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METABOLIC STUDIES ON DIPHENYLSULFONE DERIVATIVES IN CHICK MACROPHAGES

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Abstract—The activity of 1-[4-(4-sulfanilyl)phenyl]urea, a compound previously found to decrease visceral lesions and mortality in chickens infected with Marek's disease virus, was investigated using chick peritoneal macrophages. The material showed no effect on the biosynthesis of DNA, RNA or protein but markedly inhibited the synthesis of phosphatidylcholine. The site of action in this pathway was determined to be subsequent to the formation of phosphorylcholine, and on the steps involving the synthesis of either cytidinediphosphate choline or phosphatidyl choline. Several derivatives of diphenylsulfone were also examined for their effects on choline incorporation. The analogues that were found to be active in the macrophage system *in vitro* closely paralleled those that were found to be active in the Marek's disease assay *in vivo*.

Marek's disease, caused by a DNA herpes-type virus [1–3], is a neoplastic lymphoproliferative disorder in chickens affecting primarily liver, spleen, gonads, kidneys and peripheral nerves. It is generally considered to be the most serious infectious condition in domestic chickens; hence, much effort has been expended in the past toward discovering effective means of controlling the disease. In this respect, considerable progress was made recently with the development of Marek's disease virus vaccine [4, 5]. This vaccine, although highly beneficial, does not provide a complete cure, since infectious virions are known to persist in the treated chickens. The use of simple chemical compounds for treatment of the disease or to supplement the use of vaccine has met thus far with only limited success. For example, L-*O*-ethylthreonine was found in these laboratories to decrease the incidence of mortality in chickens infected with the virus.* This substance, unfortunately, was potentially toxic to the birds by virtue of its being incorporated into tissue proteins as an analog of L-isoleucine [6–8]. More recently, a totally different class of compounds, diphenylsulfone derivatives, was reported to markedly decrease visceral lesions and mortality in infected chickens at doses that were nontoxic to the host [9]. The metabolism of these substances, particularly in chickens, that may account for their therapeutic efficacy is not as yet known. The purpose of this paper is to describe some aspects of the activity of sulfone derivatives in a chick tissue and discuss possible relevance of these findings to Marek's disease.

* T. A. Maag and T. Y. Shen, unpublished observation.

MATERIALS AND METHODS

Materials

Choline-1,2-^{14}C (50 μCi/1·4 mg), glycerol-U-^{14}C (50 μCi/0·15 mg), L-leucine-1-^{14}C (250 μCi/1·28 mg), uridine-2-^{14}C (50 μCi/0·07 mg), thymidine-2-^{14}C (50 μCi/0·28 mg), ethanolamine-1,2-^{14}C (100 μCi/2·56 mg), L-serine-U-^{14}C (100 μCi/1·0 mg) and Na acetate-2-^{14}C (1 μCi/1·59 mg) were obtained from New England Nuclear Corp. Silica gel G (250 μ thickness) was purchased from Analtech, Inc. Phosphorylcholine (P-choline), cytidinediphosphate choline (CDP-choline) and L-α-lecithin (β,γ-dipalmitoyl) were obtained from CalBiochem.

The diphenylsulfone derivatives, synthesized in the laboratories of Dr. T. Y. Shen, were dissolved in dimethylsulfoxide at 20 mg/ml. In experiments using these compounds, the concentration of dimethylsulfoxide in the reaction mixture was always less than 0·2%.

Chick peritoneal cells

Two- to two-and-a-half-week-old Vantress-Arbor Acre chicks obtained from Kerr Hatcheries Co., Inc., Frenchtown, N.J., were used to obtain peritoneal cells. Eight ml of 10% soluble starch was injected into the peritoneal cavity, and after 2 days the birds were killed by asphyxiation in CO_2. The abdominal surface was sponged with 70% ethanol, the skin reflected and 10 ml of Medium 199 was injected into the peritoneal cavity. The abdomen was gently massaged in order to mix the fluid in the peritoneal cavity. The fluid was then aspirated with a sterile Pasteur pipette and kept at 0–5°. Under these conditions, 2–5 × 10^7 cells were collected

from each chick. Exudate cells from four to five chicks were combined and used for each experiment.

