Inhibition of Mammary Carcinoma 755 by Malonic Acid Derivatives.* (22534)

B. L. FREEDLANDER, F. A. FRENCH AND ARTHUR FURST.†
Research Laboratories, Mount Zion Hospital, San Francisco, Calif.

This paper presents an evaluation of a number of substituted malonic esters and amides which were tested as carcinostatic agents against mammary carcinoma 755 in C57 black mice. Malonic acid is a classical inhibitor of succinic dehydrogenase in the Krebs cycle. This inhibition may offer a basis for evaluating new types of malonic derivatives which are more selective in their action on cancer tisues than on normal tissues. Previous investigators have confined their efforts largely to malonic acid and amide on a single tumor. Boyland(1) found orally administered malonic acid, its ethyl ester and amide to be carcinostatic in dba mice with grafted mouse sarcoma. Gal(2,3) and coworkers reported several malononitriles including ethoxymethylene, p-nitrobenzilidene and 5-nitrofurilidene malononitrile moderately effective against the C3HS carcinoma. Other investigators(4) have found a number of malonic derivatives inactive on sarcoma 180. Petrakis(5) tested these compounds on human neoplasms with no therapeutic effect. The malonic esters and amides tested here were of the types $R\text{-}CH = CH(COOR)_2$ and $R\text{-}CH = CH(CONR_2)_2$. Five of these compounds, administered orally, showed some degree of activity (Tables I and II). These active compounds were also tested on other mouse tumors with negative results.

Methods. From 12 to 60 C57 black mice were weighed and then inoculated subcutaneously by trocar with mammary adenocarcinoma 755. These mice were divided into 2 groups; the first group was untreated and served as the control. For the second group, the drugs were mixed in the diet (Purina Laboratory Chow) at the maximum percentage the mice could tolerate with a slight weight loss. This value was determined by a preliminary 5-day toxicity test. Therapy was started the day of the tumor implant. At the end of 11 to 14 days the animals were weighed, the tumors were measured in 2 diameters, and the surface area was calculated. Experiments were repeated in a similar manner with sarcoma 180 in Webster-Swiss mice and with neuroblastoma C-1300 in strain A mice. The drugs were also evaluated on myeloid leukemia C1498 in C57 black mice, and were judged negative if the average longevity was prolonged less than 2 days compared to that of the controls.

The *results* are summarized in the Tables. The data for the inhibitory effect of 8-azaguanine (No. 17), a known inhibitor of mammary carcinoma 755 is given for comparison. Diethyl ethoxymethylenemalonate (No. 3) showed a definite carcinostatic effect; the treated mouse tumors were 16.5% as large as the untreated controls. This compound is relatively low in toxicity; the mice tolerated 1.2% of the drug in the diet. If therapy was deferred one day the positive effect was considerably decreased. Diethyl formylmalonate (No. 5) was also active, somewhat less than compound No. 3. The β-pyridylene derivative (No. 12) also showed some activity. The weight loss that accompanied the administration of these drugs was in general less than 10% of the body weight.

Of the malonamides, the amide of the most active malonic ester, namely ethoxymethylenemalonamide (No. 22) was only moderately active. The N, N, N′, N′-tetramethyl malonamide (No. 24) showed significant inhibitory effect on the tumor growth, whereas the tetramethyl amide of the ethoxymethylene compound (No. 26) had only slight activity. None of the substituted amino malonates had any effect on the transplanted tumor. These include the urea (No. 7), the thiourea (No.

*This work was supported in part by grants from American Cancer Society, and Damon Runyan Memorial Fund for Cancer Research.

† Department of Pharmacology, Stanford Univ. School of Medicine.

8), the N-formyl (No. 10) and the N-acetyl (No. 11) derivatives. None of the compounds active against mammary carcinoma 755 showed any inhibitory effect against the other tumors tested (*i.e.* Sarcoma 180, Neuroblastoma 1300 and Myeloid Leukemia C1498).

Discussion. It is possible to draw some conclusions on structure-activity relationships from these results. The activity of diethyl ethoxymethylenemalonate (No. 3) may be related to the formation of the hydrolysis product diethyl formylmalonate (No. 5), which is also active. The inactivity of the formyl succinate ester (No. 16) implies that the malonate moiety is required. Unsaturation *per se* is not a requisite for activity.

TABLE I. Malonic Derivatives.

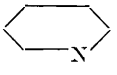
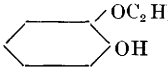
No.	$RC<^{COOC_2H_5}_{COOC_2H_5}$ Compound R	Dose % in diet	Surface area of tumor relative to controls, %	Ratio wt gain, treated/ controls	Ratio living, treated/ controls
1	$=H_2$	1.2	> 80	+0.5/+0.9	15/15
2	$=C_2H_5$	1.2	"	—1.1/+1.0	23/23
3	$=CH \cdot O\,C_2H_5$	1.2	16.5	—1.6/+2.1	32/33
4	$=CH_2$	.4	> 80	—0.4/+0.8	11/11
5	—CHO	1.2	30	—1.2/+1.6	13/13
6	$=CH\,CH_3$	.4	66	—0.2/+2.1	6/ 6
7	$=CH \cdot NH \cdot CO \cdot NH_2$	1.2	> 80	+0.4/+2.1	6/ 6
8	$=CH \cdot NH \cdot CS \cdot NH_2$	.9	"	—0.5/+2.4	6/ 6
9	$-NO_2$	.3	"	—0.5/+2.4	6/ 6
10	—NHCHO	1.5	"	—0.5/+2.4	6/ 6
11	$-NH \cdot COCH_3$	1.5	"	+0.5/+2.4	6/ 6
12		.4	40	—1.2/+1.4	20/21
13		1.2	62	—0.1/—1.0	7/ 7
	Related compounds				
14	$CH_3COC(=CH_2)-COOET$ α,Methylene acetoacetic ester	1.2	> 80	—1.7/+2.4	6/ 6
15	$ETOOC \cdot C(=CH_2)-CH_2COOET$ Ethyl itaconate	1.0	"	—3.7/+2.4	6/ 6
16	$ETOOC \cdot CH(CHO) \cdot CH_2COOET$ Diethyl formyl succinate	.6	"	—0.5/+2.5	6/ 6
17	8 Azaguanine	50 mg/kg i.p.	15	—2.3/+0.9	14/14

