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Choline and halogen derivatives of CMA (9-oxo-10-acridine acetic acids) as tools for monitoring the interaction of the interferon inducers via a specific receptor

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Summary

New choline and halogen derivatives of CMA (9-oxo-10-acridine acetic acid) were investigated as interferon (IFN) inducers in mice and in the mouse bone marrow-derived macrophage cultures. Two of the choline derivatives, DMCMA and CSCMA, were active IFN inducers presumably because they were hydrolyzed so as to release CMA. The halogen analogues of CMA were inactive or weak IFN inducers in vivo and in vitro. On the contrary, the Br and I derivatives of CMA were potent inhibitors of IFN induction by CMA in vitro. The behavior of the agonists and antagonists of CMA suggests that the induction of interferon may occur indirectly via a specific CMA-receptor complex.

Interferon inducers; IFN-inducer receptors; Agonists and antagonists of IFN inducers

Introduction

In our previous work on synthesis and mode of action of the low molecular weight interferon (IFN) inducers related to tilorone and 9-oxo-10-acridine acetic acid (CMA) we found that the activity of CMA was enhanced by administration in vivo or in vitro of some of its inactive analogues (Szulc et al., 1984, 1985; Inglot et al.,

1985). We also observed that CMA strongly bound to serum albumins and only small amounts of IFN inducer were able to reach as such the target cells (Piasecki et al., 1985). We suggested that CMA induced IFN indirectly via a specific receptor, whereas albumin played the role of a transport protein (Inglot et al., 1985; Piasecki et al., 1985). Recently, we have synthetized a number of novel CMA analogues including compounds that could be bioactivated (Szulc and Mlochowski, 1985).

Our experimental approach has been similar to that generally known in chemotherapy, and has already been adopted to antiviral agents. For example, acyclovir (Colla et al., 1983) and salicylates (Inglot and Kochnam, 1966) have been used in the form of water-soluble esters as prodrugs and some of them showed antiviral activity higher than that shown by the parent compounds.

In this paper we report that one of the CMA derivatives, DMCMA, is active as an IFN inducer presumably because of its ability to be hydrolyzed so as to release CMA. We also describe the biological properties of several new CMA analogues three of which proved as active as IFN inducers as the parent compound.

Materials and Methods

Compounds

CMA and analogues were synthetized as described previously (Inglot et al., 1985; Szulc and Mlochowski, 1985). The compounds were dissolved in water. For use in the biological experiments the dilutions of the drugs were prepared in the maintenance medium.

Abbreviations

CMA: 9-oxo-10-acridine acetic acid sodium salt ($R=Na^+$); CSCMA: cholinium 9-oxo-10-acridine acetate ($R=CH_2CH_2N^+(CH_3)_3$); CCMA: cholinium 9-oxo-10-acridine acetate chloride ($R_1=OCH_2CH_2N(CH_3)_3Cl$, $R_2=R_3=H$); DMCMA: (*N,N*-dimethylamino)ethyl ester of 9-oxo-10-acridine acetic acid hydrochloride ($R_1=OCH_2CH_2N(CH_3)_2\cdot HCl$, $R_2=R_3=H$); DEAMA: (*N,N*-diethylamino)ethylamide of 9-oxo-10-acridine acetic acid hydrochloride ($R_1=NHCH_2CH_2N(C_2H_5)_2\cdot HCl$, $R_2=R_3=H$); MCLCMA: sodium 2-chloro-9-oxo-10-acridine acetate ($R_1=ONa$, $R_2=Cl$, $R_3=H$); DCLCMA: sodium 2,7-dichloro-9-oxo-10-acridine acetate ($R_1=ONa$, $R_2=R_3=Cl$); MBCMA: sodium 2-bromo-9-oxo-10-acridine acetate ($R_1=ONa$, $R_2=Br$, $R_3=H$); DBCMA: sodium 2,7-dibromo-9-oxo-10-acridine acetate ($R_1=ONa$, $R_2=R_3=Br$); MICMA: sodium 2-iodo-9-oxo-10-acridine acetate ($R_1=ONa$, $R_2=I$, $R_3=H$); DICMA: sodium 2,7-diiodo-9-oxo-10-acridine acetate ($R_1=ONa$, $R_2=R_3=I$).

Determination of stability CCMA and DMCMA

CCMA, DMCMA and CMA were subjected to thin-layer chromatography on silica gel plates (60 F_{254}, Merck) developed in methanol/ethyl acetate (1:1, v/v). Under these conditions the R_f value of CMA was 0.8, whereas CCMA and

DMCMA remained at the starting points. Both CCMA and DMCMA were highly unstable when kept in buffered solution at pH 7.0, they were readily hydrolized to CMA.