Cell culture

The peritoneal cells were cultured as monolayers in Medium 199 containing 100 units penicillin/ml, 100 μg streptomycin/ml and 10% fetal calf serum. Specified numbers of peritoneal cells as described in the legends were transferred to either 16 × 125 mm glass tubes or 30-ml Falcon plastic bottles and incubated at 37° under 5% CO_2 95% air mixture. After 1 hr, the medium containing non-adherent cells was aspirated and discarded when adherent cells were being studied. The cells attached to the glass or plastic surfaces, designated macrophages, were used in most experiments. For comparative purposes, a few experiments were also done with non-adherent cells. One ml of fresh Medium 199 containing appropriate amounts of radioactive precursors and compounds under investigation was added to the macrophage monolayer culture. After incubation at 37° for a specified time, the medium was discarded and the macrophage monolayers were processed for radioactivity measurements as follows. The adherent cells were detached from the surface with 0·5 ml concentrated formic acid, mixed with 5 ml cold 5% trichloroacetic acid (TCA) and transferred to Millipore filters (0·8 μm). The precipitates were washed thoroughly with cold 5% TCA, dried and radioactivity was determined in a Packard liquid scintillation spectrometer.

Chromatography and analysis of phospholipids

To determine the effects of a diphenylsulfone derivative on the biosynthesis of phospholipids, the intermediates present in both the acid-soluble and acid-insoluble fractions from experiments as described under "Cell culture" were separated and analyzed as follows. The cold 5% TCA-soluble fraction was extracted three times with 3–4 vol. of ether and the aqueous phase was evaporated to dryness under a stream of N_2 at 30–35°. The residue was dissolved in 0·2 ml H_2O and 0·025 ml was applied on Whatman 3 MM filter paper and developed with solvent (A) *n*-butanol–acetic acid–H_2O (5:2:3) according to Schneider *et al.* [10] or solvent (B) isopropanol–H_2O (8:2) according to Schneider and Rotherham [11]. Standard phosphorylcholine or CDP-choline was used as markers. Phosphorylcholine was identified by phosphate analysis [12] and by R_f values compared to the standard compound. Cytidinediphosphate choline was identified by its fluorescence under ultraviolet light and by R_f values as compared to the standard substance.

For measurement of phosphatidylcholine synthesis, the cold 5% TCA-insoluble precipitate was extracted twice with 3 ml of 80% ethanol and twice with 3 ml ethanol–ether (3:1) at room temperature. The lipid extracts were combined and evaporated to dryness under N_2 at 30–35°. The residue was taken up in 0·2 ml of 80% ethanol and 0·025 ml was applied on Silica gel G and developed with solvent (C) chloroform–methanol–H_2O (85:35:4) according to Wagner *et al.* [13], or solvent (D) *n*-propanol–12 N ammonia (80:20) according to Jatzkewitz and Mehl [14]. Standard L-α-lecithin was co-chromatographed as marker. Lecithin was identified by its migration in two solvent systems as compared to the standard and also by staining with rhodamine [14].

One-cm sections of paper or Silica gel G containing the appropriate phospholipid intermediate were transferred to vials containing toluene–liquifluor and measured for radioactivity.

RESULTS

Since 1-[4-(4-sulfanilyl)phenyl]urea (AUS, compound 1 in Table 3) was previously found to be efficacious in the Marek's disease assay *in vivo* [9], it was chosen for our initial exploratory studies. To serve as chick cells for these experiments, peritoneal macrophages were selected not simply because the peritoneal cavity is a convenient source of cells but also because macrophages have been implicated to play an active role in immune processes of herpes simplex virus infections [15].

The effects of AUS on the biosynthesis of various macromolecules in macrophages, as measured by the incorporation of appropriate precursors into cold 5% TCA-insoluble precipitates, are shown in Table 1. The compound showed no effect on the biosynthesis of DNA or protein and only a slight inhibitory effect at higher concentrations on the formation of RNA. The most conspicuous aspect of this chart was the marked suppression of choline uptake, presumably into phosphatidylcholine. This antagonistic action against the metabolism of choline was, in addition, extremely selective, since several other precursors of phospholipid or neutral lipid synthesis such as L-serine, ethanolamine and acetate were unaffected by AUS.

