Biochemical Pharmacology, 1965, Vol. 14, pp. 1605–1619. Pergamon Press Ltd., Printed in Great Britain.

3′,5′-DIESTERS OF 5-FLUORO-2′-DEOXYURIDINE: SYNTHESIS AND BIOLOGICAL ACTIVITY

YOSHIHIKO NISHIZAWA* and JOHN E. CASIDA†

Division of Entomology and Acarology, University of California, Berkeley, Calif.

and

SANDRA W. ANDERSON and CHARLES HEIDELBERGER‡

McArdle Memorial Laboratory, The Medical School, University of Wisconsin, Madison, Wis., U.S.A.

Received 14 *November* 1964; *accepted* 21 *May* 1965

Abstract—Twenty-seven esters of 5-fluoro-2′-deoxyuridine (FUDR) were prepared and their toxicity and/or carcinostatic activity assayed by oral administration to mice with adenocarcinoma-755 tumors. 3′,5′-Diesters of FUDR with C_4 to C_{16} aliphatic acids had a greater effect on the mice or the tumors than equivalent dosages of unesterified FUDR. The dibutyryl, dihexanoyl, and dioctanoyl were the most effective antitumor agents of the FUDR diesters with aliphatic acids. Carboxylic diesters with nonaliphatic acids, and certain sulfonyl, carbamoyl, and phosphoryl esters, offered no distinct advantage over the aliphatic esters, owing to either low carcinostatic activity or high toxicity. The toxicity and possibly also the antitumor potency of 3′,5′-dibutyryl FUDR were increased by simultaneous administration of an organophosphate inhibitor for esterases, effecting its hydrolysis. 3′,5′-Dibutyryl thymidine also increased the toxicity of FUDR to mice. The FUDR esters were less potent than FUDR in inhibiting the reproductive potential of houseflies and pea aphids.

5-FLUORO-2′-DEOXYURIDINE (FUDR) has significant carcinostatic activity in certain solid human tumors and in transplanted mouse and rat tumors.[1, 2] The carcinostatic activity of FUDR is considered to be primarily the result of inhibition of DNA biosynthesis by blocking thymidylate synthetase, the enzyme that catalyses the methylation of 2′-deoxyuridylic acid to thymidylic acid.[3, 4] In this action FUDR must be phosphorylated intracellularly to yield 5-fluoro-2′-deoxyuridine-5′-monophosphate (FUDRP), the specific competitive inhibitor for thymidylate synthetase.[4–6] The efficiency with which the cell can metabolize FUDR to FUDRP is decreased by cleavage of the glycosyl bond by nucleoside phosphorylase, yielding 5-fluorouracil (FU).[4, 7]

Attempts to modify the phosphate group of FUDRP so that the substituted compounds, mostly phosphodiesters, might have a facilitated entry into the cell and release of FUDRP have proved unsuccessful.[8, 9] However, nucleoside phosphorylase was less active on FUDRP and certain of its esters than on FUDR in cleaving such

* Permanent address: Pesticide Research Laboratory, Sumitomo Chemical Co., Osaka, Japan.

† This study was conducted while these authors were in the Department of Entomology, University of Wisconsin, Madison, Wis.

‡ American Cancer Society Professor of Oncology.

compounds to FU.[10] The action of FUDR might also be altered by administration in the form of neutral esters which could be hydrolyzed intracellularly and then metabolized to FUDRP. The effective chemotherapeutic and toxic doses might be altered by changes in tissue specificity resulting from variations in any of the following: tissue distribution because of the liposolubility of the esters; selectivity patterns in the esterases hydrolyzing the esters of FUDR; and susceptibility of the esters to cleavage by nucleoside phosphorylase. Studies comparing FUDR with 3′,5′-diacetyl FUDR, and 6-azauridine (AzUR) with 2′,3′,5′-triacetyl AzUR, after their administration to cancer patients have established the greater stability of the acetylated derivatives *in vivo*, with the 5′-acetyl monoesters of FUDR and AzUR occurring as the major persisting intermediates in the release of free FUDR and AzUR.[11–14] Nucleoside phosphorylase is much less effective *in vitro* in freeing FU from acetylated FUDR than from free FUDR unless the esterases active in de-esterification are also present in the nucleoside phosphorylase preparation.[9, 10]

Many purine and pyrimidine antimetabolites are also active as insect chemosterilants.[15, 16] FUDR induced sterility with no oviposition when incorporated into the adult diet of houseflies, while FU gave temporary sterility with oviposition.[17]

Twenty-seven esters of FUDR were prepared and subjected to various types of bioassay to ascertain the influence of the neutral ester groupings. The properties of esterases in human, mouse, and insect tissues effecting the hydrolysis of these esters were also investigated.[18]

MATERIALS AND METHODS

Synthesis of FUDR esters

FUDR and thymidine were provided by Dr. S. A. Schepartz, Cancer Chemotherapy National Service Center, Bethesda, Md. The twenty-seven esters of FUDR were prepared by the following six esterification procedures:

1. *From acid anhydride.* To 1 g FUDR dissolved in 20 ml anhydrous pyridine (previously dried over potassium hydroxide) was added at room temperature 2·1 molar equivalents of acid anhydride. The reaction mixture was then heated to 80°–85° for 2 hr, cooled, poured into ice water, and the esters recovered by extraction three times with equal volumes of chloroform. The chloroform was then washed with ice-cold 0·01 N sulfuric acid, 1% aqueous sodium bicarbonate, and finally water. After drying with sodium sulfate, the chloroform was evaporated and the residual oil or crystals subjected to chromatography.

2. *From acid chloride.* To 1 g FUDR in 20 ml anhydrous pyridine was added at 5° 2·1 M equiv. of acid chloride. The mixture was held at room temperature overnight, at which time a large precipitate of pyridine hydrochloride was evident. The reaction mixture was then added to ice water and worked up as indicated above.

