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In vitro evaluation of the antineoplastic activity of combretastatin A-4, a natural product from *Combretum caffrum* (arid shrub)

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Combretastatin A-4 is a natural product which was isolated from the South African tree *Combretum caffrum*. In this study, the cytotoxic activity of combretastatin A-4 was tested in radiometric and human tumor cloning assays against eight different tumor cell lines and against 15 patient tumors in the human tumor cloning assay. To test the preferential cytotoxicity of combretastatin A-4 against tumor cells versus non-tumor cells, it was also tested in the radiometric assay against both normal human diploid fibroblasts and human bone marrow cells. Of the eight cell lines used, combretastatin A-4 showed preferential cytotoxicity for six of them. In addition, combretastatin A-4 showed a concentration-dependent cytotoxicity against a variety of human tumors. Based on the data generated in this study, combretastatin A-4 should be further tested in *in vivo* preclinical models.

Key words: Arid shrub, combretastatin A-4, *Combretum caffrum*.

Introduction

A large number of the known antimitotic agents are derived from plant components. These include the colchicum alkaloids colchicine[1] and cornigerine,[1–3] the *Catharanthus* (Vinca) alkaloids vinblastine and vincristine,[4] *Podophyllum*-derived compounds such as podophyllotoxin,[5] steganacin and congeners from *Steganotaenia araliacea*,[6,7] maytansine and congeners from *Maytenus serrata*,[8,9] and taxol from *Taxus brevifolia*.[10,11]

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Recently, a large number of closely related compounds derived from the South African tree *Combretum caffrum* have been investigated by Pettit *et al.*[12] In South Africa, this tree is known by the Zulu as 'Mdubu' (used as charm), and is otherwise known as bushveld willoe, bushwillow and 'rooiblaar'. Many of the compounds derived from *C. caffrum* are known to be the simplest natural products with potent antimitotic activity. They have been found to have inhibitory effects on both tubulin polymerization and the binding of colchicine to tubulin, and were active against the NCI murine P388 lymphocytic leukemia cell line. The first of these series of compounds was (−)-combretastatin which was separated from extracts of the shrub guided by its high activity in the NCI astrocytoma 9 ASK bioassay.[12,13] The basis of the assay was to test the ability of combretastatin to reverse the differentiation of immature AC rat glioma cells to astrocytes with mature morphology.

Combretastatin is a simple compound with two phenyl rings linked by a two-carbon bridge.[12–14] The obvious need to obtain more quantities of combretastatin for evaluation against NCI *in vivo* systems led Pettit *et al.*[15] to develop a method for synthesis. The other components of *C. caffrum* belong chemically to dihydrostilbenes, *cis*-stilbenes, phenathrenes, dihydrophenanthrenes and 17-membered macrocyclic lactones (e.g. combretastatin D-1 and D-2).[16–22] Among the *cis*-stilbenes, combretastatin A-4 was isolated[23] and found to be the most powerful inhibitor of tubulin polymerization of all the naturally isolated or chemically synthesized compounds (Figure 1).[24–26] The antimitotic combretastatin A-4 was also found to be effective against two P388 cell lines with acquired

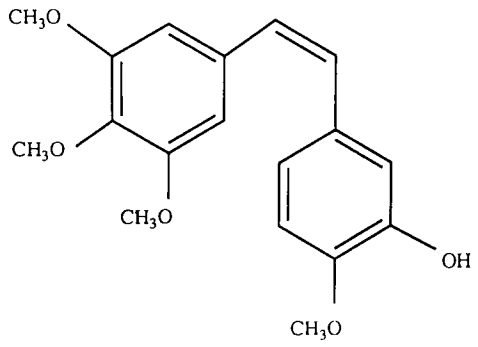

Figure 1. Structure of combretastatin A-4.

