Cancer Chemother Pharmacol (1982) 8:41–46

Cancer Chemotherapy and Pharmacology

Plasma Kinetics of Aclacinomycin A and Its Major Metabolites in Man

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Summary. *The plasma pharmacokinetics of the antineoplastic anthracycline antibiotic aclacinomycin A (Acm) and its metabolites were studied in 12 patients treated with 60–120 mg/m² during a phase I clinical trial. Total plasma drug fluorescence initially declined very rapidly, but from 2 to 24 h after injection, fluorescence rose progressively to intensities greater than those measured 1 min after Acm injection. Plasma total drug fluorescence slowly declined from 24 to 72 hours after Acm administration. These events reflected the rapid disappearance of Acm and the subsequent appearance of two highly fluorescent metabolites. One metabolite co-chromatographed with and had a fluorescence spectrum identical to known metabolite F_1 (bisanhydroaklavinone). The other metabolite did not co-chromatograph with any previously described Acm metabolite. This metabolite had a fluorescence spectrum unlike any previously described Acm metabolite and was not altered by treatment for 60 min with 0.2 N HCl at 100° C or by treatment for 24 h at 37° C with bacterial β-glucuronidase or limpet aryl sulfatase.*

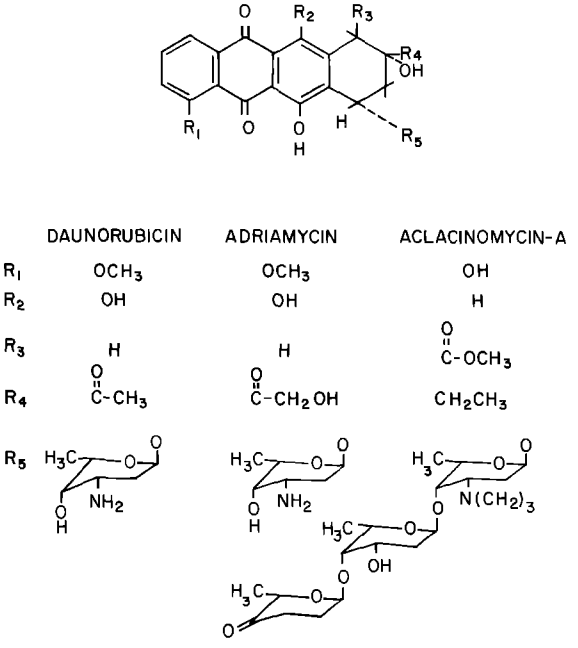

Fig. 1. Structures of daunorubicin, adriamycin, and aclacinomycin A

Introduction

Aclacinomycin A (Acm) is an anthracycline antineoplastic antibiotic discovered and tested in clinical trials in Japan [24, 25]. Acm differs from the commonly employed anthracyclines daunorubicin (Dnr) and adriamycin (Adr) both structurally (Fig. 1) and, to some extent, mechanistically, inhibiting RNA synthesis more efficiently than DNA synthesis [8, 23]. On the basis of its inhibition of radionucleoside incorporation, Acm has been described as a class II anthracycline [8, 23], and as such it is the first of this group of drugs to be used clinically. Although the in vitro and animal metabolism of Acm have been defined [18, 23, 25], there is a paucity of published information concerning this drug's plasma pharmacokinetics and metabolism in humans [26]. We have investigated these two problems in patients receiving Acm in a phase I clinical trial at our institution [11].

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Abbreviations used: Acm, aclacinomycin A; Dnr, daunorubicin; Adr, adriamycin; TLC, thin layer chromatography; MLAB, on-line modeling laboratory, Division of Computer Resources and Technology, National Institutes of Health; C × T, concentration times time; C_p, plasma concentration; t, Time after administration of drug; R_f, relative retardation factor; HPLC, high performance liquid chromatography

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Materials and Methods

Patient Population. All patients gave written informed consent before being studied. Acm, provided by the Developmental Therapeutics Branch, DCT, NCI, Bethesda, MD, was administered as an IV bolus to 12 patients, 4 female and 8 male, aged 43–70 years (median 52 years), at dosages of 60–120 mg/m^2 body surface area. All patients had advanced stage neoplasms that were not amenable to control by surgery, radiation therapy, hormonal therapy, or accepted chemotherapeutic agents. All patients had received prior chemotherapy and eight had received Adr. No patient was of Oriental or Hispanic descent.