The thermal stability of CCMA and DMCMA was therefore quantitatively investigated in 5 mM Tris-HCl (pH 7.0) at 37°C by measuring the relative increase of fluorescence intensity versus time. Relative fluorescence was measured at 445 nm (one of the maxima of CMA fluorescence spectrum (Szulc and Mlochowski, 1985)) after exitation at 380 nm. The half-life time was estimated as 36 h and 2.2 h for CCMA and DMCMA, respectively.

Media

The macrophage growth medium consisted of Eagle's minimum essential medium (MEM) supplemented with 20% heat-inactivated calf serum, 200 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol (Loba-Chemie), 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamycin. For the macrophage cultures 20% of conditioned medium from L_{929} cell cultures was added as a source of colony-stimulating factor (van Furth et al., 1982). For the experiments with IFN inducers the maintenance medium contained 2.5% heated calf serum, L-glutamine and antibiotics.

Induction of IFN in mice

Mice were obtained from the Animal Breeding Center of this Institute. Male Balb/c mice, 6–10 weeks old, weighing 24–28 g were given various doses of the compounds either intraperitoneally (i.p.) or perorally (p.o., by gavage). Serum samples were collected by retroorbital bleeding of animals, usually 2 h after i.p. or 24 h after peroral administration of the compounds.

Induction of IFN in bone marrow-derived macrophages

Bone marrow cells were obtained from the femurs of 6–12-week-old male C57B1/6 mice, as described by Meerpohl et al. (1976) and modified by Klimetzek and Remold (1980). The bones were removed and dissected from adherent tissues. A syringe tip was inserted into the bone ends and the marrow was flushed out with 2 ml of Hank's balanced salt solution (HBSS). A single cell suspension was prepared by repeated forcing of the cell clusters through a thin needle. The cells were washed twice with cold HBSS, centrifuged at 500 × g for 10 min at 4°C and suspended in the macrophage growth medium. The viable cell counts were performed with trypan blue exclusion method. Bone marrow cells were seeded into plastic plates (Costar) at a density of 2×10^6/ml per well, and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. The nonadherent cells were washed twice with warm HBSS, and one ml fresh medium was added. Cell cultures were fed twice a week starting from the fourth day. Ten-day-old esterase-positive macrophage cultures were used. Bone marrow macrophages were washed twice with warm HBSS. The cells were incubated in one ml of the maintenance medium containing the appropriate amounts of the compounds for 2 h at 37°C in a CO_2 incubator. After treatment with the compounds, the cells were again washed twice

with warm HBSS and resuspended in 1 ml fresh maintenance medium. The supernatants were collected from cultures incubated for 20 hr at 37°C and assayed for IFN activity.

IFN assay

The Borgen subline of L_{929} cells, very sensitive to mouse IFN, was used for the determination of antiviral activity. The cell line was obtained from Dr. E. Lundgren (Umeå, Sweden). Vesicular stomatitis virus (VSV)-induced cytopathogenicity was assessed in 96-well microtrays, according to well established procedures (Stewart, 1979). In our assays, one IFN unit was approximately equal to one reference IFN unit. The US National Institutes of Health reference mouse IFN G002-904-511 was used to standardize our laboratory IFN preparation obtained from C_{243} cells induced with Newcastle disease virus.

Results

Activities of new CMA analogues

Table 1 shows the results for IFN induction by our new CMA analogues in vivo and in vitro. Only three compounds (DMCMA, CSCMA and MCLCMA) induced IFN in mouse bone marrow-derived macrophage cultures. However, three mono-

TABLE 1

Activity of CMA analogues as IFN inducers

Compound	LD_{50}[a] (mg/kg)	Cytotoxicity[b] (μg/ml)	IFN induced[c] in the mouse serum (log_{10} units/ml ± S.D.)	IFN induced[d] in the supernatant fluid of macrophage cultures (log_{10} units/ml ± S.D.)
CCMA	~ 200	1660	< 1.0	< 1.0
DMCMA	~ 500	830	2.5 ± 0.41	2.0 ± 0.0
CSCMA	N.D.	1660	N.D.	2.25 ± 0.25
MCLCMA	> 400	830	3.07 ± 0.32	1.75 ± 0.43
DCLCMA	~ 400	200	< 1.0	< 1.0
MBCMA	> 400	400	3.00 ± 0.41	< 1.0
DBCMA	~ 600	200	1.50 ± 0.87	< 1.0
MICMA	> 400	1660	2.67 ± 0.37	< 1.0
DICMA	> 400	400	< 1.0	< 1.0
DEAMA	~ 300	200	< 1.0	< 1.0
CMA	1516	>1100	4.1 ± 0.23	3.5 ± 0.50

[a] Determined in Balb/c mice after i.p. administration of the compounds. The LD_{50} for CMA is taken from reference (Kramer et al., 1981).
N.D.: not done. The other values are only approximate.

