P_3 (i.e., cord factor = TDM) to produce regression of the guinea pig line 10 hepatoma, after intralesional injection. Some synthetic analogues (for instance, 8) were also active.[86c,d] More recently they have replaced part of the activity of ET by MDP(Ser) (the serine analogue of MDP) and have obtained 100% regression with a purified ET preparation called B_4.[106] Yarkoni et al.[90] had shown that the synthetic C_{76} analogues (9) of TDM are just as active as TDM itself (with ET). Then they replaced ET by MDP and obtained 100% cures using 10% paraffin oil or, more recently, 2 to 10% squalane.[107]

At Orsay, the sequential immunostimulation of peritoneal macrophages was evaluated by (1) thymocyte mitogenic protein production in vitro and (2) cytostatic activity against a syngeneic mastocytoma P815.[108] TDM-stimulated macrophages are cytostatic in vitro against mastocytoma cells. This activity decreases during in vitro cultivation but can be maintained by MDP, MPP, or LPS.

Resident macrophages can be made cytostatic against the mastocytoma cells by sequential stimulation in vitro, first by TDM and then by MDP or MPP, thus showing a direct synergistic action of both types of immunostimulants on macrophages.[108]

Conclusions

Future research in this field will develop along several lines leading, hopefully, to applications in veterinary and human medicine: (a) In view of the rapid excretion of MDP, it will be necessary to prepare slow-release forms of MDP or its analogues, using, for instance, minicapsules, minipumps,[109] liposomes,[70,110] macromolecular carriers,[64] etc. (b) It should be possible to modulate the structure of MDP so as to produce specific molecules with high affinity to certain organs or target cells; the length of the peptide moiety, the substitutions of the glycosidic part, and, finally, the nature, position, and length of a lipid moiety can all be modified so as to improve the biological activities desired. (c) Combined immunostimulation is certainly a field with great promise; it will be necessary to find the best combinations and dosage of immunostimulants (such as trehalose diesters plus MDP) so as to produce efficient and long-lasting effects.

One important advantage of the use of these adjuvants in vaccines will be to use less antigen (these being very often expensive and difficult to prepare in large quantities). Especially the future viral vaccines will use synthetic vaccinating subunits which are much less immunogenic than the whole virus; they will need a strong adjuvant.

The nonspecific antibacterial activity of MDP (or its derivatives and combinations) will be particularly useful in cases of bacterial infections by antibiotic-resistant strains.

Experimental and clinical applications of cancer immunotherapy are now widely studied. It is hoped that, here too, the synthetic immunostimulants described in this review might be used, either in vaccines (with tumor antigens) or for stimulation of nonspecific resistance, especially for treatment of immunodepressed patients.

It is still doubtful whether the immunosuppressive properties of MDP and some of its derivatives will find useful applications (for instance, for the treatment of autoimmune diseases).

To end this review with a word of caution, let us quote Gisler et al.[78] enumerating the potential risks of therapy with immunostimulants (Table II).

Acknowledgment. The author thanks Arlette Adam, Jean-François Petit, Pierre Lefrancier, Adam Bekierkunst, and Eli Yarkoni for critical reading of the manuscript and for their helpful suggestions for improvement.

The author's work was supported over the last 20 years by funds from the C.N.R.S. and grants from the D.G.R.S.T., La Fondation de la Recherche Médicale, La Ligue Nationale Française contre le Cancer, and the Cancer Research Institute (N.Y.).

(106) E. E. Ribi, R. Parker, S. M. Strain, Y. Mizuno, A. Nowotny, K. B. von Eschen, J. L. Cantrell, C. A. McLaughlin, K. W. Hwang, and M. B. Goren, *Cancer Immunol. Immunother.*, 7, 43 (1979).
(107) E. Yarkoni, E. Lederer, and H. J. Rapp, unpublished results.
(108) J. P. Tenu, E. Lederer, and J. F. Petit, *Eur. J. Immunol.*, in press.
(109) P. J. Blackshear, *Sci. Am.*, 241(6), 52 (1979).
(110) K. Mašek, M. Zaoral, J. Ježek, and R. Straka, *Experientia*, 1363 (1978).