3. *N-Methylcarbamate.* One g of FUDR was suspended in methyl isocyanate (5 g) in a sealed tube. After heating at 100° for 5 hr, the tube was cooled and opened. Crystals of the 3′, 5′-di(N-methylcarbamoyl) FUDR were evident on evaporating the excess methyl isocyanate. The product was recrystallized from acetone without chromatographic purification.

4. *Diformyl FUDR.* To 0·5 g FUDR dissolved in 10 ml 98% formic acid at room temperature was added 2·0 g anhydrous sodium formate. The mixture was cooled to

5°, 1·1 g conc. sulfuric acid added by drops, and the reaction mixture held overnight at room temperature. Excess formic acid was evaporated and the residue extracted with chloroform. The chloroform-soluble materials were subjected to chromatography.

5. *5′-Acyloxy esters of FUDR.* The procedure was the same as 1 except that 1·0 M eq of the acid anhydride was utilized. On chromatography the major product was the 5′-substituted ester, but some 3′-substituted and diester were also recovered.

6. *3′-Acyloxy esters of FUDR.* The procedure used by Michelson and Todd[19] with thymidine esters was found to be directly applicable to preparation of the FUDR esters. The 5′-trityl FUDR (m.p. 150°–151°)[8] was esterified to yield the 5′-trityl-3′-acetyl FUDR (m.p. 90°–91°)[8] and the 5′-trityl-3′-butyryl FUDR (m.p. 107°–108°), and the trityl group was removed by refluxing with acetic acid.

The yields given in Table 1 are based on the amount of FUDR used in the initial reaction. Acid anhydrides were used for preparing the shorter chain length esters, since the acid chlorides were found to result in very poor yields. With the aliphatic acid chlorides the yield appeared to increase with chain length, such that the chlorides were practical for the pentanoyl and higher esters only. The relatively low yield with some of the higher esters resulted from a large amount of mono-substituted esters recovered. Only the mono-(*p*-toluenesulfonyl) ester, and no diester, was recovered from reaction of FUDR with *p*-toluenesulfonyl chloride.

3′,5′-Diacetyl, dipropionyl, and dibutyryl esters of thymidine were prepared in the same manner as the corresponding esters of FUDR. Five carbonyl-^{14}C-labeled FUDR esters were made from acetic-^{14}C anhydride for use in another study.[18] FUDR reacted with 2·1 M eq of acetic-^{14}C anhydride according to procedure 1 yielded 3′,5′-diacetyl-^{14}C FUDR. Similarly, 1·0 M equiv. of acetic-^{14}C anhydride was reacted with 3′-acetyl FUDR to yield 3′-acetyl,5′-acetyl-^{14}C FUDR, and with 5′-acetyl FUDR to yield 3′-acetyl-^{14}C,5′-acetyl FUDR. Procedure 5 was used to prepare 5′-acetyl-^{14}C FUDR and procedure 6 to prepare 3′-acetyl-^{14}C FUDR.

Chromatography and characterization of products

Silica gel columns were used to isolate the pure esters of FUDR. The columns were prepared by thoroughly mixing 100 g silicic acid (100 mesh powder, Mallinckrodt Chemical Works, N.Y., for chromatographic analysis) with 50 ml water and then packing the gel suspended in hexane to yield a 3·5 × 30-cm column. The columns were generally developed with 200 ml of each of the following solvents in the order indicated: hexane, chloroform, ethyl acetate, and methanol. In the case of the monoacetyl and monobutyryl FUDR esters, the solvent series was hexane, chloroform, chloroform:ethyl acetate (1:1), ethyl acetate, and methanol. Unreacted FUDR was eluted in the methanol. Fifteen-ml aliquots were collected and the elution position of the FUDR derivatives ascertained by determining the absorbance of a small aliquot from each tube at 266 mμ in methanolic solution. The fraction tubes were combined, based on the peaks for elution of ultraviolet-absorbing materials, evaporated, and the residue recrystallized from the solvents indicated in Table 1.

The structures assigned are based on elemental analyses and interpretation of infrared spectra in addition to the synthetic route utilized. Chromatography and crystallization procedures employed ruled out possible contamination of the FUDR diesters by FUDR or its monoesters.

TABLE 1. SYNTHETIC METHOD, PURIFICATION, AND PROPERTIES FOR FUDR ESTERS

FUDR Ester	Synthetic method	Solvents*		M.p. (°C)	Yield (%)	Analyses	
		Elution	Recryst.			Calcd. (%)	Found
3′,5′Diformyl	4	$CHCl_3$	$CHCl_3$	117–118	56	C 43·71 H 3·64 N 9·27	C 44·18 H 4·14 N 9·52
3′,5′-Diacetyl	1	$CHCl_3$	benz	149–150	96	C 47·27 H 4·58 N 8·48	C 46·95 H 4·54 N 8·53
3′-Acetyl	6	$CHCl_3$-EtAc (1:1)	H_2O	200–201	20	C 45·83 H 4·51 N 9·72	C 46·01 H 4·61 N 9·65
5′-Acetyl	5	EtAc	H_2O	146	29	C 45·83 H 4·51 N 9·72	C 46·02 H 4·57 N 9·61
3′,5′-Dipropionyl	1		hex-benz	78–79	96	C 50·27 H 5·34 N 7·82	C 50·06 H 5·30 N 7·80
3′,5′-Dibutyryl	1	$CHCl_3$	hex-benz	115–116	99	C 52·84 H 6·00 N 7·25	C 52·80 H 6·01 N 7·18
3′-Butyryl	6	$CHCl_3$-EtAc (1:1)	H_2O	135–136	21	C 49·37 H 5·38 N 8·86	C 49·32 H 5·26 N 9·03
5′-Butyryl	5	EtAc	H_2O	109	16	C 49·37 H 5·38 H 8·86	C 49·03 H 5·11 N 8·70
3′,5′-Dipentanoyl	2	hex		oil	16	C 55·07 H 6·52 N 6·76	C 55·71 H 6·82 N 7·03