Sample Collection. Before and after Acm administration, blood was collected in heparinized tubes and was immediately centrifuged. The resulting plasma was frozen immediately and stored at −20° C until analyzed.

Plasma Extraction and Assay. Plasma was assayed for total Acm-derived fluorescence and for individual Acm metabolites. Total Acm-derived fluorescence was determined in plasma samples that had been extracted with 0.45 *N* HCl in 50% ethanol as previously described [3]. Fluorescence was determined with an Aminco SPF 125 spectrofluorometer (American Instrument Co., Silver Spring, MD, USA) at 450 nm excitation and 585 nm emission [10], and plasma Acm equivalents were determined by comparison with a series of freshly prepared Acm standards.

For separate measurements of Acm and its metabolites, plasma was extracted with 2 volumes of chloroform : isopropanol (1 : 1) as previously described [4]. This procedure did not alter the chromatographic behavior of standard of Acm, M_1, N_1, C_1, F_1, S_1, or E_1 extracted from control plasma. The chloroform : isopropanol extract was analyzed by TLC on 250 μm silica gel G plates (E. Merck, Darmstadt, FRG) that were developed first in diethyl ether, air-dried, and then developed in an ascending fashion to a solvent front of 15 cm with chloroform : methanol : water (80 : 20 : 3) as a solvent system (solvent system I). In some cases, aliquots of plasma extracts were also developed in chloroform : methanol : glacial acetic acid (100 : 2 : 2.5) (solvent system II). Chromatographic standards of Acm and metabolites M_1, N_1, C_1, F_1, S_1, E_1, deoxypyrromycin and aklavinone [23] (Fig. 2), kindly provided by Dr T. Oki of the Sanraku-Ocean Co., Ltd. Fujisawa, Japan, were run on each plate. Drug fluorescence on the TLC plates was detected with 2537 Å light (UVS-54 Mineralight, Ultra-Violet Products, San Gabriel, CA) and fluorescent regions of the plates were scraped. Drug was then eluted from the silica gel with 0.3 *N* HCl in 50% ethanol and quantified fluorometrically by comparison with a series of fresh Acm standards [1]. When clinically administered Acm was analyzed by these TLC systems, all fluorescence was associated with parent compound.

Fluorescence Spectra. Fluorescent excitation and emission spectra of solutions of Acm and purified metabolites in 0.154 *M* NaCl and 0.3 *N* HCl in 50% ethanol were obtained with an absolute spectrofluorometer (model SPF 1000 CS, American Instrument Co.).

Kinetic Analysis. The early decline in plasma fluorescence, expressed as Acm equivalent concentrations, was analyzed with the programs of MLAB (an on-line modeling laboratory, Division of Computer Resources and Technology, NIH, Bethesda, MD). Curves were fit to the sum of three exponentials by a non-linear fitting technique. Concentration times time (C × t) for the 0–60 min portion of patients' studies was estimated by calculating the area under the curve by means of the INTEGRAL operator in MLAB.