[b] Determined in mouse L cells by the microassay described in Methods.

[c] Determined in Balb/c mice 2 hrs after p.o. or i.p. administration of 400 mg/kg of the compounds, except for DBCMA, which was used at a dose of 600 mg/kg.

[d] Determined in the bone marrow-derived macrophage cultures treated with 500 μg/ml of the compounds, except for DBCMA, which was used at a concentration of 200 μg/ml.

halogen derivatives of CMA (MCLCMA, MBCMA and MICMA), one disubstituted analogue (DBCMA), and DMCMA were found to induce IFN in mice. The disubstituted analogues DCLCMA and DICMA, and compound DEAMA, were inactive in vivo and in vitro.

The ability to induce IFN was not related to toxicity of the compounds. For instance, CCMA which is inactive as an IFN inducer in vivo and in vitro was more toxic than CMA. Only the approximate LD_{50} values were determined, because of the limited supply of compounds and animals.

The IFN response to CMA and analogues was dose-related both in vivo and in vitro. The optimal concentration of CMA, DMCMA, CSCMA and MCLCMA for interferon induction in the macrophage cultures was approximately 500 μg/ml (Table 1).

Effects of combined administration of CMA and analogues on the IFN response in vivo and in vitro

Recently, we observed that several inactive analogues of CMA enhanced serum IFN response after the administration of a suboptimal dose of CMA (Inglot et al., 1985; Szulc et al., 1985). Therefore, it seemed interesting to determine whether the new analogues of CMA also had such activity. CCMA was selected for the experiments in vivo because it was inactive as an IFN inducer when administered alone. Results shown in Table 2 indicate that when CCMA (100 mg/kg, p.o.) was combined with CMA (500 mg/kg, p.o.) the 4-h serum IFN response to CMA was enhanced by approximately 60-fold. Another CMA analogue, DBCMA, was the most active enhancer following i.p. administration (Table 2).

All three CMA analogues CSCMA, DMCMA and CCMA showed an additive effect on the IFN response to CMA in macrophage cultures (Table 3). Similar activity was also displayed by DEAMA (data not shown).

The di-bromo analogue of CMA (DBCMA) completely inhibited the IFN re-

TABLE 2

Serum IFN response in mice following peroral or intraperitoneal administration of CMA and its analogues

Exp. No.	Inducer	Dose (mg/kg)	Serum IFN (log_{10} units/ml ± S.D.)
1	CMA	500	1.5 ± 0.0
	CCMA	100	< 1.0
	CMA + CCMA	500 + 100	3.33 ± 0.47
2	CMA	200	3.11 ± 0.52
	DBCMA	200	1.25 ± 0.25
	CMA + DBCMA	200 + 200	4.25 ± 0.25

In exp. 1 serum was collected for IFN assay 4 hrs after p.o. administration of the compounds at the indicated doses.
In exp. 2 serum was collected for IFN assay 2 hrs after i.p. administration of the compounds at the indicated doses.

TABLE 3

IFN response of mouse bone marrow-derived macrophage cultures following exposure to CMA and its choline substituted analogues

Compound	Concentration (μg/ml)	IFN (log_{10} units/ml ± S.D.)
CMA	50	<1.00 ± 0.00
	100	1.0 ± 0.50
	200	1.66 ± 0.47
CSCMA	200	1.83 ± 0.23
DMCMA	200	2.16 ± 0.62
CCMA	200	<1.0 ± 0.0
CMA + CSCMA	50 + 200	2.5 ± 0.0
CMA + DMCMA	50 + 200	2.25 ± 0.43
CMA + CSCMA	100 + 200	2.66 ± 0.23
CMA + DMCMA	100 + 200	2.37 ± 0.41
CMA + CSCMA	200 + 200	2.83 ± 0.47
CMA + DMCMA	200 + 200	2.33 ± 0.55
CMA + CCMA	200 + 200	2.66 ± 0.23

Supernatant fluids from the control untreated cultures contained < 10 units of IFN/ml.

