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Activity of *N*-benzyl-adriamycin-14-valerate (AD198), a new anthracycline derivate, in multidrug resistant human ovarian and breast carcinoma cell lines

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The new lipophilic anthracycline *N*-benzyl-adriamycin-14-valerate (AD198) was evaluated for its activity in comparison to doxorubicin in P-glycoprotein (Pgp)-positive and -negative cell lines. AD198 and doxorubicin showed comparable antitumor activity in the Pgp-negative breast cancer cell line MCF-7 and the Pgp-negative ovarian carcinoma cell line A2780. By contrast, AD198 was significantly more active than doxorubicin in the Pgp-positive breast cancer cell line MCF7AD (IC_{50} values 2.5 and 0.15 μM for 96 h continuous exposure) and the Pgp-positive ovarian carcinoma cell line A2780 DX5 (IC_{50} values 0.6 and 0.07 μM, respectively). Unlike doxorubicin, the activity of AD198 was not increased by concommittant application of cyclosporin A in cell line MCF7AD. Flow cytometry studies showed that, in contrast to doxorubicin, AD198 was not transported by Pgp and that verapamil did not change the intracellular pharmakokinetics of this new anthracycline. These data provide evidence that AD198 possesses high activity in human solid tumor cell lines expressing the classical multidrug resistant phenotype. Its further clinical development appears to be warranted.

Key words: AD198, breast cancer, MDR, ovarian cancer.

Introduction

Anthracyclines are among the most frequently used anticancer agents with documented clinical activity in leukemia, lymphoma, breast, ovarian, lung and gastric carcinoma.[1] However, most responses to anthracycline-based chemotherapy are of limited duration and patients usually develop anthracycline-resistant disease. Many mechanisms which appear to be responsible for acquired resistance have been identified in tumor material obtained from relapsing patients and *in vitro* models. The most consistent finding is an over-expression of the 170 kDa membrane glycoprotein (P-glycoprotein, Pgp), which functions as an energy-dependent efflux pump and establishes multidrug resistance (MDR) to anthracyclines, vincalkaloids, epipodophyllotoxins, actinomycin D and taxanes.[2–4] After the first description of Tsuruo and colleagues that calcium channel blockers can inhibit the function of Pgp and thus reverse resistance *in vitro*, numerous Pgp-inhibiting agents have been identified, including tamoxifen, calmodulin inhibitors, quinine, amiodarone, cyclosporins and progesterone.[5–7] Though all these agents are capable of reversing drug resistance *in vitro*, the clinical application has been not very successful so far. This is mainly due to the fact that most modulators are too toxic to allow the application of doses high enough to achieve plasma concentrations that appear to be necessary for optimal reversal of Pgp-mediated resistance.[8,9] Furthermore, the clinical evaluation of resistance modulators is hampered by their influence on the pharmacokinetics of Pgp-dependent cytostatics.[10] Finally, thus far it is not clear whether these modulators will increase the toxicity of cytostatic drugs due to an inhibition of Pgp in normal cells.

An alternative way to overcome Pgp-based cellular resistance might be the development of anthracyclines which are not substrates for the pump and thus will not be exported from the cell. *N*-benzyl-adriamycin-14-valerate (AD198) (Figure 1) is one of a series of lipophilic anthracyclines synthesized in an attempt to overcome multidrug resistance. The drug has shown promising activity against multidrug resistant human and mouse leukemia cell lines *in vitro* and *in vivo*.[12] However, data on AD198 in human solid tumors expressing the multidrug resistance phenotype are limited so far.[13] We have evaluated this new anthracycline in multidrug resistant human ovarian and breast carcinoma cell lines in comparison with doxorubicin, given either alone or with an efficient modulator of Pgp.