Table 1. Effects of AUS on the incorporation of labeled precursors into cold 5% TCA-insoluble materials of chick macrophages*

Labeled precursors	% Inhibition of incorporation AUS (μg/ml) 10	20	40
Choline	46	53	67
Uridine	0	5	19

* Monolayer cultures of chick peritoneal macrophages in 1 ml containing 8 × 10^5 cells were incubated for 18–20 hr at 37° with indicated amounts of radioactive precursors with or without AUS. The amounts of labeled substances used/ml were: choline (0·005 μCi/0·14 μg), glycerol (0·01 μCi/0·03 μg), ethanolamine (0·01 μCi/0·20 μg), acetate (0·20 μCi/0·32 mg), L-serine (0·10 μCi/0·7 μg), thymidine (0·005 μCi/0·03 μg), uridine (0·01 μCi/0·02 μg) and L-leucine (0·005 μCi/0·01 μg). After incubation, the macrophages were processed as described in the text. Although not shown below, there was no effect on the uptake of glycerol, ethanolamine, acetate, L-serine, thymidine and L-leucine.

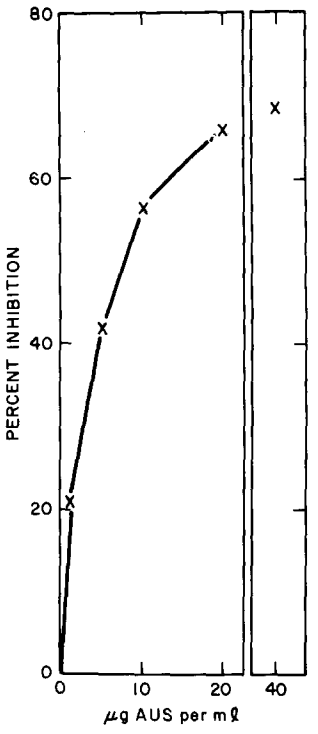


Fig. 1. Effects of increasing concentrations of AUS on the incorporation of labeled choline into cold 5% TCA-insoluble materials in chick macrophages. Monolayer cultures of chick peritoneal macrophages in 1 ml containing 8×10^5 cells were incubated for 20 hr at 37° with choline-1,2-^{14}C (0·005 μCi/0·14 μg) and varying amounts of AUS. After incubation, the macrophages were processed as described in text.

A dose response study demonstrated that the inhibitory effect on choline uptake could be detected at concentrations as low as 1 μg AUS/ml (Fig. 1). For comparative purposes, similar studies were done with the non-adherent cell population of chick peritoneal exudate. At 1, 10 and 20 μg AUS/ml, the per cent inhibition of choline incorporation into non-adherent cells was 10, 38 and 50 respectively. One may conclude from these observations that AUS is a selective inhibitor of choline incorporation and furthermore, the various types of cells in chick peritoneal cavity were equally sensitive to the inhibitory action of this substance.

Site of action of AUS

The results obtained thus far suggested that the inhibitory activity of AUS was primarily on the biosynthesis of lecithin via the salvage pathway; the *de novo* route in which the incorporation of ethanolamine was followed by methylation of phosphatidylethanolamine to form lecithin appeared not to be affected by AUS. Hence, the site of action of the inhibitor on the incorporation of labeled choline into phosphatidylcholine in intact macrophages was investigated.

The data from three experiments are shown in Table 2A. Lecithin was separated by elution with solvent (C). The R_f values of both standard lecithin and the radioactive material varied from 0·31 to 0·34. The results showed that the synthesis of lecithin was markedly suppressed by AUS, and the degree of inhibition obtained in the first two experiments corresponded to those observed earlier for the inhibition of choline incorporation into cold 5% TCA-insoluble precipitates. The slightly lower per cent inhibitions in Expt. 3 was probably due to the use of 2-fold increase in cell concentration. Similar results were obtained with solvent (D) in which the R_f of lecithin was 0·25. In the absence of the inhibitor, the per cent of added choline converted to phosphatidylcholine under these conditions was calculated to be 9–10 per cent. Radioactive materials corresponding to lysolecithin or sphingomyelin were not detected.