resistance to daunorubicin; thus it may play a role in the treatment of tumors with acquired resistance to anthracycline antibiotics and to agents associated with the MDR phenotype.[27] In the present investigation, we have studied the *in vitro* cytotoxicity of combretastatin A-4. The preferential cytotoxicity of combretastatin A-4 {(*cis*-1-3,4,5,-trimethoxyphenyl) - 2 - (3′ - hydroxy - 4′ - methoxyphenyl)ethene} was tested against three colon cancer cell lines (OM-1, HT 29 and COLO 320 DM), three lung cancer cell lines (SK-MES-1, SK-LU-1 and CALU-3) and two breast cancer cell lines (MCF-7M and HS5678T) using a radiometric system that utilizes the inhibition of [^{14}C] glucose conversion to $^{14}CO_2$ as an index of cytotoxicity[28–30] and the soft agar human tumor cloning system.[31,32] A human fibroblast cell line and normal human bone marrow were used to determine the preferential toxicity of combretastatin A-4 for the tumor versus the non-tumor cells. Combretastatin A-4 was also tested against tumors taken directly from patients and plated in a soft agar human tumor cloning assay.

Materials and methods

Tumor/non-tumor cell lines

Eight tumor cell lines were used in the radiometric system while only seven cell lines were used in the soft agar cloning system since the breast cell line HS578T did not readily form colonies in soft agar. The tumor cell lines, obtained from the American Type Culture Collection, included three lung cancer cell lines (SK-MES-1, SK-LU-1 and CALU-3), three colon cancer cell lines (OM-1, COLO 320 DM and HT 29) and two breast cancer cell lines (MCF-7M and HS578T). The colon cell lines were grown in RPMI 1640 medium with 10% heat inactivated fetal calf serum (FCS), except OM-1 which was grown in 20% heat inactivated FCS. The lung cell lines were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% non-heat inactivated FCS, 1% non-essential amino acids, 1% sodium pyruvate, 1% glutamine and 1% minimal essential medium vitamins. The breast cell lines were grown in Iscove's modified Eagle's medium (IMEM) with 5% non-heat inactivated calf serum and 10^{-9} M insulin for the MCF-7M cell line, and in 10% heat inactivated FCS plus 10^{-8} M insulin for the HS578T cell line. The non-tumor cell line used in the radiometric assay was WI-38 (normal human diploid fibroblast), which was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated FCS. All the cell lines were grown as attached cells (except for the suspension cell line COLO 320 DM, which was subcultured biweekly at a 1 : 10 ratio). Subculturing was done biweekly by removing the old media, overlaying the cells with 0.25% trypsin until the cells released and then adding the cells to new media in a 1 : 10 ratio. They were grown in a 5% humidified CO_2 incubator at 37°C.

Media/solutions

Transport medium. The transport medium was composed of 500 ml McCoy's 5A medium, 50 ml heat inactivated newborn calf serum, 5 ml penicillin/streptomycin (5000 units/ml stock), 10 ml HEPES buffer and 5 ml sodium pyruvate (200 mM stock for a final concentration of 2 mM). The medium was adjusted to pH 7.2–7.3.

McCoy's Wash. The wash was similar in composition to transport medium except that the heat inactivated newborn calf serum was replaced by an equal amount of heat inactivated fetal calf serum.

Enriched McCoy's medium. This medium was prepared by supplementing 500 ml McCoy's 5A medium with 25 ml heat inactivated horse serum, 50 ml heat inactivated FCS, 5 ml sodium pyruvate (2.2% stock), 1 ml L-serine (21 mg/ml stock) and 5 ml penicillin/streptomycin (5000 units/ml stock).

Enriched CMRL 1066. This was prepared by supplementing 400 ml CMRL 1066 medium with 75 ml heat inactivated horse serum, 10 ml heat inactivated FCS, 10 ml insulin (100 units/ml stock),

5 ml vitamin C (30 mM stock) and 5 ml penicillin/streptomycin (5000 units/ml stock).

Double enriched CMRL 1066. This was prepared by supplementing 100 ml enriched CMRL with 1.5 ml asparagine (6.6 mg/ml stock) and 2 ml glutamine (200 mM stock). The medium was adjusted to pH 7.0–7.2 using 1N hydrochloric acid.

Lysing buffer. The lysing buffer was prepared by dissolving 8.29 g ammonium chloride, 1 g potassium bicarbonate and 0.0371 g of EDTA in 1 l of distilled water and filter sterilizing.

Bone marrows

After centrifuging the marrow specimens (obtained from patients with bone marrow histologically negative for tumor cells) at 1000 r.p.m. for 7 min, the buffy coat layer was removed and was treated with lysing buffer and washed twice with McCoy's Wash as described below and suspended in 20 ml of RPMI 1640 supplemented with 10% heat inactivated FCS. To ensure the absence of any bulky particles, the suspension was run through a 200 mesh sieve, followed by drawing up the suspension through a 25 g needle. The cells were suspended in an adequate amount of media and were then counted.