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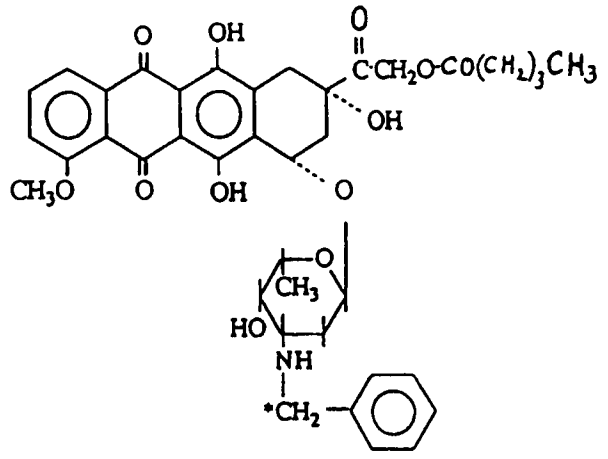

Figure 1. Formula of *N*-benzyladriamycin-14-valerate (AD198).

Materials and methods

Chemicals

N-benzyl-adriamycin-14-valerate (AD198) was obtained from Dr M Israel (Memphis, TN). Doxorubicin was obtained from Pharmacia (Freiberg, Germany). Verapamil was supplied by Knoll AG (Ludwigshafen, Germany). All other agents were from Sigma (Deisenhofen, Germany).

AD198 was dissolved in pure dimethylsulfoxide at a concentration of 2 mg/ml and further diluted in medium; doxorubicin was dissolved in saline and further diluted in medium. Cyclosporin A was dissolved in cornoil and further diluted in medium. For all experiments, controls were treated with the respective vehicle alone.

Cell lines

The ovarian carcinoma cell line A2780 was established by Rogan *et al.* from a non-pretreated patient with adenocarcinoma of the ovaries.[9] Several multidrug resistant lines have been generated by Jamali[14] using a continuous exposure to stepwise increasing concentrations of doxorubicin. Two of the resulting cell lines, A2780 DX1, which expresses low amounts of Pgp and is 4- to 5-fold resistant to doxorubicin, and A2780 DX5, which is highly P-glycoprotein positive and 75-fold resistant to doxorubicin, were used for these experiments. The breast carcinoma cell line MCF7 and the highly doxorubicin resistant subline MCF7AD were obtained from Dr K Cowan at NCI. All cell lines were grown as monolayers in RPMI 1640 with 10% fetal calf serum at 37°C and 5% CO_2 in a humidified incubator. The resistance to doxorubicin was stable even without further drug exposure for at least 3 months in all cell lines. The cells had not been exposed to doxorubicin for at least 4 weeks prior to the experiments.

Cytotoxicity assay

A modified sulforhodamine B assay was used as described by Skehan *et al.*[15] In brief, cells were seeded into 96-well microtiter plates and allowed to attach overnight. Then appropriate dilutions of the anthracyclines were added and the cells were exposed to the drugs for 96 h. The medium was aspirated and the cells were fixed in 10% trichloroacetic acid for at least 1 h. The staining procedure using sulforhodamine (0.4% in 1% acetic acid) was done as described originally. The absorbance was determined at 550 nm in a Dynatech plate reader and the percentage of growth inhibition compared with untreated controls was calculated. Eight wells were used for one concentration of drug and all experiments were repeated twice.

For the combination studies, cyclosporin A was added at the highest non-toxic concentration which was found to be 2 μg/ml. Cyclosporin was incubated together with the anthracyclines for 96 h. The percent of growth inhibition was determined using controls which were treated with cyclosporin alone.

Flow cytometry studies

Cells in log phase were trypsinized and adjusted to a concentration of 1×10^6/ml. They were incubated with rhodamine 123 or AD198 at a concentration of 5 μg/ml, either in the presence or in the absence of verapamil at a final concentration of 10 μM. After 15 min of incubation at 37°C cells were washed twice with ice-cold RPMI 1640 with 10% FCS and resuspended in RPMI 1640 with or without verapamil (10 μM). The cellular fluorescence was determined in an Epics flow cytometer. The reduction of cellular fluorescence after incubation in drug-free medium was recorded over 20 min and converted to percent using the fluorescence intensity at the end of the loading period as 100%.