Communications to the Editor

Prevention of Ames Test Mutagenicity by Chemical Modification in a Series of Monoamine Oxidase Inhibitors

Sir:

Potential drugs sometimes surface, especially in medicinal chemistry and insecticidal research, which, while of acceptably low acute toxicity, must be abandoned when found to be carcinogenic on chronic administration to test animals. The possibility of a rational approach to removing carcinogenicity is therefore of practical, as well as of theoretical, interest. That this possibility exists was thought likely after consideration of the current understanding of the succession of events believed necessary for a great proportion of chemically induced carcinogenicity. For example, eq 1 shows the succession of events which the work of J. A. and E. C. Miller, among others,[1] shows

$$\mathrm{ArNH_2} \rightleftharpoons \mathrm{ArNHC(O)CH_3} + [\mathrm{O}]\ (\text{"microsomal enzymes"}) \rightarrow \underset{\mathbf{1}}{\mathrm{ArN(OH)C(O)CH_3}} \rightarrow \underset{\mathbf{2}}{\mathrm{ArN(OZ)C(O)CH_3}} \rightarrow \underset{\mathbf{3}}{\mathrm{Ar\overset{+}{N}C(O)CH_3}} \quad (1)$$

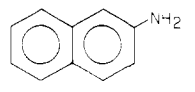
apparently to be required for an aryl amide, such as 2-acetaminofluorene [*N*-(2-fluorenyl)acetamide; 2-AAF], to cause cancer to experimental animals. A similar sequence is thought to apply to carcinogenic arylamines either directly or after in vivo acylation. An arylamine or aryl amide must first be oxidized to the arylhydroxyamine or arylhydroxamic acid 1. This oxidation is catalyzed by

(1) Miller, J. A. *Cancer Res.* **1970**, *30*, 559. Weisburger, J. H.; Yamamoto, R. S.; Williams, G. M.; Grantham, P. H.; Matsushima T.; Weisberger, E. K. *Ibid.* **1972**, *32*, 491.

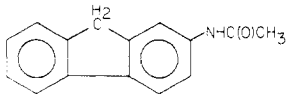
0022-2623/80/1823-0825$01.00/0

mixed-function oxidases present in greatest amount in liver cell endoplasmic reticulum and which can be concentrated in microsomal preparations. Compounds 1 then are converted to acetoxy, sulfonyloxy, or other similarly functionalized compounds [2, Z = $CH_3C(O)$ or SO_3H] in which OZ is a good leaving group. The particular 2 is then converted to an electrophile 3, which may have a free nitrenium ion as shown or be part of an ion-pair complex.[2] Such compounds are extremely reactive electrophiles. When 3 reacts with DNA, the resulting modified DNA in some yet undefined way is thought to lead to tumor initiation as well as other consequences, notably mutagenesis. Other compounds potentially leading to electrophilic species also appear to be carcinogenic by similar mechanisms.[3]

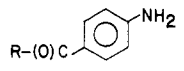
An early and still generally valid formulation of the structural requirements for arylamine and aryl amide carcinogenesis[4] was that arylamines and aryl amides with nonmetabolizable groups para to the amine or amide function (e.g., 4a and 4b) would be carcinogenic. The



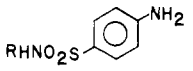
2-Naphthylamine
4a



2-AAF
4b



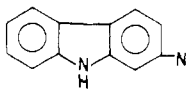
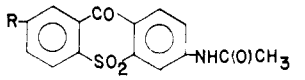
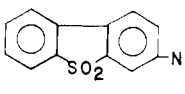
5



6

obvious exceptions to this "rule" include *p*-aminobenzoic acid (PABA, 5) derivatives in the folic acid cycle and the antibacterial sulfonamides (6). The former are necessary for all known forms of life, and the latter have been given to many animal species for prolonged periods with few if any signs of carcinogenesis or mutagenesis. An initial "explanation", that polycyclic structures are required for cancer production,[5] can be ruled out.[6] An explanation[7] accepted at present is that sulfonamides and PABA derivatives partition too much in favor of water compared to lipid and so do not reach the microsomal oxidizing systems believed responsible for hydroxylation to form 1. The author preferred a theory that the common presence of electron-withdrawing groups in 5 and 6 would slow the oxidation of aryl amides (oxidation is the loss of electrons) and would also slow the ionization or charge separation required to lead to the electrophilic species 3. A test of this hypothesis ideally would require a biologically active (to indicate penetration into cells, if not necessarily into microsomes) aryl amide carcinogen and a reasonably rapid test for carcinogenesis. The Ames mutated *Salmonella typhimurium* strains with metabolic activation[8] supply an approach to carcinogenic potential with positive results reported as correlating about 90% with carcinogenicity in whole animals. A biologically active aryl amide has recently come out of our work in another area, fulfilling the proof of penetration requirement. This is *N*-(2-carbazolyl)acetamide (7). Compound 7 was found[9] to inhibit monoamine oxidase (MAO) both in vitro and in vivo. In