TABLE 4

IFN response of mouse bone marrow-derived macrophage cultures following exposure to CMA and its halogen-substituted analogues

Exp. No.	Compound	Concentration (μg/ml)	IFN (log_{10} units/ml)
1	CMA	200	2.5
	MBCMA	200	<1.0
		20	<1.0
	CMA + MBCMA	200 + 200	<1.0
		200 + 100	2.0
		200 + 50	2.5
		200 + 25	2.5
		200 + 12.5	2.0
2	CMA	200	2.0
	DBCMA	200	<1.0
		50	<1.0
	CMA + DBCMA	200 + 200	<1.0
		200 + 100	<1.0
		200 + 50	<1.0
		200 + 5	<1.0
		200 + 2.5	1.0
		200 + 1.25	2.0
		200 + 0.6	2.0
3	CMA	200	2.0
	DICMA	200	<1.0
	CMA + DICMA	200 + 200	<1.0

Supernatant fluids from the control untreated cultures contained < 10 units of IFN/ml.

sponse to CMA in the concentration range from 5.0 to 200 μg/ml. At these doses DBCMA was non-toxic for the macrophages as well as mouse L cells. Two other halogen derivatives of CMA, MBCMA and DICMA, were weaker as inhibitors of CMA-induced IFN synthesis than was DBCMA (Table 4).

Discussion

A series of novel derivatives of CMA have been tested as IFN inducers in view of the possibility of their hormone-like, i.e. receptor-mediated, mode of action (Inglot et al., 1985; Piasecki et al., 1985; Szulc et al., 1985). It appears that the parent compound, CMA, is the most potent IFN inducer and none of its analogues surpasses its activity in vitro or in vivo. The monohalogens (Cl, Br or I) derivatives of CMA were better IFN inducers in mice than the bi-substituted halogen derivatives. Presumably, the former compounds fit better with the putative cellular receptor than the latter (Inglot et al., 1985; Piasecki et al., 1985).

DBCMA was a potent inhibitor of IFN induction in the bone marrow-derived macrophages cultured in vitro. This effect can best be explained in terms of competition at the cellular receptor, as suggested previously (Inglot et al., 1985; Piasecki et al., 1985; Szulc et al., 1985). MBCMA and DICMA were weaker inhibitors of IFN induction by CMA than DBCMA. We have already described the characteristic binding of CMA and its analogues with serum albumins using three different biochemical assays. We have also suggested that albumins play a role as transport protein for the compounds (Piasecki et al., 1985).

The inactive analogues of CMA can enhance the IFN response to CMA, as shown in this and our previous papers (Inglot et al., 1985; Szulc et al., 1985). As a rule, biologically active drugs react with target cells via membrane, cytoplasmic or intranuclear receptors. Transport proteins, such as albumin, having a high affinity for the ligand do not play a role in cell activation but only bind and transport the ligand. The cellular receptor not only has high affinity for the ligand but is also capable of cell activation. We have previously suggested that the induction of IFN by CMA and analogues is such a receptor-mediated event. The enhancement of the IFN response by the inactive analogues may be due to a competitive effect for binding to the transport molecule(s) (albumin) for CMA (albumins) (Inglot, 1984), making more ligand (CMA) available for interaction with the specific cellular receptor.

Our observations are compatible with the concept that CMA and its analogues act via a putative IFN-inducer receptor. A relatively short pulse with CMA (analogues) suffices for eliciting an IFN response in macrophages, the main target cells (Storch and Kirchner, 1982; Szulc et al., 1985). CMA also induces interferon-β in interleukin-2-stimulated mouse T cells (Storch et al., 1986). Differences in the interferon-inducing activities in vivo and in vitro can be rationalized in terms of the relatively greater number of receptors available in vivo, and the relatively greater chance for biotransformation, which would lead to metabolites fitting with the putative receptor.

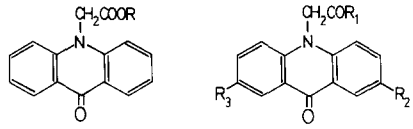

Fig. 1. Chemical structures of CMA and analogues.

The results discussed above shed some light on the relationship between molecular structure and biological response. The IFN response decreased in the order CMA > CSCMA > DMCMA > CCMA > DEAMA. This order is consistent with the susceptibility toward hydrolysis and release of active form. For example, DMCMA is more readily hydrolyzed and also more potent as an inducer of IFN than CCMA. The active compounds must have free acetate groups which are easily regenerated from the protected form (Fig. 1).

Structural modification of CMA other than halogenation (Fig. 1) leads to complete loss of activity. Among the halogen substituted derivatives of CMA, steric factors are of crucial importance. An increase in the size and number of halogen atoms leads to a decrease in both the IFN-inducing ability and antagonizing effect towards CMA.

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