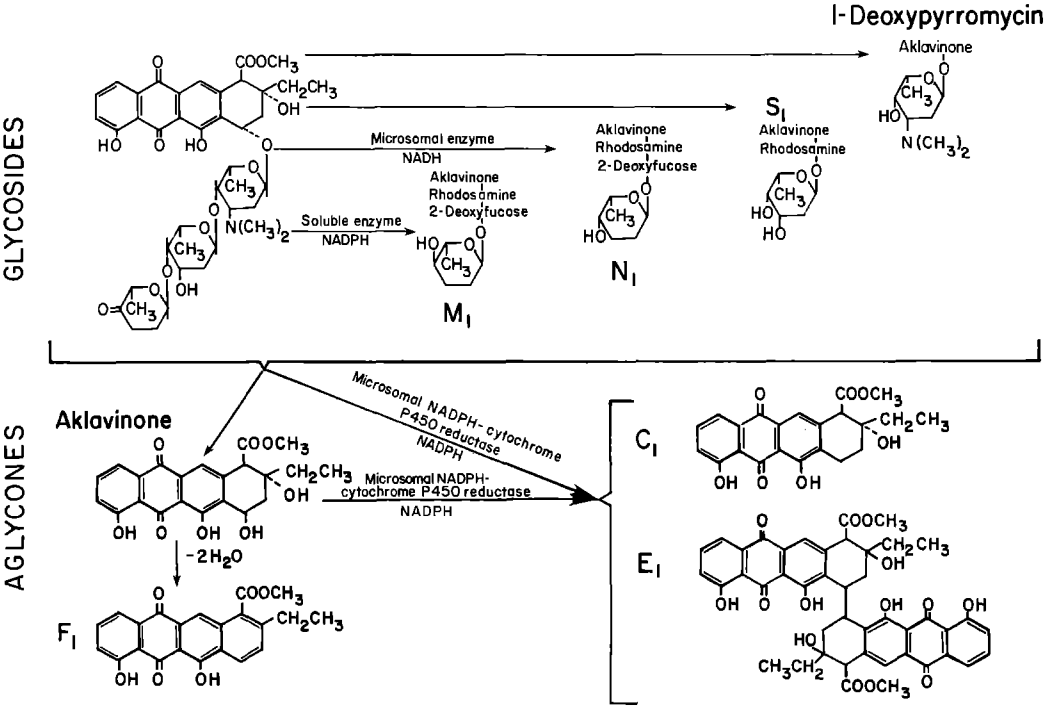

Fig. 2. Structures of the known metabolites of aclacinomycin A

Results

Plasma Total Fluorescence

Plasma total drug fluorescence initially declined very rapidly but then rose progressively from 2 to 24 h before finally declining slowly (Fig. 3 and 4). The early decline in plasma drug fluorescence was well described by a triexponential decay defined by the equation, plasma concentration $C_P = 0.989e^{-0.0519t} + 0.345e^{-0.0668t} + 0.190$.

As can be inferred from the composite curve presented in Fig. 3, there was close agreement among the individual patients' studies, and there was close agreement in the C × t for the 0–60 min portion of each patient's studies (18.42 ± 2.0 μM · min, mean ± SD). This type of analysis is valid only if plasma fluorescence, expressed as nmole equivalents of Acm/ml, reflects a single fluorescent entity. Similar analyses of total plasma fluorescence that reflect multiple fluorescent species, such as parent compound and metabolites, are much less useful and in fact may be misleading [9, 16]. Our analysis of plasma fluorescent components (see below) shows that plasma drug fluorescence from 0 to 60 min is due only to parent compound, Acm. This rapid decline in plasma fluorescence agrees with earlier reports of plasma studies done in rabbits [17, 26], a dog [21], and humans [19, 26].

In every patient studied, a secondary peak occurred in plasma drug fluorescence, which exceeded the amount of total fluorescence measured in the first sample drawn after Acm administration (Fig. 4). This rebound in plasma total drug fluorescence has not been reported for animals treated with Acm.

When the plasma samples were extracted and analyzed for metabolites by TLC, we found that the rebound in plasma drug fluorescence was not due to reappearance of parent Acm, but rather represented the appearance of two metabolites. One of these (spot A) was less polar than Acm and co-chromatographed with known metabolite F_1 (Fig. 2, Table 1). The other metabolite (spot D) was much more polar than Acm in solvent system I but less polar than Acm in solvent system II, and did not co-chromatograph with any previously described Acm metabolite (Table 1).