Results

Initially the activity of AD198 was evaluated in the highly doxorubicin resistant cell line MCF7AD. This

line is approximately 125-fold more resistant to doxorubicin than the corresponding wild-type (IC_{50} values are 0.02 and 2.5 μM, respectively). In the wild-type, AD198 showed comparable activity to doxorubicin (IC_{50} of 0.06 μM), but it was significantly more active than doxorubicin in the multidrug resistant cell line MCF7AD.

These observations were further extended in the two multidrug resistant ovarian carcinoma cell lines A2780 DX1 and A2780 DX5. Again, AD198 was as active as doxorubicin in the Pgp-negative wild-type but showed significant higher activity in the highly doxorubicin resistant line A2780 DX5. Surprisingly, the cell line A2780 DX1, which has only a weak expression of Pgp and possesses a low level of doxorubicin resistance, showed also some resistance to AD198 (Table 1).

Table 1. IC_{50} (μM; 96 h continuous incubation)

	Doxorubicin	RF[a]	AD198	RF[a]
MCF7	0.02	—	0.06	—
MCF7AD	2.5	125	0.15	2.5
A2780	0.009	—	0.008	—
A2780 DX1	0.03	3.5	0.03	4
A2780 DX5	0.6	66	0.07	9

[a] RF, resistance factor.

Inhibition of Pgp

In order to assess the influence of Pgp-mediated drug efflux on the cytotoxic activity of AD198, MCF7 wild-type and MCF7AD cells were exposed to cyclosporin A at a concentration of 2 μg/ml concurrently with the anthracyclines. As expected, cyclosporin A had no effect on the cytotoxicity of either anthracycline in the Pgp-negative wild-type line. However, the inhibition of Pgp resulted in a marked augmentation of the activity of doxorubicin in MCF7AD cells whereas no effect was seen for AD198 (Figure 2).

Flow cytometry studies

The Pgp-mediated efflux of AD198 was further evaluated by flow cytometry in MCF7 and MCF7AD cells. Rhodamine 123, which has been shown to be a good substrate for Pgp, was used as a control. The Pgp-negative MCF7 cells lost approximately 25% of their initial rhodamine 123 or AD198 content after an incubation period of 20 min in drug-free medium. This efflux was not changed by verapamil (Figure 3A). In contrast, MCF7AD cells exported more than 80% of their initial rhodamine 123 content with 20 min. This Pgp-mediated efflux was reduced to 33% in the presence of 10 μM verapamil. In contrast, only 35% of AD198 was lost during the

