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Daunomycin-arachidonic acid complex as a potential new antitumor agent

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Summary. *A daunomycin-arachidonic acid complex (DM-$C_{20:4}$) administered IV had a marked antitumor effect on the intraperitoneal growth of the AFP-producing hepatoma cell line, AH66. However, the daunomycin-arachidic acid (DM-$C_{20:0}$) saturated fatty acid complex showed only the same antitumor effect on the AH66 cells as free daunomycin. It was demonstrated that the DM-$C_{20:4}$ preparation maintained its chemical properties for some time in both plasma and liver homogenate.*

Introduction

One of the major limitations of effective cancer chemotherapy is that effective killers of neoplastic cells are usually also toxic to rapidly proliferating normal cells. One way of improving the selectivity of a chemotherapeutic agent is to use antibodies directed against the tumor cells as specific carriers of the drugs [4, 6, 10, 13]. However, this approach has a serious limitation in that it can only be employed for a limited time since it elicits foreign protein reactions in the recipient. An alternate and more attractive approach that has applications for the chemotherapy of hepatoma appears to be utilization of the ability of AFP to strongly bind polyene fatty acids [7, 8]. In previous studies [3] we tested the effects of daunomycin-polyene fatty acids complexes on the growth of several rat tumor hepatoma lines and found them to be effective. The present work entails the use of only the IV route for administration of these complexes.

Materials and methods

Determination of the number of double bonds. Iodine numbers of the unsaturated fatty acids and their daunomycin complexes were determined essentially by the method of Yasuda [14] to determine the levels of unsaturated fatty acid. About 2–9 mg $C_{18:1}$, $C_{20:4}$, DM-$C_{20:4}$, or DM-$C_{20:0}$ in chloroform was added to 5 ml pyridine dibromide in a glass-stoppered flask and incubated in the dark for 15 min at room temperature. Then 0.5 ml 10% KI and 0.5 ml H_2O were added to each flask and the liberated I_2 was titrated with 0.02 *N* $Na_2S_2O_3$, starch being used as the endpoint indicator.

H-NMR spectra were recorded at room temperature on a Nihondenshi spectrophotometer at 400 MHz. The measurement was carried out on a solution of deuterated chloroform. Chemical shifts (σ) were measured relative to internal TMS and recorded as parts per million (ppm).

Drug analysis by HPLC. A Hitachi model 655 HPLC connected to a Hitachi model 650-10LC high-sensitivity fluorescence detector was used to assay daunomycin derivatives. The results were recorded with a Hitachi model 056 printer. The HPLC experiments were carried out in a 4 × 155 nm column packed with 5-μm-diameter Hitachi C_{18} gel particles, and elution was performed with a mixture of 0.05 *M* Na H_2PO_4 and methanol (1 : 4 by volume) at a flow rate of 1.0 ml per min. The excitation and emission wavelengths were 482 and 580 nm, respectively [12]. Studies were conducted to determine whether modifications of the daunomycin or of the daunomycin-fatty acid complexes were effected as the result of mixing them with rat plasma or with rat liver homogenate. A 0.1-ml aliquot of 0.1 *M*, pH 9.8, borate buffer containing 4 μg of the substance being investigated was added to 0.1 ml plasma. The mixture was incubated for 20 min at 37° C and the drug was extracted with 1.8 ml chloroform-methanol (4 : 1) mixture [2]. Then 1 ml of the chloroform layer was evaporated and the residue was dissolved in the mobile phase used in the HPLC analysis. A similar extraction procedure was carried out on 20 μl of an ethanol solution containing a 4-μg equivalent of daunomycin as a fatty acid complex which, after being added to 0.1 ml plasma and 0.1 ml borate buffer, was vortexed and incubated at 37° C for 20 min. Similar experiments were carried out with rat liver homogenized in four volumes of pH 7.4, 0.05 *M* Tris-HCl buffer. Daunomycin (4 μg) in 0.1 ml Tris buffer was added to 0.1 ml homogenate and the mixture incubated at 37° C for 20 min. Following this, 50 μl pH 9.8, 0.5 *M* borate buffer was added and the daunomycin extracted in the same manner as the plasma sample described previously. A similar experiment on the homogenate was carried out with a 4-μg daunomycin equivalent of DM-$C_{20:4}$ in 20 μl ethanol added to the same level of homogenate as used in the experiment with free daunomycin. All the extracts described above were subjected to HPLC analysis.

Evaluation of antitumor activity. The in vivo therapeutic effects of daunomycin and of its fatty acid complexes were tested in Donryu rats weighing approximately 200 g. Following IP

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Abbreviations. AFP, α-fetoprotein; DM, daunomycin; $C_{20:4}$, arachidonic acid; $C_{20:0}$, arachidic acid; $C_{18:1}$, oleic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TMS, tetramethyl silane

injection of 10^4 AH66 cells the rats were given an IV injection of 1.0 mg free daunomycin in saline at 3, 5, and 8 days. Similar experiments with daunomycin-fatty acid complexes were performed with a 1.0-mg equivalent of daunomycin in 0.3 ml 95% ethanol. The survival of these animals was noted.