Table I. Log Octanol-Water Partition Coefficient (log *P*) and Ames Test Averaged Results in Revertants/Plate with Strains Shown[a] at Compound Amount per Plate Given

	log P	Amount (mg/plate)	Revertants/Plate[b,c] TA1538	TA98
		.13	679	725
		.25	762	732
		.50	699	468
7	3.02	1.0	775	456
		.1	0	0
		.5	0	0
	2.35	2.5	0	0
8: R = H		5	0	0
9: R = CH_3[d]	2.76	.13	0	1
		.25	2	0
		.5	0	0
		1.0	5	8
10: R = C_2H_5[e]	3.44	.13	1	0
		.25	0	0
		.5	1	0
		1.0	2	0
11: R = N-C_3H_7[e]	3.51	.13	0	0
		.25	0	0
		.5	0	0
		1.0	0	0
		.13	0	0
		.25	0	0
		.5	2	2
12		1.0	2	4

[a] Strain TA1537 was negative for all compounds. TA100 was positive for 7 and negative for 8-11, but the background for the tests with 9-11, averaging 388 revertants per plate (n = 10), was higher than recommended in a recent publication: de Serres, F. J.; Shelby, M. D. *Mutat. Res.* 1979 *64*, 159-165. [b] Revertants per plate after subtracting solvent control. Duplicate results were averaged. Mutagenicity results essentially by the method of Ames.[8] Results supplied by Dr. William J. Suling of Southern Research Institute, except those for 8 which were from E. G. and G. Mason Research Institute and had comparable control values and positive values for 7. Two other laboratories' results for 8 were in agreement. See text. [c] Results are given for tests preincubated with liver microsomal enzyme (9000*g* supernatant induced in male Sprague-Dawley rats with Aroclor 1254, supplied by Litton Bionetics). Without this "activation" all of the tests were negative except for the carbazole analogue 7, which was slightly positive but far below values for tests run with activation. [d] Compound 9 gave the correct elemental analysis but had several spots indicating traces of impurities on thin-layer chromatography (TLC). An adequate high-performance LC system could not be found. [e] The samples of compounds 10 and 11 used for mutagenicity testing were purified by high-performance LC and each gave a single spot on TLC.

(2) Gutschke, D.; Heesing, A.; Heuschkel, U. *Tetrahedron Lett.* **1979**, *16*, 1363.
(3) Maugh II, T. H. *Science* **1974**, *183*, 940.
(4) Clayton, D. B. *Br. J. Cancer* **1953**, *7*, 460.
(5) Arcos J. C.; Argus, M. F. "Chemical Induction of Cancer"; Academic Press: New York and London, 1974; Vol. IIB, p 282.
(6) Reference 5, pp 1-8.
(7) Miller, J. A., private communication.
(8) Ames, B. N.; McCann, J.; Yamasaki, E. *Mutat. Res.* **1975**, *31*, 347-364.
(9) Made by Charles Joyner, MAO data from Dr. Helen White, both of these laboratories. A full report will be submitted for publication in due course. The author is indebted to his colleagues for permission to use their data.

the latter tests, it was given orally to rats, and their brain MAO became inhibited. It therefore passes through both the gut wall and the "blood-brain barrier". Since 7 has a nonmetabolizable group para to the amide nitrogen, it was sent for mutagenicity testing; 7 was found to be mutagenic in the Ames test with activation, performed by three different laboratories independently. It should be noted that 7 has been reported to give a slight incidence of tumors in 8-month rat carcinogenicity testing.[10]

The effect of electron withdrawal on mutagenicity was checked by preparing and subjecting to the Ames test the thioxanthen-9-one 10,10-dioxide analogue 8. This compound has two strongly electron-withdrawing groups and was found nonmutagenic with strains in which 7 was mutagenic (Table I). However, octanol-water partition coefficients [log P = log (concentration in octanol/concentration in water)][11] showed that 8 has a lower log P value (i.e., is less lipophilic) than 7. The 7-methyl, 7-ethyl, and 7-propyl homologues of 8, i.e., 9, 10, and 11,[12] were therefore prepared to ensure a lipophilicity range including that of 7 and subjected to the Ames test. The log P values of these[13] were shown (see Table I) to straddle that of 7, and all were found nonmutagenic in the systems in which 7 was mutagenic. To ensure that nonmutagenicity was not causally related specifically to the thioxanthone dioxide ring system present in 8-11, 2-(acetylamino)dibenzothiophene 9,9-dioxide (12) was also subjected to the Ames test and also was found to show no mutagenicity. This compound was negative in limited carcinogenicity testing.[10] Compounds 8-11 were found to be inhibitors of the MAO in mouse brains after intraperitoneal administration and so penetrated the "blood-brain barrier". Of course this does not prove their penetration into microsomes or into the strains of *Salmonella typhimurium* used in the mutagenicity testing. However, since penetration into the brain appears more restricted than microsomal oxidation in general, it is likely that these compounds would penetrate into the microsomal oxidation region.[14] Penetration of 7 into the test strains of *Salmonella* obviously occurs as shown by dose-related toxicity. Penetration of 8-12 was not proved, since toxicity due to these compounds did not occur at the highest dosage used. Penetration is commonly assumed in the Ames test; the tester strains have genetically defective cell walls, to ensure permeability to chemicals which are tested.