TABLE 1.—*continued*

FUDR Ester	Synthetic method	Solvents*		M.p. (°C)	Yield (%)	Analyses	
		Elution	Recryst.			Calcd. (%)	Found
3′,5′-Dihexanoyl	2	hex		oil	39	C 57·01 H 7·01 N 6·33	C 57·83 H 6·99 N 6·42
3′,5′-Diheptanoyl	2	hex		oil	40	C 58·72 H 7·45 N 5·96	C 58·74 H 7·59 N 6·25
3′,5′-Dioctanoyl	2	hex		oil	61	C 60·24 H 7·83 H 5·62	C 59·96 H 7·84 N 5·71
3′,5′-Dinonanoyl	2	hex		oil	60	C 61·60 H 8·17 N 5·32	C 63·06 H 8·12 N 5·24
3′,5′-Didecanoyl	2	hex		oil	62	C 62·82 H 8·48 H 5·05	C 63·83 H 8·51 N 5·14
3′,5′-Dilauroyl	2	hex	pent	49–50	20	C 64·81 H 9·17 N 4·58	C 65·23 H 9·43.. N 4·54
3′,5′-Dimyristoyl	2	hex	pent	65–67	68	C 66·67 H 9·46 N 4·20	C 66·29 H 9·64 N 4·58
3′5,′-Dipalmitoyl	2	hex	pent	42–43	41	C 68·14 H 9·83 N 3·88	C 68·31 H 10·35 N 3·95
3′,5′-Distearoyl	2	hex	pent	71–73	24	C 69·41 H 10·15 N 3·60	C 68·74 H 10·24 N 3·71

TABLE 1.—*continued*

FUDR Ester	Synthetic method	Solvents*		M.p. (°C)	Yield (%)	Analyses (%)	
		Elution	Recryst.			Calcd.	Found
3′,5′-Dioleoyl	2	hex	pent	80–83	30	C 69·77 H 9·70 N 3·62	C 69·83 H 9·63 N 3·45
3′,5′-Dicyclohexylcarbonyl	1		hex	189–190	90	C 59·48 H N 6·03	C 59·55 H N 5·91
3′,5′-Dibenzoyl	1		pent-acet	234–235	43	C 60·79 H 4·19 N 6·18	C 60·55 H 4·52 N 6·22
3′,5′-Diphenoxyacetyl	1	$CHCl_3$	pent-$CHCl_3$	61–62	36	C 58·37 H N 5·45	C 58·30 H N 5·71
3′,5′-Di(phenylacetyl)	1	$CHCl_3$	pent-benz	42–43	62	C 62·24 H 4·77 N 5·81	C 62·38 H 4·90 N 5·80
3′,5′-Di(N-methylcarbamoyl)			acet	212–213	66	C 43·33 H 4·72 N 15·56	C 43·39 H 4·80 N 15·18
3′,5′-Di(O,O-diethylphosphoryl)	2	hex		oil	72	P 12·02	P 11·82
3′,5′-Di(methylsulfonyl)	2		EtOH	159–160	87	C 32·84 H 3·73 N 6·97	C 32·83 H 3·67 N 6·82
5′-(*p*-Toluenesulfonyl)†	2		EtOH	157–158	84	C 48·12 H 4·01 N 7·00	C 48·26 H 4·33 N 6·83

* Solvents for elution from silica gel columns or recrystallizations are abbreviated as follows: pent = pentane; hex = hexane; benz = benzene; acet = acetone; EtOH = ethanol; EtAc = ethyl acetate; $CHCl_3$ = chloroform; and H_2O = water.

† Monoester presumed to be the 5′-(*p*-toluenesulfonyl) derivative.

Bioassays

The tumor-inhibitory activity of the FUDR esters was assayed against the transplanted mouse tumor, adenocarcinoma 755; a described strain and assay procedure were used.[1, 20] FUDR was administered at 5 to 40 mg/kg daily by stomach tube for seven days, starting one day after transplantation of the tumor. Dosages for the esters were the same in mmoles/kg as for FUDR and were therefore expressed as FUDR-equivalent doses. Compounds were dissolved or suspended in a fine state of subdivision in 0·5% carboxymethylcellulose in isotonic NaCl, or propylene glycol or corn oil, dependent on the solubility characteristics of the compound. Oral administration involved 0·20 ml of solution per 20-g mouse weight each day. A typical experiment involved adenocarcinoma 755 in mice with 10 mice per group. One group was a control, another received only the solvent used to dissolve the FUDR derivatives, a third was given FUDR, and the remaining groups received equivalent doses of the FUDR esters. Survival, tumor volume, and mouse weight were recorded on days 10, 15, and 20. Results are presented for only day 15, as they serve to illustrate the activity of the compounds and are generally averaged from at least two different experiments.

The chemosterilant assay with houseflies, *Musca domestica* L., was based on that of LaBrecque and Gouck[21] and involved incorporation of the test compound into the adult diet. Solutions of 0·25 mmole of the compounds in 5 ml acetone, aqueous acetone, or methanol were added to 10 g of food mixture (6 parts sugar, 6 parts powdered nonfat dry milk, and 1 part powdered egg). The treated food was allowed to dry for 24 hr, repulverized, and placed in the test cages; 100 housefly pupae were placed in each cage. The emerging flies were exposed to treated food only for 4 days. Thereafter, treated and untreated food were available. Egg collections were made daily from days 4 through 9 by placing a container of C.S.M.A. housefly media[22] in each cage for a 90-min period. The seeded media was then transferred to holding cages for emergence. The 4–6 day egg collections were kept separate from the 7–9 day group. Adult mortality was recorded at 4 days and again at 7 days.