When activated by 450 nm light and measured at 585 nm, more fluorescence was measured in spot A than in spot D (Fig. 5). However, when TLC plates were examined with 2537 Å UV light prior to scraping, spot D appeared brighter than did spot A. As might be expected, this discrepancy, to some

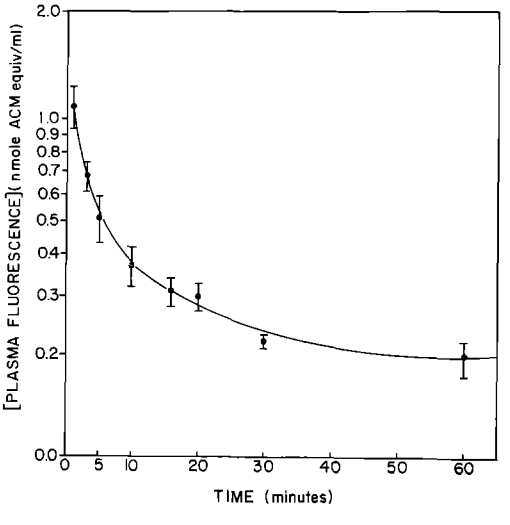

Fig. 3. Aclacinomycin A-derived total fluorescence in plasma 1–60 min after drug injection. Acm was injected as an IV bolus at a dosage of 120 mg/m^2. Plasma was obtained and extracted with 0.3 *N* HCl in 50% ethanol as described in *Materials and Methods*. Fluorescence was measured and Acm equivalents were determined as described in *Materials and Methods*. The *points* represent the means ± SE from three patients' studies, each done in duplicate

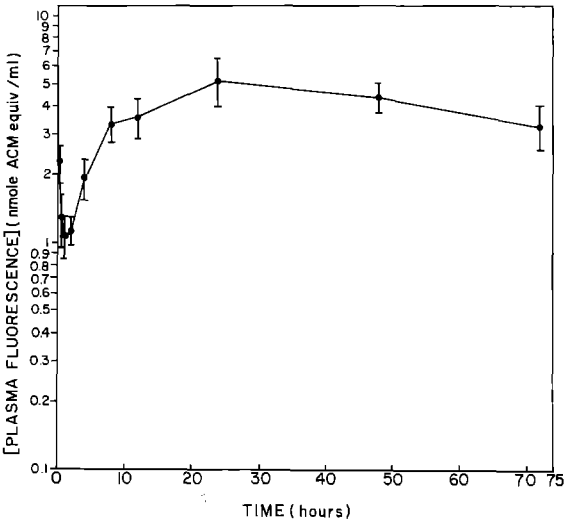

Fig. 4. Total drug-related fluorescence in plasma of patients treated with aclacinomycin A. Patients received Acm as an IV bolus at dosages of 60–120 mg/m^2 and plasma was obtained and extracted with 0.3 *N* HCl in 50% ethanol as described in *Materials and Methods*. Fluorescence was measured and Acm equivalents were determined as described in *Materials and Methods*. The points represent the means ± SE of 13 studies done in 9 patients

Table 1. Values for R_f of aclacinomycin and its metabolites in TLC

Compound	R_f	
	System I[a]	System II[b]
F_1	0.89	0.77
E_1	0.86	0.38
C_1	0.83	0.49
Aklavinone	0.80	0.40
Acm	0.77	0.00
M_1	0.64	0.00
N_1	0.57	0.00
S_1	0.36	0.00
Aklavin	0.26	0.00
Spot A	0.87	0.77
Spot D	0.24	0.34

[a] Chloroform: methanol: distilled water (80: 20: 3)
[b] Chloroform: methanol: glacial acetic acid (100: 2: 2.5)