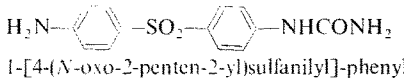
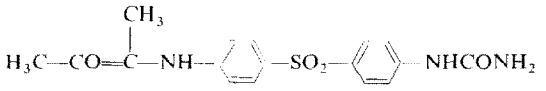
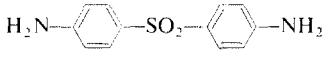
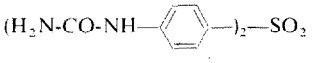
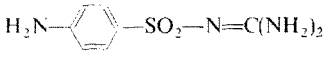
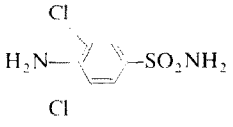
In the same experiment, attempts were made to isolate the acid-soluble intermediates of lecithin synthesis (phosphorylcholine and cytidinediphosphate choline) by means of paper chromatography. Solvent (A) was used for these experiments (Table 2B), and the R_f of both standard P-choline and radioactive zone from the reaction mixtures varied from 0·38 to 0·39. The substances in the R_f 0·38 regions also reacted with molybdate [10]. In the absence of AUS, approximately 50

Table 2. Effects of AUS on the incorporation of choline-^{14}C into phospholipids in macrophages*

	Expt. 1		Expt. 2		Expt. 3	
	(cpm)	(% Inhib.)	(cpm)	(% Inhib.)	(cpm)	(%Inhib.)
A. Lecithin						
Control	1190		1190		1270	
Plus 10 μg/ml AUS	760	36	710	40	880	31
Plus 20 μg/ml AUS			460	61	640	50
B. Phosphorylcholine						
Control	1180		1710		1410	
Plus 10 μg/ml AUS	1140	0	2000	0	1840	0
Plus 20 μg/ml AUS			2070	0	1880	0

* Monolayer cultures of chick macrophages (2 ml containing 1×10^7 cells for Expts. 1 and 2; 2×10^7 cells for Expt. 3) were incubated for 20 hr at 37° with 8·8 μg choline-1,2-^{14}C (0·125 μCi) and indicated amounts of AUS. The phospholipids were isolated as described in text.

Table 3. Comparative effects of diphenylsulfone derivatives in macrophage *in vitro* and Marek's disease tests *in vivo*.*

No.	Compound	% Inhibition of incorporation of choline	Marek's disease assay
1	1-[4-(4-Sulfanilyl)phenyl]urea 	56, 66, 46, 55	0·0005
2	1-[4-(*N*-oxo-2-penten-2-yl)sulfanilyl]-phenyl urea 	47, 47, 50, 42	0·0010
3	*p,p'*-Diaminodiphenyl sulfone 	60, 53, 49, 53	0·0050
4	4,4'-Bisureidodiphenyl sulfone 	24, 25, 25, 24	0·0050
5	Sulfaguanidine 	0, 0, 0	0·0050
6	3,5-Dichlorosulfanilamide 	0, 0, 2	0·0200
7	*p,p'*-Sulfanyl-bis(1-*p*-hydroxyphenyl)-3-phenyl urea 	0, 3, 0	0·0200

* Macrophage experiments *in vitro* were done in the same manner as described in the legend to Table 1, using 10 μg/ml of test substance. Each tube contained 0·005 μCi/0·14 μg of choline-^{14}C. The results obtained from several experiments are listed for each compound. The Marek's disease experiments *in vivo* were done by Drs. B. J. Skelly and T. A. Maag, and a detailed account of this procedure will be described in a separate publication. The numbers in the column under "Marek's disease assay" represent the per cent composition of each derivative in the diet that gave arbitrarily chosen "positive index" or "cure". The values are statistically significant with P equal or less than 0·05.

per cent of the added choline-^{14}C was converted into P-choline. As shown in the table, the synthesis of this intermediate was not affected by AUS. Instead, the presence of the sulfone caused a significant accumulation of P-choline (Expts. 2 and 3), suggesting that the site of action of AUS was subsequent to the formation of phosphorylcholine. One may also conclude that the transport of choline through the cytoplasmic membrane was not affected by AUS. Similar results were obtained with solvent (B) in which the R_f of phosphorylcholine was 0·25 to 0·27.