In a preliminary study it was found that fourth-instar or apterous adult pea aphids, *Acrythosiphon pisum* (Harris), treated with FUDR failed to produce as many nymphs as untreated aphids. These aphids were exposed to the FUDR by feeding through a membrane into a 0·1% aqueous solution of FUDR for 20 hr, by feeding on pea plants (*Pisum sativum* L., Early Perfection variety) previously injected with 500 μg FUDR per plant, or by applying topically to the abdomen of the aphids a solution containing 2 μg FUDR in 0·4 μl of 50% aqueous acetone.

The injection of the FUDR derivatives into the pea plants was used for further bioassay. Test materials in aqueous solution or suspension were injected in volumes of 25 to 50 μl per plant by a described procedure.[23] One day after treatment each plant was infested with 10 fourth-instar pea aphids. Nymphal counts and adult mortalities were recorded six days after infestation. The remaining nymphs and adults were then removed and the plants reinfested on the seventh day with 10 aphids per plant and again checked out as previously indicated. Adult mortality during the six days averaged 33% and did not appear to be related to the chemical injected. The adult mortality occurred mostly after nymph production was essentially complete and thus did not affect the overall interpretation of results. The size of the plants at the time of injection was such that an FUDR dose of 1·0 μmole per plant yielded theoretical

ppm values (uniform distribution assumed) of 665 for the part of the plant above the injection site, or 328 for the whole plant exclusive of the seed, or 185 for the whole plant including the seed.

TABLE 2. ANTITUMOR ACTIVITY OF FUDR AND ESTERS AS ASSAYED WITH MICE AND ADENOCARCINOMA 755

Compounds administered orally for seven consecutive days and observations made on day 15 after inoculation.

Compound	Solvents*	FUDR Equiv. (mg/kg/day)	No. alive Total no.	ΔWgt. T-C (g)	Tumor vol. T (mm³)	Tumor vol. C (mm³)	T/C
FUDR	pg	40	56/59	−0·8	768	1,321	0·58
	pg	20	28/28	+0·2	768	1,080	0·71
	pg	10	18/20	−0·2	525	492	1·07
	cmc	40	28/28	−1·0	1,202	1,555	0·77
Monoesters of FUDR							
3′-Acetyl	pg	40	29/29	−0·5	645	1,161	0·56
5′-Acetyl	pg	40	27/29	−2·3	462	1,161	0·40
3′-Butyryl	pg	40	7/10	−1·5	530	1,520	0·35
	pg	20	20/20	−1·3	647	1,323	0·49
5′-Butyryl	pg	40	8/9	−1·1	910	1,520	0·60
	pg	20	20/20	−0·9	724	1,323	0·55
5′-(*p*-Toluene-sulfonyl)	pg/co	40	16/19	+0·4	544	741	0·73
3′,5′-Diesters of FUDR							
Formyl	pg	40	18/18	−1·4	424	1,313	0·32
	pg	20	29/29	+0·4	472	483	0·98
Acetyl	pg	40	67/69	−1·2	563	1,122	0·50
	pg	20	19/19	−1·3	338	420	0·80
	pg	10	18/20	−1·1	540	492	1·10
	cmc	40	26/26	−1·4	894	1,555	0·57
Propionyl	pg	40	20/20	−1·1	610	1,313	0·46
	pg	20	19/20	+0·3	896	1,313	0·68
	co	40	20/20	−1·3	517	1,072	0·48
Butyryl	pg	40	12/19	−3·4	454	1,650	0·28
	pg	25	10/10	−5·2	560	1,780	0·31
	pg	20	18/19	−1·0	543	1,323	0·41
	pg	10	17/20	−0·6	482	1,119	0·43
	co	40	16/20	−2·4	375	1,072	0·35
	co	20	19/20	−0·5	1,129	1,372	0·82
	co	10	17/20	−1·1	983	1,372	0·72
Pentanoyl	pg	10	5/10	−2·0	146	586	0·25
Hexanoyl	co	20	19/20	−0·4	547	1,324	0·41
	co	10	19/20	−0·1	970	1,372	0·71
Heptanoyl	co	20	5/10	−2·0	114	499	0·23
Octanoyl	co	40	9/20	−5·2	152	1,072	0·14
	co	20	16/20	−0·4	187	920	0·20
	co	10	20/20	−1·0	703	1,372	0·51
Nonanoyl	co	20	7/8	−3·4	224	499	0·45
Decanoyl	co	20	13/20	−1·3	327	967	0·34
	co	10	20/20	+0·5	729	1,291	0·56
Lauroyl	co	20	12/20	−1·5	392	1,243	0·32
	co	10	18/19	−0·6	662	991	0·67
Myristoyl	co	10	13/17	−1·2	452	991	0·46
Palmitoyl	co	10	19/20	−1·2	454	991	0·46
Stearoyl	co	10	17/20	+0·2	891	916	0·97
Oleoyl	co	10	18/18	+1·2	1,173	916	1·28
Cyclohexylcarbonyl	pg	10	6/10	−0·3	550	1,095	0·50
Benzoyl	pg/co	20	24/28	−2·6	280	558	0·50
Phenoxyacetyl	pg/co	40	18/20	−3·2	190	813	0·23

TABLE 2.—*continued*

Compound	Solvents*	FUDR Equiv. (mg/kg/day)	No. alive Total no.	ΔWgt. T-C (g)	Tumor vol. T (mm³)	Tumor vol. C (mm³)	Tumor vol. T/C
Phenylacetyl	pg/co	40	4/19	−3·9	76	813	0·09
N-Methylcarbamoyl	pg	40	19/20	−2·6	1,203	1,510	0·80
O,O-Diethylphosphoryl	pg	40	10/10	+1·1	159	241	0·66
Methylsulfonyl	pg/co	40	16/18	−1·1	812	813	1·00

* Abbreviations used: pg = propylene glycol; cmc = carboxymethylcellulose; co = corn oil; and pg/co = half of group tested with pg and the other half with co solvent and results averaged since no apparent difference results from solvent effect. Compounds were in solution except those tested with both solvents (pg/co) where the administered dose was part in solution and part in suspension.