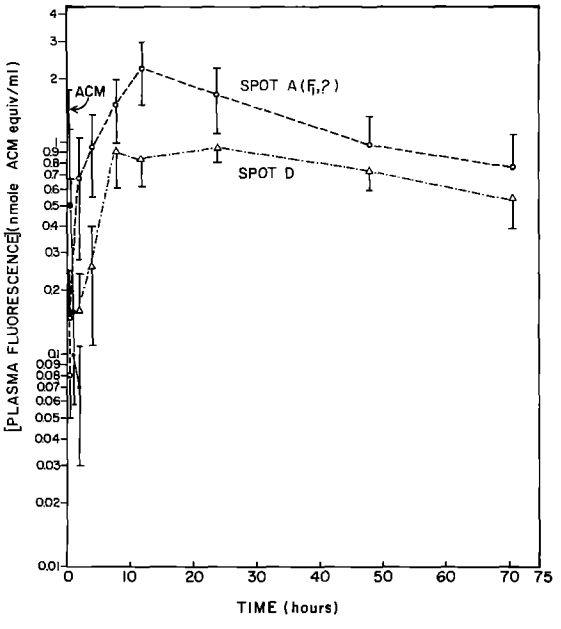

Fig. 5. Fluorescence of aclacinomycin A and metabolites in plasma of patients treated with aclacinomycin A. Patients received Acm as an IV bolus at dosages of 60–120 mg/m^2. Acm and metabolites were extracted from plasma with chloroform : isopropanol and separated by TLC as described in *Materials and Methods*. Fluorescence was measured and Acm equivalents were determined as described in *Materials and Methods*. *Points* represent the means ± SE of eight studies done in seven patients

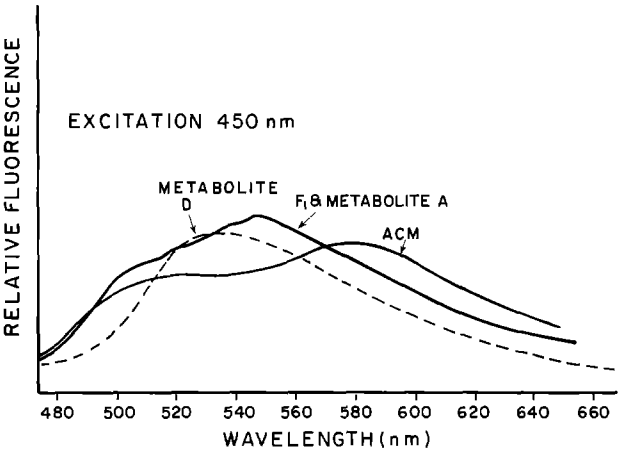

Fig. 6. Fluorescence spectra of aclacinomycin A and plasma metabolites A and D. Plasma metabolites A and D were isolated from pooled plasma by chloroform : isopropanol extraction and TLC as described in *Materials and Methods*. Spectra were obtained with a 1 cm path length and 450 nm exciting light

degree, reflects different fluorescent spectra for the metabolites (Fig. 6). The fluorescent spectrum of the metabolite in spot A was identical to that of metabolite F_1, with maximum excitation at 450 nm and maximum fluorescence at 552 nm (Fig. 6). On the other hand, the metabolite in spot D, while exciting maximally at 450 nm, had a maximum emission at 538 nm, emitting much less at 585 nm, the wave length measured in our studies. Small amounts of metabolites 'A' and 'D' were isolated and purified from pooled plasma by extraction with chloroform : isopropanol and subsequent TLC. The TLC behavior of these materials was not altered by treatment at 100° C with 0.2 *N* HCl, or by incubation at 37° C for 24 h with bacterial β-glucuronidase (type VII, Sigma Chemical Co., St Louis, MO, USA) or limpet aryl sulfatase (type V, Sigma Chemical Co., St. Louis, MO, USA). Unfortunately, the quantities of pure metabolites A and D that were isolated were insufficient for mass spectral analysis. Both Acm metabolites persisted in plasma for long periods of time and their slow decline was not associated with the appearance of any new fluorescent species.

Discussion

Since the introduction into clinical use of Dnr and Adr, a number of other anthracycline antibiotics have been promoted on the basis of different spectra of activity or modified toxicity [2, 5, 6, 7, 14, 15]. Acm, an anthracycline with major structural differences from Dnr and Adr, has demonstrated significant activity against a number of animal and human tumors [12, 13, 20, 22] and does not appear to produce the alopecia and extravasation necrosis associated with Dnr and Adr [11, 20, 22]. In addition, the mechanism of cytotoxic activity may not be the same for Acm as for Dnr and Adr since Acm's inhibition of nucleic acid synthesis [8, 10, 23] and

certain aspects of its cellular pharmacology differ greatly from those of Dnr and Adr [10].