In these experiments with intact macrophages, there was no trace of CDP-choline accumulation in the soluble fraction either in the presence or absence of AUS. For this reason, in order to determine if the site of inhibition was on the synthesis of CDP-choline or on the subsequent step leading to the formation of phosphatidylcholine, attempts were made to study these two reactions in a cell-free system. The procedure described by Kennedy and Weiss [16] for rat liver supernatant fluid and mitochondria was used. These two reactions, however, could not be measured in macrophage cell-free preparations, possibly due to the liberation of lysosomal enzymes during homogenization of macrophages. Hence, further elucidation of the site of action of AUS may require purification of the appropriate

enzyme phosphorylcholinecytidyl transferase or phosphorylcholine-glyceride transferase from chick macrophages.

Diphenylsulfone derivatives

A number of compounds structurally related to AUS that were previously examined for efficacy in the Marek's disease assay *in vivo* [9] were tested for their effects on the incorporation of labeled choline into cold 5% TCA-insoluble precipitates in whole macrophages (Table 3). The numerical values under "Marek's disease assay" represent the per cent composition of sulfone derivatives in the diet required to achieve an arbitrarily chosen level of "positive index". For clarity, the compounds in the table are listed in order of decreasing efficacy in the Marek's disease assay *in vivo*; the results from the assay *in vivo* were then compared with those from the macrophage assay *in vitro*. With respect to the macrophage test system, compounds 2 and 3 were found to be almost or equally as active as AUS (compound 1). Compound 4 was moderately active, while the remainder of the derivatives were essentially inactive. It can be seen that there is fairly good correlation between efficacy in the assay *in vivo* and the inhibitory effects on choline uptake in the tests *in vitro*.

DISCUSSION

The comparative results shown in Table 3, in conjunction with the previously demonstrated ineffectiveness of AUS against DNA, RNA and protein synthesis, suggested that the disease in chickens caused by the herpes-type virus may in some manner be related to or dependent upon uninterrupted synthesis of lecithin in host cells, presumably of the synthesis of lipoproteins present in various membranous components of the cell. The indispensable role of membrane lipids in the normal physiology of virus growth or the close relationship of membrane turnover with virus multiplication has been shown on a number of occasions, both in animal cells [17–19] and in bacteria [20, 21]. For example, lecithin synthesis in rat hepatoma cells infected with mengovirus [18] and acetate-^{14}C incorporation into neutral lipids in HeLa cells infected with adenovirus [19] were found to increase soon after infection. Furthermore, membranes containing lipids appeared to be involved in all stages of virus infection, from penetration into the cell to the final assembly of infectious virions. In the present work, one may speculate that interference of normal choline metabolism by AUS resulted in lack of formation of lipoprotein membranes essential for Marek's disease virus growth. The precise manner in which AUS and other diphenylsulfone derivatives may suppress growth or function of Marek's disease virus remains to be elucidated.

In another area of chemotherapy, it is interesting to note that one of the sulfones (*p,p'*-diaminodiphenyl sufone, DDS) examined in this study is currently the drug of choice for treatment of leprosy. Although the mode of action of this compound against *Mycobacterium leprae* is not fully understood, it is generally assumed to behave like *p*-aminosulfonamide in antagonizing the utilization of *p*-aminobenzoate [22]. However, *p*-aminosulfonamide itself appeared to be ineffectual against *M. leprae*; it was also without effect against chick cells (results not shown). Thus, the observation that DDS is a potent inhibitor of phospholipid biosynthesis suggested the possibility that its efficacy in the treatment of leprosy may also be related to its inhibitory effect on lipid synthesis.

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