Preparation of a single cell suspension of the patients' tumor specimens

The *in vitro* antineoplastic activity of combretastatin A-4 was tested against human tumors of different types in addition to the tumor cell lines mentioned above. The specimens were first processed to single cell suspensions as described below.

Solid specimens. The patient's tumor specimen was immediately placed in 25 ml of transport medium. To prepare single cell suspensions, the tissues were minced through a 100 mesh sieve, placed into a Petri dish and cut into the smallest possible pieces (1–2 mm) using sterile scissors. A glass pestle was used to push the tissue pieces through the sieve. Using a syringe with a 22 g needle, the tumor suspension was drawn up and placed in a 50 ml conical tube. After centrifugation at 1000 r.p.m. for 7 min, the fluid was aspirated off leaving the pellet. Then 35 ml of McCoy's Wash was added to the pellet and gently vortexed. The suspension was spun at 1000 r.p.m. for 7 min, the supernatant was aspirated, and the tumor cells were suspended in an appropriate amount of McCoy's Wash and counted.

Pleural fluids. The fluid was collected using standard techniques with 10 units of preservative-free heparin added per milliliter of malignant fluid. The fluid specimens were centrifuged at 1000 r.p.m. for 7 min. After aspirating the fluid, the pellet was suspended in lysing buffer to lyse red blood cells and respun. The lysing buffer was aspirated and the pellet was washed twice with 35 ml aliquots of McCoy's Wash before the final suspension in an appropriate amount of McCoy's Wash and counting.

Human tumor cloning assay

The human tumor cloning assay was performed using the two-layer system described by Hamburger and Salmon (with several modifications[31]). Each base plate layer (underlayer) contained 0.4% agar (Difco, Detroit, MI, USA) in plating medium which was composed of enriched McCoy's medium supplemented with 0.5% soy broth, 66 μg/ml asparagine, 250 μg/ml DEAE dextran and 1.3 mM glutamine. All the above ingredients were added prior to use. The top layer contained 0.25% agar in double enriched CMRL 1066. Tumor cells and combretastatin were added to this top layer. Since the drug was dissolved in methanol, the final solvent concentration was adjusted so as not to exceed 1%. For control plates, cells with the same percentage of the solvent were plated without the drug. Each test was done in triplicate and each experiment included a positive control with orthosodium vanadate[32] to assure the presence of a single cell suspension. Plates were incubated at 37°C, 5% CO_2. After 14 days, colonies were counted with an inverted microscope, and the percent survival was calculated by dividing the average number of colonies in the three experimental plates by the average number of colonies in the control plates and multiplying by 100. Generally, 10^4 cells of the tumor cell lines were inoculated per plate except for the MCF-7M and COLO 320 DM cell lines, which were inoculated at 3×10^4 and 5×10^4 cells/plate, respectively. Primary tumors were inoculated using 0.5×10^6 cells/plate.

An experiment was considered evaluable when the solvent control had at least 20 colonies/plate and the positive control showed at least 70% inhibition of colony formation compared with the solvent

control. A tumor or cell line was considered sensitive to combretastatin A-4 if colony survival was 50% or less of the solvent control.