The metabolism of Acm has been well studied in vitro and in animals and has been shown to proceed in two main directions [11, 18, 23, 26]. There may be enzymatic reduction of the terminal keto sugar to form the stereoisomeric metabolites M_1 and N_1, which possess antitumor activity [26]. Alternatively, there may be enzymatic cleavage of the trisaccharide to liberate the aglycone, 7-deoxyaklavinone (C_1), or its dimer, metabolite E_1 (Fig. 2).

There is a certain amount of sequential cleavage of Acm's sugars, producing first the disaccharide metabolite, S_1, and then the monosaccharide glycoside, aklavin, which is also called deoxypyrromycin (Fig. 2). Further, the aglycones, aklavinone and bisanhydroaklavinone (F_1) have been described (Fig. 2). Despite these extensive metabolic studies, there is little information on the disposition of Acm in animals and on the metabolism and disposition of Acm in humans [19, 25]. Oki and co-workers demonstrated a very rapid decline in the concentration of radioactivity in the plasma of a rabbit treated IV with 5 mg Acm/kg when the drug was labeled with ^{14}C in the C-10 methyl ester and in the terminal sugar, cinerulose A [25] or in the C-9 position, the C-10 methyl ester, and the C-3′ position [26]. In other studies, Oki and co-workers at Sanraku-Ocean Co., Ltd used a reversed-phase HPLC system coupled to a fluorescence detector to demonstrate a very rapid decline in the concentrations of Acm and metabolites M_1 and S_1 in the plasma of a dog that received 5 mg Acm/kg IV [21]. This study, however, included only the first 3 h after injection. Using TLC and subsequent fluorescent scanning, Oki and co-workers documented the rapid disappearance of Acm from the plasma of rabbits following 10 mg/kg IV [17], and the similar behavior of Acm in a single patient who received 2 mg Acm/kg IV [26]. They also detected small amounts of M_1 and F_1 in the plasma of this patient [26]. Malspeis and co-workers have used HPLC to study the plasma pharmacokinetics of Acm and have confirmed the rapid disappearance of Acm from patients' plasma [19]. Our studies represent the most extensive study of the human plasma pharmacokinetics of Acm and its metabolites. As with previous studies, we observed a very rapid disappearance of Acm from the plasma. On the other hand, every one of our patients demonstrated a dramatic rebound in plasma fluorescence; this was due to two metabolites of Acm, one of which has not been described previously in plasma.

There may be several explanations for why plasma fluorescence due to Acm metabolites reaches levels greater than the amount of Acm fluorescence measured 1 min after IV injection of Acm. It is not likely that one mole of Acm molecules is converted to more than one mole of fluorescent metabolites. Rather the two Acm metabolites observed may have smaller volumes of distribution than does Acm, and they appear to be produced more rapidly than they are excreted and metabolized. Alternatively, the two metabolites may have greater quantum fluorescence efficiencies than does Acm. At this time we are unable to define whether either, or both, of these mechanisms can actually explain our observations.

In addition to previously undescribed plasma pharmacokinetics, we have observed what appear to be two major plasma metabolites of Acm, which were present in the plasma of all 12 patients studied. One of these appears to be the previously described metabolite, F_1. The other metabolite did not correspond to any previously described metabolites of Acm. It may be that 'metabolite D' is a metabolite not produced by rabbits or dogs. Furthermore, since our study included no Oriental patients, we cannot exclude the possibility that our results may reflect racial or genetic differences in the human disposition of Acm. Alternatively, the structure of metabolite D may be such that it was not detected by the analytical methods used by others. It is possible that both the terminal cinerulose A sugar residue and the methyl group esterified at carbon 10 are absent, thereby removing the radiolabel. Also, due to the altered fluorescence spectrum, metabolite D may have eluded detection by either TLC fluorescence scanning or HPLC fluorescence detection. Procuring enough metabolite A for structural confirmation and enough metabolite D for structural identification and toxicology and antitumor activity studies seems imperative in view of the amounts of these materials present and their persistence in human plasma. Studies are under way in our laboratory to accomplish these ends as rapidly as possible.

Acknowledgements. We thank Drs. Charles E. Riggs, Jr. and Jerry Collins for their assistance with pharmacokinetic analyses.

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Received August 3/Accepted November 3, 1981