TABLE II. Malonamide Derivatives.

No.	$RC<^{CONR^1}_{CONR^2}$ Compound R	R^1	R^2	Dose % in diet	Surface area of tumor relative to controls, %	Ratio wt gain, treated/ controls	Ratio living, treated/ controls
21	H_2	H_2	H_2	.9	73	—0.1/+1.0	8/ 8
22	$=CH-OC_2H_5$	H_2	H_2	.8	48	—1.3/+0.4	16/16
23	H_2	CH_3	CH_3	.8	61	—0.8/+2.0	10/10
24	H_2	$[CH_3]_2$	$[CH_3]_2$	1.2	32	—0.1/+1.1	12/13
25	$=CH-OC_2H_5$	CH_3	CH_3	.9	>80	—0.7/+2.0	10/10
26	$=CH \cdot OC_2H_5$	$[CH_3]_2$	$[CH_3]_2$	.9	70	—0.6/+0.8	8/ 8

Neither the diethyl ethylidinemalonate (No. 2) nor the diethyl methylenemalonate (No. 4) is active; the unsaturated aceto acetic ester (No. 14) and itaconic ester (No. 15) are also inactive. The activity of the tetramethyl malonamide (No. 24) may be related to its greater lipid solubility. The ethoxymethylene grouping (No. 22) may impart polarity for a favorable partition coefficient.

The mode of action of these malonates might be explained by their interference with either of 2 biological pathways. Diethyl ethoxymethylenemalonate may be an inhibitor of succinic acid dehydrogenase, and consequently interfere with the tumor energy supply. Busch and Potter(6) found that succinate accumulated in tumor tissues of animals treated with sodium malonate. Another possible explanation for the activity may be considered relative to nucleic acid synthesis. It has been postulated, tentatively, that urea and oxaloacetic acid are involved in the synthesis of pyrimidines *via* orotic acid. It is conceivable that diethyl ethoxymethylenemalonate could either (a) block the condensation of urea and oxaloacetate, thus blocking orotic acid formation, or (b) condense with urea to give rise to an isomer of orotic acid, namely uracil-5-carboxylic acid. Either pathway for the utilization of diethyl ethoxymethylenemalonate might interfere with the build up of nucleic acids necessary for tumor growth.

Summary. Diethyl ethoxymethylenemalonate inhibited the growth of transplanted adenocarcinoma 755 in C57 black mice. The formyl and β-pyridylene malonic ethyl esters as well as two malonamides gave moderate inhibitory values. These compounds were inactive when tested against other transplanted tumors. Some possible modes of action are discussed.

1. Boyland, E., *Biochem. J.*, 1940, v34, 1196.
2. Gal, E. M., and Greenberg, D. M., *J. Am. Chem. Soc.*, 1951, v73, 502.
3. Gal, E. M., Fung-Haan Fung, and Greenberg, D. M., *Cancer Res.*, 1952, v12, 565.
4. Stock, C. C., Editor *Cancer Res.* Supp. Nos. 1 and 2, 1953, and 1955.
5. Petrakis, N. L., Bierman, H. R., and Shimkin, M. B., *Cancer Res.*, 1952, v12, 573.
6. Busch, H., and Potter, V. R., *ibid.*, 1952, v12, 660.

Received May 17, 1956. P.S.E.B.M., 1956, v92.

Preparation of Human Amnion Tissue Cultures.* (22535)

HASKELL J. WEINSTEIN, CAROLYN ALEXANDER, GEORGE M. YOSHIHARA, AND WILLIAM M. M. KIRBY.
Department of Medicine, University of Washington School of Medicine and King County Hospital System, Seattle.

Successful growth of human amnion in monolayer tissue culture was first reported by Zitcer *et al.*(1). A subsequent report from the same laboratory verified the usefulness of this tissue for culture of poliomyelitis virus (2). Because of the ready availability of placental tissue, attempts to culture human amnion were undertaken in this laboratory. Placentas from both vaginal and Caesarean section deliveries were used. These preliminary attempts were unsuccessful, however, and modifications of the method were undertaken, including a number suggested by Hok (3). The procedure here described evolved from these studies, and has proven to be reliable, simple, and economical. It requires little equipment that is not readily available in the average hospital laboratory. The tissue cultures are of uniformly good quality, and require a minimum of attention.

Method. 1) Human placentas from both Caesarean and vaginal deliveries have been collected by the obstetrical service of the King County Hospital System. Good growth

* This study was supported in part by grant from Office of Naval Research.