Radiometric (Bactec) assay

The Bactec system 460 (Johnston Laboratories, Towson, MD) is a clinical instrument which was developed to detect bacteria in blood cultures. The instrument has been used to screen for new antineoplastic agents.[28–30] The radiometric system is a rapid, semiautomated system which utilizes inhibition of the conversion of [^{14}C]glucose to $^{14}CO_2$ as an index of cytotoxicity. Single cell suspensions of the eight tumor cell lines and the two non-tumor cell types were aseptically put in 2 ml of the tissue culture media containing 2 μCi of [^{14}C]glucose, and injected into 15 ml rubber-stoppered Bactec vials which contained a mixture of 5% CO_2 and air, and incubated at 37°C for 6–9 days depending on the tumor type used. After this period of incubation, the vials were removed from the incubator and inserted into the Bactec instrument for determination of the amount of $^{14}CO_2$ produced by the tumor cells upon metabolizing the [^{14}C]glucose. The Bactec machine automatically flushes out the $^{14}CO_2$ into an ion chamber where the signal of the radiolabelled CO_2 is changed into a proportional electrical signal known as the growth index. The signal was read on a meter scale of 0–1000. Combretastatin A-4, dissolved in methanol, was added to the tumor cells in the final concentrations described below taking care that the solvent concentration did not exceed 1%. The number of cells inoculated per vial was 7.5×10^4 for the three colon cell lines and MCF-7M (breast), 12.5×10^4 for the three lung cell lines and HS578T (breast), and 2×10^5 for WI-38 and 4×10^6 for bone marrow. The growth index of combretastatin A-4 treated cells was compared with the growth index of non-treated cells and the percent survival was calculated. The percentage survival was calculated by dividing the growth index of drug-treated cells by the growth index of untreated cells and multiplying by 100. To determine the selectivity of combretastatin A-4 as an antineoplastic agent, we tested the compound against two normal cell types in the same concentrations used against the eight tumor cell lines. Both the non-tumor human diploid fibroblasts cell line, WI-38, and normal human bone marrow cells were utilized, and both the growth indices and the percentage survivals were calculated as above. The concentration of the compound which inhibited 90% of the tested cells (IC_{90}) was calculated for both the tumor and the non-tumor cells.

The therapeutic index was calculated by taking the IC_{90} value of the drug activity against the tumor cells divided by the IC_{90} of the drug against the non-tumor cells (the human diploid fibroblasts or the bone marrow). Any ratio less than 1 was a favorable ratio and indicated probable selective cytotoxicity to the tumor cells.

Combretastatin A-4

Combretastatin A-4 (see Figure 1) was obtained from Dr George Pettit at Arizona State University. The compound was dissolved in methanol, and tested at final concentrations of 0.01, 0.1, 1.0, 10 and 100 ng/ml.

Clinical drugs

In addition to combretastatin A-4, the clinical agents doxorubicin and 5-fluorouracil (5-FU) were tested in both human cloning and radiometric assays. The two water-soluble compounds were tested for continuous exposures of 14 days at final concentrations of 0.004, 0.04 and 0.4 μg/ml for doxorubicin, and 0.6, 6 and 60 μg/ml for 5-FU.

Statistical analysis

Rates of sensitivity for the different concentrations of combretastatin A-4 used in the human tumor cloning assay were compared using McNemar's test for paired proportions. Due to the small numbers of specimens, the *p*-values were computed using the exact binomial distribution.

Results

The IC_{90}s of combretastatin A-4 against each of the eight tumor cell lines and the two non-tumor control cells are displayed in Table 1, together with the IC_{90}s for doxorubicin and 5-FU (where these agents were tested). Also shown in Table 1 are the therapeutic indices of each tumor cell line relative to fibroblasts and to bone marrow.

Of the eight cell lines, combretastatin A-4 showed selective cytotoxicity towards six of them

Table 1. Cytotoxicity of combretastatin A-4 and the clinical agents doxorubicin and 5-FU against different tumor cell lines in the radiometric assay

Cell line and controls	Compound	IC_{90}[a]	Therapeutic index	
			fibroblast	human marrow
MCF-7M breast	combretastatin A-4	6.562	0.030	0.080
	doxorubicin	0.098	0.030	0.145
HS578T breast	combretastatin A-4	5.617	0.023	0.070
	doxorubicin	0.040	0.014	0.059
COLO 320DM colon	combretastatin A-4	4.571	0.019	0.050
	5-FU	3.219	0.006	0.690
HT 29 colon	combretastatin A-4	950.472	3.910	11.400
	5-FU	1.976	0.004	0.430
OM-1 colon	combretastatin A-4	40.597	0.170	0.490
	5-FU	1.814	0.004	0.390
CALU-3 lung	combretastatin A-4	65.561	0.270	0.800
	doxorubicin	0.010	0.003	0.015
SK-MES-1 lung	combretastatin A-4	8.199	0.033	0.100
	doxorubicin	0.397	0.140	0.590
SK-LU-1 lung	combretastatin A-4	215.434	0.900	2.600
	doxorubicin	0.284	0.099	0.420
WI-38 (fibroblasts)	combretastatin A-4	242.811		
	doxorubicin	2.867		
	5-FU	503.970		
Bone marrow	combretastatin A-4	83.489		
	doxorubicin	0.675		
	5-FU	4.639		

[a] Combretastatin A-4 values are in ng/ml while both the clinical drugs, doxorubicin and 5-FU, are in μg/ml.