RESULTS

Antitumor activity and toxicity to mice of FUDR esters

Growth of adenocarcinoma-755 tumors was inhibited by many FUDR esters administered by stomach tube to the mice (Table 2). Interpretation of inhibitory studies with this tumor is complicated by the decrease in tumor volume when there is marked weight loss in treated animals This can be illustrated by two starvation experiments in which the tumor volume and animal weight were compared on day 15 after tumor transplantation for mice on normal feed and mice that were fed only every third day. In the first experiment with 10 mice per group the tumor volume averaged 1,473 mm³ for mice on full feed and 602 mm³ for mice partially starved, resulting in a 3·9-g weight difference between the two groups. In the second experiment with 15 mice per group the tumor volume averaged 1,700 mm³ for full-fed and 770 mm³ for partially starved mice, resulting in a 4·5-g weight difference. The weight loss resulting from partial starvation was almost identical for mice with and without the transplanted tumors. Thus a weight difference of 3·9 to 4·5 g due to starvation resulted in a tumor volume ratio (T/C, volume of tumor in partially starved mice divided by volume of tumor in mice on full feed) of 0·41 to 0·45. A decrease in the tumor volume ratio could also result from weight loss initiated by the toxic effect of a chemical as well as by starvation. Selective carcinostatic activity for a test compound would be indicated by reduction in the tumor volume ratio without marked weight loss or mortality for the mice.

Certain of the esters of FUDR with aliphatic acids gave marked carcinostatic activity independent of weight loss, others were not greatly different from unesterified FUDR in activity or toxicity, and many were sufficiently toxic that their potential carcinostatic activity could not be readily interpreted because of the weight loss at the dosages used. Esters with aliphatic acids of intermediate chain length (C_4, C_6, and C_8) were in the first group, shorter chain length acid (C_1 to C_3) esters were in the second group, and the C_5, C_7, C_9, and longer chain acid esters were in the last group. Diformyl, diacetyl, dipropionyl, and monoacetyl esters of FUDR were not greatly different in activity from unesterified FUDR. The dibutyryl through the dipalmitoyl esters of FUDR gave reduced T/C values at lower FUDR-equivalent doses (10 or 20 mg/kg/day) than esters of shorter chain length. Toxicity also increased with the

longer chain lengths as was evidenced by the higher mortality and less efficient weight gain resulting from the 40-mg FUDR-equivalent dose of the butyryl esters, the 20-mg FUDR-equivalent dose of the esters with C_7 and C_9 to C_{12} acids, and the 10-mg FUDR-equivalent dose of the dipentanoyl and dimyristoyl esters. However, three diesters of FUDR gave T/C values of 0·45 or less with 1·0 g or less weight difference between the treated and control group. These diesters were the butyryl at 10 and 20 mg, and the hexanoyl and octanoyl at 20 mg FUDR-equivalent dosages. Further testing of the dibutyryl, dihexanoyl, or dioctanoyl esters of FUDR would appear warranted as a possible means of reducing the total FUDR-equivalent dose necessary to inhibit tumor growth.

Esters of FUDR derived from other than aliphatic acids offered no distinct advantage over the aliphatic esters due to either low carcinostatic activity or high toxicity, although further testing of lower doses of the dibenzoyl, diphenylacetyl, and diphenoxyacetyl esters might be justified.

Assays of the acids which would be released on hydrolysis of the FUDR esters *in vivo* showed that they probably do not contribute greatly to the toxicity or the antitumor activity with adenocarcinoma 755. The dibutyryl, dihexanoyl, and dioctanoyl esters of FUDR at 20 mg FUDR-equivalent/day and the didecanoyl, dilauroyl, dimyristoyl, and dipalmitoyl esters at 10 mg FUDR-equivalent/day were much more potent than equivalent amounts of the acids formed on their complete hydrolysis. Only caproic, caprylic, and capric acids gave any indication of reduction in tumor volumes at these doses. Phenoxyacetic and phenylacetic acids were also less potent than their FUDR esters at equivalent doses. An experiment with 3′,5′-dipalmitoyl FUDR (Table 3) illustrates the greater potency of this diester than of the hydrolysis products administered individually or in combination, but does not differentiate between tumor inhibition due to weight loss caused by toxicity of the material and that which resulted from selective carcinostatic activity. The toxicity in many cases and the carcinostatic activity in certain instances (dibutyryl, dihexanoyl, and dioctanoyl esters) for the FUDR diesters are greater than that due to either the FUDR or the acid component alone. It is less certain that the diester is more toxic or of

TABLE 3. ANTITUMOR ACTIVITY OF 3′,5′-DIPALMITOYL FUDR COMPARED TO ITS HYDROLYSIS PRODUCTS (FUDR AND PALMITIC ACID) AS ASSAYED WITH MICE AND ADENOCARCINOMA 755

Compound	FUDR-Equiv.* (mg/kg/day)	No. alive / Total no.	ΔWt.† T-C (g)	Tumor vol.‡ (mm^3) T/C
FUDR	15	9/10	0·0	0·93
Palmitic acid	15	8/10	−1·8	1·04
FUDR + Palmitic acid	15	7/10	+0·6	1·25
3′,5′-Dipalmitoyl FUDR	15	8/10	−3·3	0·47

* Compounds administered orally in propylene glycol for seven consecutive days after inoculation. The molar equivalent dose of palmitic acid used was twice that of either FUDR or 3′,5′-dipalmitoyl FUDR; it represented the theoretical amount of palmitic acid released on total hydrolysis of the diester.