Results

The structure of the DM-$C_{20:4}$ complex used in these experiments is shown in Fig. 1.

The effects of free daunomycin and of its complexes with $C_{20:4}$ and $C_{20:0}$ on the longevity of rats inoculated with AH66 rat hepatoma cells are shown in Fig. 2. Nontreated control rats that received the same amounts of AH66 cells died within 20 days. It can be seen that the complex with saturated fatty acid ($C_{20:0}$) had essentially the same effect as free daunomycin, although a rather wide range of survival times, 12–64 days, was noted. The results with the $C_{20:4}$ complex are striking, 80% of the animals being cured by treatment with this compound.

It was important to ascertain whether or not arachidonic acid had undergone any change during reaction with daunomycin or whether some modification occurs upon mixing of the complex with plasma. The complex appears to remain unsaturated, as evidenced by the results of iodine number determinations summarized in Table 1. As anticipated, a control sample of oleic acid ($C_{18:1}$) gave the theoretical iodine value. The levels for $C_{20:4}$ and DM-$C_{20:4}$ are lower than the theoretical values expected for polyunsaturated fatty acids. However, results based on the NMR spectra shown in Fig. 3

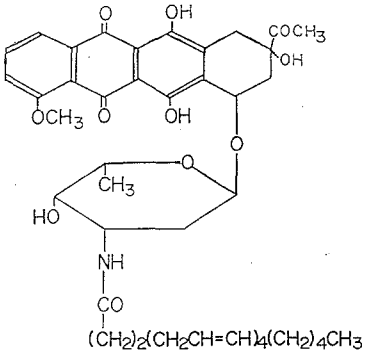

Fig. 1. Structure of daunomycin-arachidonic acid complex (DM-$C_{20:4}$)

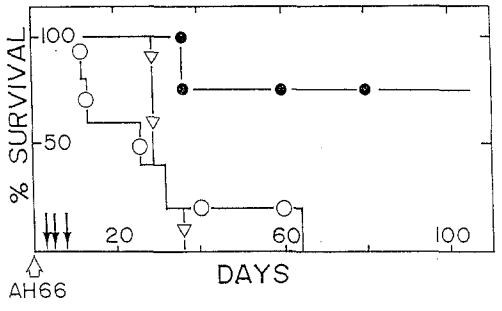

Fig. 2. Chemotherapeutic effectivity of DM-$C_{20:4}$. The *arrows* at the lower left of the figure indicate the times at which injections were given. Five to six rats were used in each group. (●——●), DM-$C_{20:4}$; (▽——▽), DM-$C_{20:0}$; (○——○), DM

Table 1. The number of double bonds determined from iodine values[a]

Fatty acids	$C_{18:1}$	$C_{20:4}$	DM-$C_{20:4}$	DM-$C_{20:0}$
No. of double bonds	0.98	3.3	2.9	0.07

[a] The theoretical number of double bonds is indicated by the last value of the formula in each case

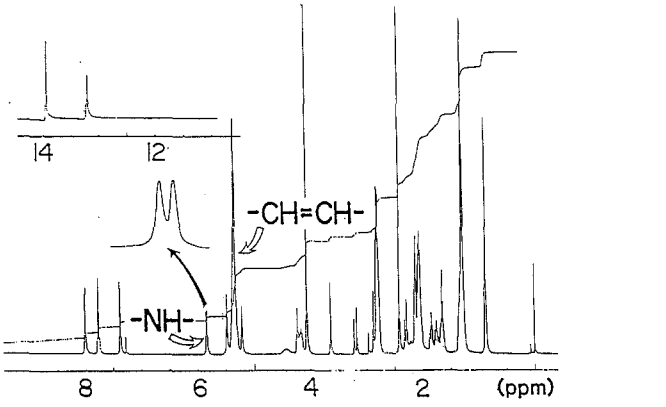

Fig. 3. NMR spectrum of DM-$C_{20:4}$ in deuterated chloroform solution (30 mg/ml) at 400 MHz (TMS as internal standard)

