, 1 h, 37° C, 5% CO_2, [DnR] = 10 μ*M* [Combretastatin A1 or A4] = 50 μ*M*). Cells were then washed twice in ice-cold saline, digested [H_2O_2-$HClO_3$ (2:1, v/v), 40 min, 65° C] and counted using Ecoscint A (Mensura Technology, Wigan, UK) as the scintillant. All experiments were done in triplicate and the results were expressed as means ± standard deviations.

Results

The effects of Combretastatins A1 and A4, vincristine, vinblastine, vindesine, amphethinile (a synthetic anti-microtubular agent) and daunorubicin on the parental and resistant cell lines are shown in Table 1. It can be seen that resistance to daunorubicin was accompanied by extensive

Table 1. Drug sensitivity pattern of parental and MDR P388 cells in vitro

Cell line	Daunorubicin	Vinblastine	Vincristine	ID_{50} (n*M*): Vindesine[2]	Amphethinile	Combretastatin A4	Combretastatin A1
P388	19 ± 1	1.7 ± 0.5	2.2 ± 0.3	0.18 ± 0.09	45 ± 4	120 ± 19	88 ± 10
P388 R8/13	650 ± 23	47 ± 9	35 ± 6	30 ± 10	45 ± 2	70 ± 20	425 ± 75
P388 R8/22	2,700 ± 160	–	–	150 ± 22	–	22 ± 5	576 ± 180

[2] Continuous drug challenge; all other data represent one cell-cycle period

Table 2. Intracellular daunorubicin levels following a 1-h incubation with drugs at 37° C

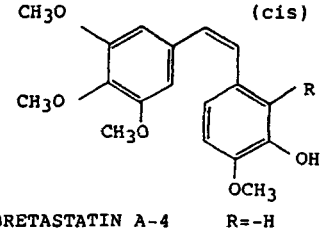
Daunorubicin (/10^{-15} mol)/cell (± SD):			
Cell line	Daunorubicin (10 μ*M*)	Daunorubicin (10 μ*M*) + Combretastatin A4 (50 μ*M*)	Daunorubicin (10 μ*M*) + Combretastatin A1 (50 μ*M*)
P388	1.38 (0.02)	1.39 (0.06), $P > 0.5$	1.30 (0.04), $P > 0.5$
P388 R8/13	0.53 (0.03)	0.57 (0.02), $P = 0.5$	0.80 (0.04), $P < 0.01$
P388 R8/22	0.40 (0.02)	0.51 (0.01), $P < 0.01$	0.72 (0.05), $P < 0.01$

P values were calculated using Student's *t*-test and refer to daunorubicin levels in the corresponding cell line treated with daunorubicin alone

cross-resistance to the vinca alkaloids; this cross-resistance was also seen towards Combretastatin A1. However, no resistance was observed for Combretastatin A4; indeed, a small enhancement in sensitivity to this agent was seen in resistant cell lines. The small enhancement of sensitivity seen towards A4 was shown to be statistically significant in the P388 R8/13 cell line ($P = 0.031$) using data obtained from four separate experiments. Similarly, the decrease in drug sensitivity in the P388 R8/13 line observed for A1 was also significant ($P = 0.022$) according to data from separate experiments.

This small collateral sensitivity was further investigated in the parental and the P388R8/13 cell lines by a clonogenic assay of toxicity. Again, although the absolute ID_{50} (growth-inhibitory dose 50%) concentration was higher in the growth inhibition assay than in the clonogenic assay, the small degree of collateral sensitivity was still observed towards A4, along with cross-resistance to A1 (Table 1) [ID_{50} (combretastatin A1) for P388, 3.5 ± 1.5 n*M*; P388 R8/13, 56.2 ± 5 n*M*; ID_{50} (combretastatin A4) for P388, 1.6 ± 0.5 n*M*; P388 R8/13, 0.8 ± 0.3 n*M*].

The effects of A1 and A4 on daunorubicin accumulation in parental and resistant cell lines are shown in Table 2. From these data it can be seen that the level of drug accumulation was greater in the parental than in the resistant cell lines. Also, co-incubation caused no increase in the anthracycline content of the parental (P388) cell line ($P > 0.5$, Student's *t*-test). However, Combretastatin A1 was capable of elevating intracellular levels of daunorubicin in both resistant cell lines (151% ± 21% for P388 R8/13; 180% ± 22% for P388 R8/22) relative to daunorubicin alone (100%); these increases were highly significant ($P < 0.01$). Combretastatin A4 showed no effect on daunorubicin levels in the parental or P388 R8/13 cell lines ($P = 0.5$). An increase was observed in the more resistant cell line (P388 R8/22); although it was highly significant ($P < 0.01$), it was much lower (127.5% ± 2.5%) than that seen for Combretastatin A1 (180% ± 22%).



COMBRETASTATIN A-4 R=-H NSC 817373

COMBRETASTATIN A-1 R=-OH NSC 600032

Fig. 1. Structures of Combretastatins A1 and A4

Discussion

Multi-drug resistance has been described in many cell types and has been implicated as a cause of the failure of contemporary chemotherapy. Several strategies are being investigated to overcome this problem, including co-administration of agents such as verapamil [3], some of which are proposed to act directly on the P170 glycoprotein associated with MDR, thus decreasing the cell's ability to efflux drug [2].

The novel agents Combretastatin A1 and A4 are potent inhibitors of microtubule assembly [5, 6]. Combretastatin A4 [(*cis*-1-3,4,5-trimethoxyphenyl)-2-(3′-hydroxy-4′-methoxyphenyl)ethene (CRC 87-09)] differs from the A1 analogue only in its lack of a hydroxyl group (Fig. 1). This small alteration in structure is sufficient to alter the spectrum of activity of these two agents in daunorubicin-resistant cell lines. The A1 analogue shows the typical cross-resistance pattern observed for a variety of agents (mainly natural products) in cells exhibiting MDR characteristics. In contrast, Combretastatin A4 showed no evidence of cross-resistance, but instead a small but reproducible level of collateral sensitivity. This phenomenon has been reported for a number of agents including the vinca alkaloids [8]. It is interesting to note that the resistance-sensitivity patterns of both Combretastatins can be shown by both growth-curve

and clonogenic assays. Hence, these differences cannot be explained by alterations in the cytotoxic/cytostatic profiles of these agents.

Evidence for the mechanisms by which these agents work can be derived from the drug-transport studies. Combretastatin A1 is more potent than the A4 analogue in its ability to increase daunorubicin levels in resistant cell lines (Table 2). Indeed, Combretastatin A4 can only elevate anthracycline levels in the more resistant cell line (P388 R8/22), and then only to a much smaller extent than that observed for the A1 derivative (20% vs 80%). The concentration of combretastatins used (50 μ*M*) was comparable to that of daunorubicin (10 μ*M*). Although high, these concentrations do not cause cell death (as measured by membrane permeability) on the time scale used in the drug accumulation study.

We propose that of the two Combretastatin analogues tested, A1 is the better substrate for the drug-efflux mechanism known to operate in these cells [4]. This would account for the cross-resistance seen towards A1 in these cells. However, Combretastatin A4 is less effective at disrupting daunorubicin efflux and is therefore, by inference, a poorer substrate for the efflux process or has reduced affinity for an associated protein. This mechanism would be consistent with the lack of cross-resistance towards this agent. The present data indicate that Combretastatin A4 may be of use in the treatment of tumours with acquired resistance to agents associated with the MDR phenotype.

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