The results reported are in agreement with our theory that mutagenicity can be removed from an otherwise mutagenicity-causing pharmacophore by reducing the electron availability. Proof that this phenomenon is general for aryl amide carcinogens and that it can be generalized to functionalities other than aryl amide and from mutagenicity to carcinogenicity awaits further studies.

A recent publication[15] has reported a lowered rate of enzymatic N-hydroxylation of *p*-(substituted styryl)acetanilides due to an electron-withdrawing nitrile group, as anticipated. Another[16] has a regression curve in which σ^+ (i.e., electron input) correlates with increased Ames test mutagenicity and L1210 leukemia for 1-(substituted phenyl)-3,3-dialkyltriazines. These are probably carcinogenic and mutagenic after mono-N-dealkylation followed by N-N cleavage and ultimately carbonium ion formation, a complex situation to interpret but at least in agreement with our results despite the differing ultimate carcinogen.

(10) Miller, J. A.; Sandin, R. B.; Miller, E. C.; Rusch, H. P. *Cancer Res.* **1955**, *15*, 188.
(11) Fujita, T.; Iwasa J.; Hansch, C. *J. Am. Chem. Soc.* **1964**, *86*, 5175.
(12) All compounds mentioned were purified to acceptable elemental analysis values; cf. Table I, footnotes *d* and *e*.
(13) I appreciate the permission of Professor Corwin Hansch to use these data, generated in his laboratory.
(14) For a partial discussion, cf. Hansch, C.; Steward, A. R.; Anderson, S. M.; Bentley, D. *J. Med. Chem.* **1967**, *11*, 1.
(15) Hanna, P. E.; Gammans, R. E.; Sehon, R. D.; Lee, M. K. "Abstracts of Papers", 178th National Meeting of the American Chemical Society, Washington, D.C., Sept 1979; American Chemical Society: Washington, D.C., 1979; Abstr. MEDI 70.
(16) Venger, B. H.; Hansch, C.; Hatheway, G. J.; Amrein, Y. U. *J. Med. Chem.* **1979**, *22*, 473.

M. Harfenist

The Wellcome Research Laboratories
Burroughs Wellcome Co.
Research Triangle Park, North Carolina 27709

Received April 14, 1980

Articles

Thrombin Inhibitors. 1. Ester Derivatives of N^{α}-(Arylsulfonyl)-L-arginine

Shosuke Okamoto, Kiyokatsu Kinjo, Akiko Hijikata,

Department of Physiology, Kobe University School of Medicine, Kobe, Japan

Ryoji Kikumoto,* Yoshikuni Tamao, Kazuo Ohkubo, and Shinji Tonomura

Central Research Laboratories, Mitsubishi Chemical Industries Limited, Yokohama, Japan. *Received December 21, 1979*

A series of N^{α}-(arylsulfonyl)-L-arginine esters was prepared and tested as inhibitors of the clotting activity of thrombin. N^{α}-Dansyl-L-arginine methyl ester was the most inhibitory of the N^{α}-(arylsulfonyl)-L-arginine methyl esters. The most potent inhibitors were the *n*-propyl and *n*-butyl esters of N^{α}-dansyl-L-arginine with an I_{50} of 2×10^{-6} M. Esters of unsaturated straight-chain alcohols with a chain length of four carbons were also as inhibitory as the *n*-butyl ester. The inhibitors were hydrolyzed by thrombin and trypsin more slowly than N^{α}-tosyl-L-arginine methyl ester.

Thrombin catalyzes the formation of fibrin and stimulates the aggregation of platelets. Synthetic inhibitors of this enzyme are of interest as potential therapeutic and prophylactic agents for thrombotic diseases as well as re-

0022-2623/80/1823-0827$01.00/0