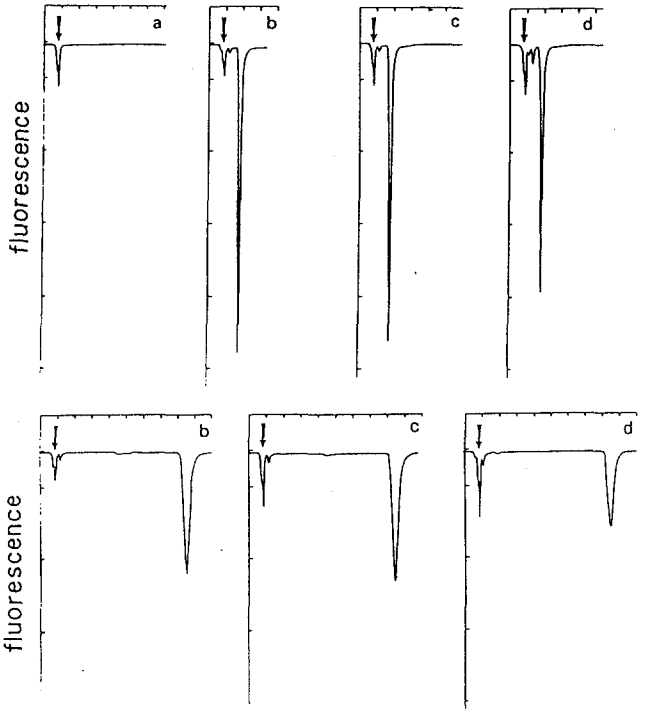

Fig. 4. HPLC resuls for DM *(upper panel)* and for DM-$C_{20:4}$ *(lower panel)*. a, plasma extract; b, standard; c, material extracted after mixing with plasms, d, material extracted after mixing with liver homogenate

indicate that the degree of unsaturation of the fatty acid in DM-$C_{20:4}$ has remained unaltered. Integration of the spectra indicates the presence of eight olefinic protons (multiplet $\sigma = 5.38$) in the complex. The doublet at $\sigma = 5.85$, indicative of an amide nitrogen proton, relates the presence of the fatty acid amido bond with daunomycin.

The actual recovery of the DM-$C_{20:4}$ complex from plasma is essentially the same as the theoretical recovery. This assertion is based on a comparison of the area of the fluorescence peaks of Fig. 4 with the emission of a standard sample. Daunomycin and DM-$C_{20:4}$ are eluted at 2 and 15.4 min, respectively. No fluorescent material is noted in the extract of the control plasma (Fig. 4a). Incubation of daunomycin or of the $C_{20:4}$ complex in plasma or in liver homogenate did not appear to generate any complexes entailing a modification of the fatty acid. In these case of the liver homogenate the recovery of the complex appeared to be about 55% of the theoretical value. However, no products were noted that indicated degradation of the complex.

Discussion

Parmelee et al. [8] first demonstrated that AFP binds long-chain fatty acids as albumin does, but in contrast to albumin, the fetal protein showed a preference for polyunsaturated fatty acids, particularly for arachidonic acid and docosahexaenoic acid. This has been further substantiated by Aussel and Masseyeff [1]. This property of AFP suggested its use in the chemotherapy of tumors. It may be reasonable to infer from the present and previous results [3] that a covalent complex of arachidonic acid with daunomycin has a preferential cytotoxicity against AFP-producing target tumor cells. In this experiment, all hepatoma cell inoculations were given IP and the treatment IV. Nevertheless, the antitumor effect of DM-$C_{20:4}$ was significantly better than that of DM-$C_{20:0}$ and that of free daunomycin. This effect and the result reported in our previous paper [3] probably depend on the affinity of AFP for this acid, which selectively brings the daunomycin complex to the hepatoma cells.

Another possible approach by which our study might be extended is the utilization of arachidonic acid as a membrane component and prostaglandin precursor. Ferber and Resch [5] have described a plasma membrane-associated enzyme that catalyzes the incorporation of coenzyme A derivatives of fatty acids into phospholipid. This enzyme has a high affinity for polyunsaturated fatty acids, particularly arachidonic acid. The ability of such an enzyme to act as a transferase could explain the preferential incorporation of arachidonate into phospholipids of cells. These phenomena may be related to the transport of DM-$C_{20:4}$ through the cell membrane of the hepatoma cell after its transport there by AFP. Alternatively, AFP may have such a membrane transport function in cells which produce this protein. Prostaglandin E derivatives are known to inhibit the growth of various tumor cell lines both in vitro and in vivo [9]. Its formation from DM-$C_{20:4}$ would require release of the arachidonic acid following its uptake by the hepatoma cell. Further experiments will be required to clarify the mechanisms of tumor inhibition by DM-$C_{20:4}$.

It appears that the arachidonic acid of the complex is an essential feature of the biological action on tumors. The iodine numbers suggest that no change in unsaturation in the fatty acid moiety results from formation of the complex or from admixture of the complex with plasma, even though low values were obtained. This determination is somewhat limited because the addition of iodine to polyethenoic acid is not complete, even though mono- and diethenoic acids give fairly quantitative results [11]. The NMR studies, however, clearly indicate that the DM-$C_{20:4}$ complex contains four double bonds.

The recovery experiments for DM-$C_{20:4}$ from plasma and liver homogenate indicated that the latter was mediating changes in the complex or that it could not be adequately extracted from this material. Its admixture with plasma caused no apparent change in the complex.

The derivatives of daunomycin or the closely related adriamycin with polyunsaturated fatty acids appear to present interesting compounds for clinical use as a new type of 'missile' for hepatomas, since the antitumor effects of daunomycin appeared to be greatly enhanced by its combination with the fatty acid moiety. Furthermore, the toxic effects of free daunomycin appear to be markedly diminished in this complex [4]. This implies that relatively large amounts of the complex can be administered over longer periods of time, with the hope of better chemotherapeutic effects.

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