† Weight gain per mouse averaged 1·7 g in the control group.

‡ Tumor volume at 15 days after inoculation in mm^3 for treated group divided by control group where average volume in controls was 2,799 mm^3/mouse.

higher antitumor activity than the combined FUDR and acid simultaneously administered, although this appears to be the case with the dipalmitoyl ester (Table 3). Potential differences in absorption, distribution, and detoxication of the fat-soluble diesters as compared with the water-soluble FUDR and acidic hydrolysis products make it difficult to establish this relationship.

S,S,S-Tributylphosphorotrithioate (DEF) was inactive as an antitumor agent and had little effect on the potency of FUDR, but it increased the toxicity and possibly also the carcinostatic activity of 3′,5′-dibutyryl FUDR (Table 4). In another experiment utilizing mice and transplanted adenocarcinoma-755 tumors, it was found that 3′,5′-dibutyryl thymidine in propylene glycol greatly increased the toxicity of FUDR in saline when both were administered intraperitoneally. Dibutyryl thymidine alone at 10, 50, or 250 mg/kg/day for seven days gave no mortality, effect on tumor volume, or weight gain. FUDR at 100 mg/kg/day for seven days gave no mortality, a slight weight loss, and a tumor volume ratio (T/C) of 0·10. However, treatment with the same dose of FUDR and 10 mg dibutyryl thymidine/kg resulted in 12% mortality, no

TABLE 4. EFFECT OF S,S,S-TRIBUTYLPHOSPHOROTRITHIOATE (DEF) ON ANTITUMOR ACTIVITY OF FUDR AND 3′,5′-DIBUTYRYL-FUDR WITH MICE AND ADENOCARCINOMA 755

FUDR Derivative	FUDR-Equiv. (mg/kg/day)*	mg/kg DEF, T/C†		
		0	10	20
FUDR	0	(1·00)	1·38	1·20
	10	0·94	1·05	
	20	0·74		0·69(90%)‡§
3′,5′-Dibutyryl	5	1·03	1·22	
	10	0·89(85%)‡	0·58(83%)‡§	0·58(74%)‡§
	20	0·67(75%)‡**		0·23(30%)‡**

* Compounds administered orally in propylene glycol for seven consecutive days after inoculation.

† Tumor volume at 15 days after inoculation in mm^3 for treated group divided by control group where average volume in controls was 2,017 mm^3/mouse.

‡ Per cent survival at 15 days for those groups with more than 5% mortality.

§ Weight gain per mouse averaged 1·0–2·0 g less than control group during 15-day experimental period.

** Weight gain per mouse averaged 2·0–2·6 g less than control group during 15-day experimental period.

tumor development, and a large weight loss. With 50 and 250 mg of dibutyryl thymidine/kg combined with the 100 mg dose of FUDR/kg, no mice survived the pronounced toxic effects of the mixture. Thymidine and diacetyl thymidine in another study also greatly increased the toxicity and carcinostatic activity of FUDR at a daily dose for each compound of 40 mg FUDR-equivalent/kg.

Very limited investigations with other tumors, using described strains and procedures,[1, 20] indicated that FUDR administered at 40 mg/kg/day by stomach tube was less active than an equivalent dose of 3′,5′-diacetyl FUDR. In each of four experiments with sarcoma 180 in mice (using carboxymethylcellulose for administration), the average tumor volume on day 15 after inoculation (982 mm^3 for controls) was greater with FUDR (494 mm^3) than with an equivalent dose of diacetyl FUDR (236

mm^3). In a single experiment with leukemia 1210 in mice (using propylene glycol for administration), the average survival times (days) were: control, 11·5; FUDR, 12·0; and diacetyl FUDR, 14·0. In a single experiment with Novikoff hepatoma in rats (using carboxymethylcellulose for administration), the average survival times (days) were: control, 8·0; FUDR, 8·0; and diacetyl FUDR, 10·5.

Effect of FU, FUDR, and FUDR esters on reproduction by insects

The reproductive potential of houseflies and pea aphids, insects with different types of reproduction, was greatly diminished by FU, FUDR, and certain FUDR esters without apparent toxic effects. With houseflies, the compounds were ingested by both adult sexes for at least four days before egg collections were begun. Ingestion of the fluoro-compounds from systemically treated pea plants by the ovoviviparous aphids began in the instar prior to the adult stage in which nymph production was initiated. In both cases the FU derivatives were present in the insect at the time of nucleic acid formation in the eggs or embryos.

TABLE 5. EFFECT OF FU, FUDR and FUDR 3′,5′-DIESTERS IN THE DIET OF ADULT HOUSEFLIES ON REPRODUCTION

Compound*	Adult mortality thru 7th day (%)	Adults from eggs laid Between days 7 and 9† (No./10 flies)	(% of control)
Control	15	228	100
FU	39	1	0·4
FUDR	28	19	8·3
3′,5′-Diesters of FUDR			
Acetyl	13	28	12
Butyryl	10	163	72
Hexanoyl	28	6	2·6
Stearoyl	11	264	116
N-Methylcarbamoyl	25	302	132
Methylsulfonyl	24	245	107

* Compounds were tested at 0·25 mmole in 10 g diet or 0·33% to 1·95%, dependent on the compound molecular weight. The solvent (20% aqueous acetone for FU and FUDR, methanol for the N-methylcarbamoyl ester, and acetone for the others) containing the compound was evaporated after mixing with the diet and before offering to the flies and did not alter the results based on controls within the experiment. The average emergence from the 100 pupae originally introduced into each cage was 88·8%. All results are averages of 3 to 5 replicates per treatment.