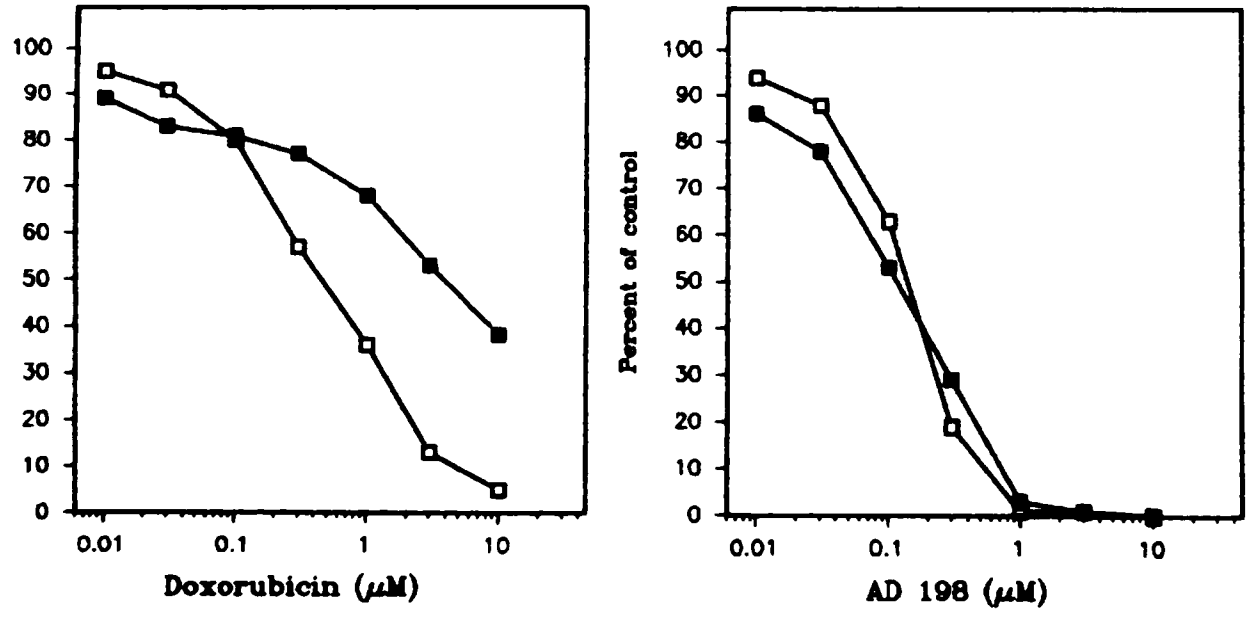

Figure 2. Infuence of cyclosporin A on the activity of doxorubicin and AD198. Cells were incubated with either doxorubicin or AD198 with (□) or without (■) cyclosporin A (2 μg/ml) for 96 h.

efflux period in MCF7AD cells incubated without verapamil and this amount was not changed by the addition of the calcium channel blocker, again indicating that Pgp was not responsible for AD198 efflux in this cell line (Figure 3B).