(i.e. therapeutic indices less than 1). As a control, the two clinical agents doxorubicin and 5-FU were tested in parallel with combretastatin A-4, and the therapeutic indices were less than 1 in all cases (Table 1).

In the human tumor cloning assay, combretastatin A-4 was tested against the same tumor cell lines used in the Bactec system. However, the HS578T breast cell line did not grow in this assay system. At a concentration of 10 ng/ml the percent survival ranged from 10 to 50% in all cell lines, while at a concentration of 100 ng/ml the compound was even more cytotoxic (Table 2). As shown in Table 2, combretastatin A-4 showed a significant concentration-dependent effect on growth inhibition.

Table 3 gives the results when combretastatin A-4 was tested at 1, 10 and 100 ng/ml against a panel of 15 tumors taken directly from patients. The panel represented tumors of different types, including endometrial, non-small cell lung, ovary, mesothelioma and other tumors of unknown primary sites. Of the 15 specimens examined, only 13 satisfied all the criteria to be called evaluable (see Materials and methods). Combretastatin A-4 demonstrated a dose–response effect and was active in 15.4, 38.5 and 77% of tumors responding *in vitro* (activity defined as a colony survival of 50% or less of the control) at final concentrations of 1, 10 and 100 ng/ml, respectively (Table 3). More tumor types showed sensitivity to combretastatin A-4 as the concentration increased from 1 to 100 ng/ml.

Discussion

Combretastatin A-4 is a simple antimitotic compound which is known to compete with colchicine in inhibiting tubulin polymerization. In our effort to further characterize the *in vitro* antitumor activity of the compound, its cytotoxicity was tested against tumor cells in the Bactec and human tumor cloning assays.

In the radiometric assay, the therapeutic indices which represent preferential cytotoxicity of the compound against the tumor cells versus the non-tumor cells were calculated. Combretastatin A-4 proved to be selective in its toxicity towards tumor versus non-tumor cells with favorable therapeutic indices in six out of eight cell lines. In general, combretastatin A-4 showed a concentration-dependent cytotoxicity against eight

Table 2. Cytotoxicity of combretastatin A-4 and the clinical agents doxorubicin and 5-FU against different tumor cell lines in the human tumor cloning assay

Concentration[a]	Percent survival (human tumor cloning assay)						
	COLO 320 DM	HT 29	OM-1	SK-MES-1	SK-LU-1	CALU-3	MCF-7M
Combretastatin A-4							
0.01	101	128	117	105	82	111	87
0.1	25	62	88	104	72	101	41
1.0	0	86	88	22	1	60	18
10	0	43	8	3	1	0	0
100	0	0	0	6	1	0	0
Doxorubicin							
0.004				94	98	38	21
0.04				25	0	0	12
0.4				3	0	0	0
5-FU							
0.6	105	0	11				
6.0	0	0	9				
60.0	0	0	0				

[a] Combretastatin A-4 values are in ng/ml while both the clinical drugs, doxorubicin and 5-FU, are in μg/ml.

Table 3. Concentration-dependent inhibition of colony formation by combretastatin A-4 against human tumors after 14 days of continuous exposure

Tumor type	Total number of specimens	Number of specimens with inhibition[a]/number of evaluable specimens		
		1 ng/ml	10 ng/ml	100 ng/ml
Endometrial	1	0/1	0/1	1/1
Lung (non-small cell)	1	0/1	0/1	0/1
Ovary	8	2/7	3/7	6/7
Mesothelioma	1	0/1	1/1	1/1
Unknown primary site	4	0/3	1/3	2/3
Total	15	2/13	5/13	10/13
Percent		15	39	77
		$p = 0.19$	$p = 0.031$	

[a] Colony survival 50% or less of control.

tumor cell lines and against 15 tumors taken directly from patients.

Based on the promising *in vitro* cytotoxicity of combretastatin A-4 results in the radiometric and human tumor cloning assays, *in vivo* testing of the agent in preclinical models should begin immediately.

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