† Eggs were collected during a 90-min oviposition period on each of days 7, 8, and 9. The numbers of adults indicated are averages from eggs laid per 10 surviving flies. The same relative potency of compounds was evident for eggs laid during days 4 to 6 of adult life.

Housefly reproduction was more greatly affected by FU than by FUDR (Table 5). The distearoyl, di(N-methylcarbamoyl) and di(methylsulfonyl) esters were inactive. The diacetyl, dihexanoyl, and probably also the dibutyryl esters of FUDR were active but did not give the graded response anticipated with chain length modification. Less effect on reproduction was noted in each replicate of the dibutyryl ester treatments than with the diacetyl and dihexanoyl esters. No explanation is available for this discrepancy. The toxicity of the compounds to adult houseflies was low and did not correlate with the potency in affecting reproduction.

Pea aphid nymph production was more sensitive to FUDR and diacetyl FUDR than FU (Table 6). FUDR and diacetyl FUDR were active at 0·1 to 0·3 μmole per pea plant or in the range of 66 to 267 ppm in the stem and leaves above the injection site (uniform distribution assumed). The FUDR appeared to be more persistent in the peas than diacetyl FUDR. In another experiment involving injection of 0·1 and 1·0 μmole per plant, it was found that di(O,O-diethylphosphoryl) FUDR was inactive, FU was active at the higher level but only during the first week, diacetyl FUDR was active at both levels but did not persist into the second week, and FUDR was active

TABLE 6. EFFECT OF FU, FUDR and 3′,5′-DIACETYL FUDR INJECTED INTO PEA PLANTS ON THE PRODUCTION OF NYMPHS BY PEA APHIDS FEEDING ON THE PLANTS

Compound	Per cent reduction in nymph production at indicated μmoles per plant*			
	0·1	0·3	1·0	3·0
First week after injection				
FU	9	16	56	83
FUDR	63	95	97	97
3′,5′-Diacetyl FUDR	73	81	93	97
Second week after injection				
FU	0	0	8	13
FUDR	0	10	71	100
3′,5′-Diacetyl FUDR	0	0	0	0

* The production of nymphs per 10 aphids on each control plant averaged 128 during the first week after injection and 94 during the second week after injection following reinfestation with an additional 10 aphids. Notes on adult mortality and approximate ppm levels of compounds in the plants are considered in the text. Results are average of 4 replicates.

at both levels the first week and persisted in active concentration into the second week with the 1·0 μmole per plant initial dose. A survey experiment with 0·5 μmole per plant showed almost complete blockage of nymph production within the first week by FUDR and diacetyl FUDR, and no activity for the di(O,O-diethylphosphoryl) FUDR. Several other FUDR diesters were also inactive [dibutyryl, dioctanoyl, dipalmitoyl, dibenzoyl, di(N-methylcarbamoyl), and di(methylsulfonyl)], but were of sufficiently low water solubility that the actual dose entering and moving within the plant was probably very low.

DISCUSSION

Certain 3′,5′-diesters of FUDR with aliphatic acids were more potent than FUDR as carcinostatic and/or toxic agents after oral administration to mice. Esterases in normal and tumor tissues of mice have been shown to hydrolyze these esters to FUDR,[18] while nucleoside phosphorylase in certain of these tissues is much less active in cleavage of 3′,5′-diacetyl FUDR than of FUDR to FU.[10]. The esters may therefore serve as FUDR precursors for slow release of higher or more persistent levels of FUDR than might be obtained by direct administration of the less stable FUDR.

The rate of FUDR release from the esters might be controlled by either modifying the ester groupings, since their nature greatly affects the hydrolysis rate, or by inhibiting the responsible esterases with appropriate organophosphate esters.[18] Inhibition of many of these esterases *in vivo* by S,S,S-tributylphosphorotrithioate[18] resulted in no effect on the carcinostatic activity of FUDR but an increased activity and toxicity for 3′,5′-dibutyryl FUDR. Thymidine and its 3′,5′-diacetyl and dibutyryl esters greatly increased the toxicity of FUDR to mice, confirming an earlier observation in respect to the effect of thymidine.[24] The increased toxicity of FUDR to mice in the presence of thymidine and thymidine esters probably results from saturation of the degradative pathway by thymine, formed from thymidine by nucleoside phosphorylase, and the consequent inability to degrade and thus detoxify FU formed from FUDR,[7] as has been discussed for somewhat analogous situations.[10] The marked toxicity increase from FUDR on continuous administration to cancer patients can be prevented by thymidine,[25] but the effect of thymidine esters under such circumstances has not been investigated.

Acetyl esters of a riboside antitumor agent, 2′,3′,5′-triacetyl AzUR, and a deoxyriboside antitumor agent, 3′,5′-diacetyl FUDR, have shown certain favorable biological properties compared with the unesterified AzUR and FUDR.[11–14] This approach may warrant investigation with other nucleoside carcinostatic agents and with longer chain length aliphatic esters involved in the esterification.