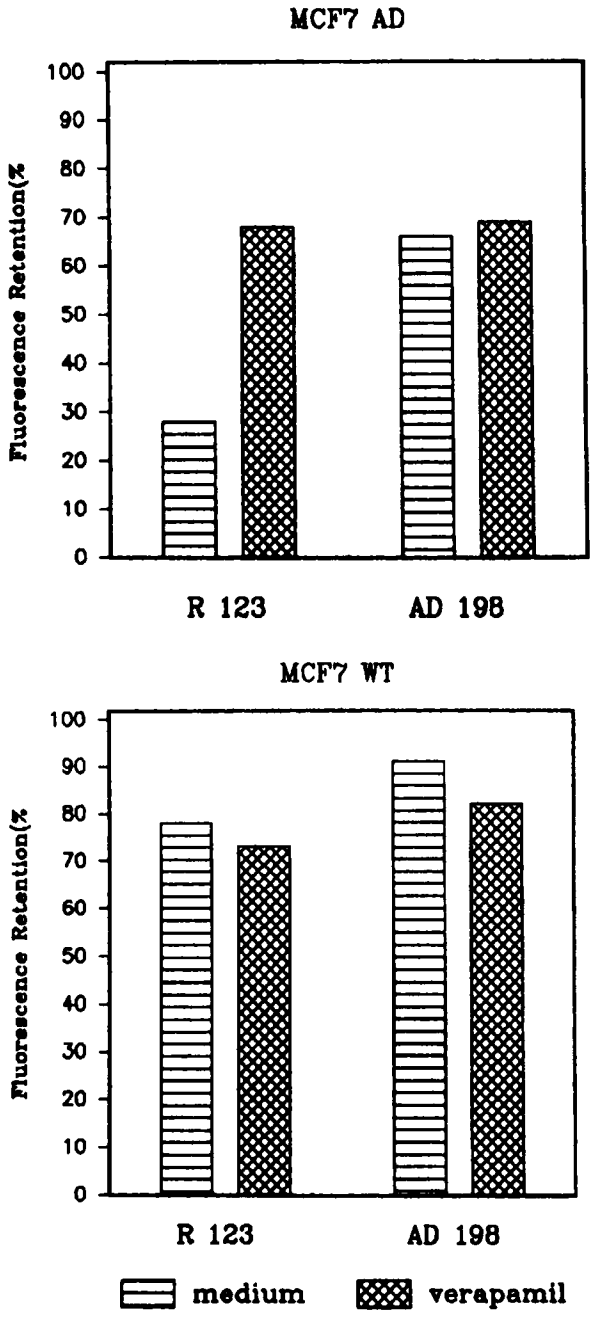

Figure 3. Influence of verapamil on the efflux of rhodamine 123 or AD198 in Pgp-negative (MCF 7) or Pgp-positive (MCF 7AD) cells. Cells were incubated with 5 μg/ml of rhodamine 123 or AD198 for 15 min, washed and resuspended in drug-free medium with or without verapamil (10 μM) for 20 min. The efflux was calculated by recording the cellular fluorescence at the end of the loading period (100%) and at the end of the efflux period.

Discussion

Acquired or intrinsic drug resistance is a major obstacle to successful chemotherapy in the majority of human tumors. Various mechanisms of drug resistance have been characterized. Experimental and clinical studies have shown that the classical multidrug resistant phenotype occurs in a variety of human hematologic malignancies and solid tumors, and appears to be a crucial mechanism for the resistance against anthracyclines, vinca alkaloids, epipodophyllotoxines, actinomycin D and taxanes in these diseases.[2–4] Various structurally non-related agents like calcium channel blockers, calmodulin inhibitors, cyclosporin A, progesterone, tamoxifen and quinine inhibit the function of Pgp and restore drug sensitivity in multidrug resistant cell lines *in vitro*.[6,7] These experimental data have prompted clinical trials with several of these modulators, usually in combination with doxorubicin or vinblastine.[7,8] However, most of these trials were unsuccessful due to the toxicity of the modulating agents which prevented the administration of doses necessary to achieve plasma levels in the range of the optimal *in vitro* concentrations.[8–10] Furthermore, several modulators significantly change the pharmokokinetics of the cytostatic drugs and therefore make it difficult to estimate the true contribution of Pgp inhibition to the overall observed effect.[10]

An alternative way to overcome multidrug resistance is the development of anthracyclines which are not substrates for Pgp. The lipophilic anthracycline AD198 is of interest in this regard. In earlier studies it could be demonstrated that AD198 has significant activity in multidrug resistant murine leukemias and B16 melanoma models.[11] Lack of cross resistance to doxorubicin was also observed in two human leukemia models, one expressing the classic multidrug resistance phenotype (CEM/VBL100) and the other showing the so called 'altered topoisomerase' type of resistance (CEM/VM1).[12] We have extended these investigations to human solid tumor cell lines expressing the classical multidrug resistance phenotype. When given continuously for 96 h, doxorubicin and AD198 had comparable activity in both Pgp-negative wild-type cell lines. However, AD198 retained almost full activity in the 125-fold doxorubicin resistant line MCF7AD and the 66-fold doxorubicin resistant line A2780 DX5. Since it has been shown that AD198 has only one metabolite (*N*-benzyladriamycin, AD288) and is not converted to doxorubicin *in vitro*, the observed activity has to be attributed either to AD198 or AD288 and

not to a doxorubicin prodrug function of AD198.[16] However, there is a certain breakdown of AD198 to doxorubicin *in vivo* in the rat which may contribute to the overall *in vivo* activity of this new anthracycline.[17] Further evidence that AD198 is not a substrate for Pgp comes from the flow cytometry and the modulation experiments. There was no augmentation of the cytotoxicity of AD198 by cyclosporin A nor a change of AD198 efflux by verapamil in MCF7AD cells, although both modulators were used in conditions capable of significantly inhibiting Pgp function.

Taken together, AD198 appears to be an interesting new anthracycline by virtue of its lack of cross-resistance to doxorubicin in classical multidrug resistant leukemia and solid tumor cell lines, its activity in altered topoisomerase resistant leukemia, its lack of direct binding to isolated topoisomerase II,[18] and its lower cardiotoxicity in experimental models.[19] Its further clinical development appears to be warranted.

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