Esterases that hydrolyze FUDR esters are present in houseflies and pea aphids.[18] Certain FUDR esters inhibited the reproductive potential of these insects, although they were less active than FUDR. FU is considered to yield temporary sterility with oviposition in houseflies, and FUDR to effect sterility with no oviposition.[17] FU-^{14}C consumed by adult flies yielded ^{14}C incorporation into the internal components of the eggs, and the amount of ^{14}C from FU in the eggs was inversely related to their viability. It was suggested without experimental evidence that FU may have replaced uracil in the egg RNA.[17, 26] Recently these same workers have isolated 5-fluorouridylic-^{14}C acid from the RNA of fly eggs after ingestion of FU-^{14}C by the adults.[27] The more permanent effect of FUDR than FU as a chemosterilant for flies might result from synthesis of FUDRP which can then inhibit thymidylate synthetase and DNA biosynthesis in a manner analogous to its action as a carcinostatic agent,[4, 6] although these reactions have not been studied with insects. The much greater effect of FUDR than FU on pea aphid reproduction might also imply an action via FUDRP for blocking DNA biosynthesis in this case. Many purine and pyrimidine antimetabolites are known to serve as insect chemosterilants,[15, 16] but very little information is available on their possible biochemical action in the insect.

Acknowledgments—The continuing interest of Drs. S. A. Schepartz and F. R. White of the Cancer Chemotherapy National Service Center, Bethesda, Md., in this study was greatly appreciated. Skilled technical assistance was provided by Miss Judith Engel, Mrs. Andrea Stencel, and Mrs. Norma Avanzado of the Department of Entomology, and Mrs. Louis Griesbach of the McArdle Memorial Laboratories, The Medical School, University of Wisconsin; and Mr. William Goetz, Entomologist, Pesticide Department, Wisconsin Alumni Research Foundation, Madison, Wis. Elemental analyses were performed by Dr. S. M. Nagy, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass. This investigation was supported by Contract PH43-62-470 from the Cancer Chemotherapy National Service Center; Grants C-2832 and C-07175 from the National Cancer Institute; Grant GM-05304 from the National Institute of General Medical Sciences; and Contract AT (11-1)-1187 from the United States Atomic Energy Commission.

REFERENCES

1. C. HEIDELBERGER, L. GRIESBACH, O. CRUZ, R. J. SCHNITZER and E. GRUNBERG, *Proc. Soc. Exp. Biol. (N.Y.)* **97**, 470 (1958).
2. C. HEIDELBERGER and F. J. ANSFIELD, *Cancer Res.* **23**, 1226 (1963).
3. L. BOSCH, E. HARBERS and C. HEIDELBERGER, *Cancer Res.* **18**, 335 (1958).
4. C. HEIDELBERGER, *Exp. Cell Res.*, Suppl. **9**, 462 (1963).
5. S. S. COHEN, J. G. FLAKS, H. D. BARNER, M. R. LOEB and J. R. LICHTENSTEIN, *Proc. nat. Acad. Sci. (Wash.)* **44**, 1004 (1958).
6. K.–U. HARTMANN and C. HEIDELBERGER, *J. biol. Chem.* **236**, 3006 (1961).
7. N. K. CHAUDHURI, K. L. MUKHERJEE and C. HEIDELBERGER, *Biochem. Pharmac.* **1**, 328 (1959).
8. D. C. REMY, A. V. SUNTHANKAR and C. HEIDELBERGER, *J. org. Chem.* **27**, 2491 (1962).
9. K. L. MUKHERJEE and C. HEIDELBERGER, *Cancer Res.* **22**, 815 (1962).
10. G. D. BIRNIE, H. KROEGER and C. HEIDELBERGER, *Biochemistry* **2**, 566 (1963).
11. K. L. MUKHERJEE, J. BOOHAR, D. WENTLAND, F. J. ANSFIELD and C. HEIDELBERGER, *Cancer Res.* **23**, 49 (1963).
12. R. E. HANDSCHUMACHER, W. A. CREASEY, M. E. FINK, P. CALABRESI and A. D. WELCH, *Cancer Chemother. Rep.* **16**, 267 (1962).
13. W. A. CREASEY, M. E. FINK, R. E. HANDSCHUMACHER and P. CALABRESI, *Cancer Res.* **23**, 444 (1963).
14. P. CALABRESI, *Cancer Res.* **23**, 1260 (1963).
15. C. N. SMITH, G. C. LABRECQUE and A. B. BORKOVEC, *Ann. Rev. Entomol.* **9**, 269 (1964).
16. A. B. BORKOVEC, *Residue Rev.* **6**, 87 (1964).
17. R. R. PAINTER and W. W. KILGORE, *J. econ. Entomol.* **57**, 154 (1964).
18. J. E. CASIDA, J. L. ENGEL and Y. NISHIZAWA. Submitted for publication.
19. A. M. MICHELSON and A. R. TODD, *J. chem. Soc.* 951 (1953).
20. C. HEIDELBERGER, L. GRIESBACH, B. J. MONTAG, D. MOOREN, O. CRUZ, R. J. SCHNITZER and E. GRUNBERG, *Cancer Res.* **18**, 305 (1958).
21. G. C. LABRECQUE and H. K. GOUCK, *J. econ. Entomol.* **56**, 476 (1963).
22. ANONYMOUS, *Soap and Chem. Specialities Blue Book*, 32nd year, 219 (1959).
23. H. W. DOROUGH and J. E. CASIDA, *J. Agric. Food Chem.* **12**, 294 (1964).
24. J. H. BURCHENAL, H. F. OETTGEN, J. A. REPPERT and V. COLEY, *Cancer Chemother. Rep.* **6**, 1 (1959).
25. E. MILLER, R. SULLIVAN, C. YOUNG and J. H. BURCHENAL, *Proc. Am. Ass. Cancer Res.*, 251 (1961).
26. W. W. KILGORE and R. R. PAINTER, *J. econ Entomol.* **55**, 710 (1962).
27. W. W. KILGORE and R. R. PAINTER, *Bull. Entomol. Soc. Am.* **10**, 175 (1964).