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A toxicological evaluation of 2-dimethylaminomethyl-1-phenyl-2-propen-1-one hydrochloride

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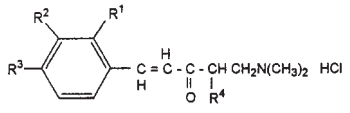
2-Dimethylaminomethyl-1-phenyl-2-propen-1-one hydrochloride (**3**) is a novel cytotoxic and anticancer agent. The objective of this study was to obtain information pertaining to possible toxic symptoms detected by *in vivo* evaluations in mice and an *in vitro* test for mutagenicity. The data obtained revealed that **3** had no effect on alanine transaminase, aspartate transaminase, HDL cholesterol and protein concentrations in sera nor were variations in the numbers of red and white blood cells detected. Furthermore autopsies of treated mice revealed no pathological symptoms in the heart, kidney, brain, spleen and testes. However elevation of the concentrations of total cholesterol, triglycerides, creatinine and urea were noted in treated mice as well as inflammation of the liver and lungs. Chromosomal aberrations were detected in a micronuclei test. In the Ames test, compound **3** was converted into one or more mutagens in the presence (but not the absence) of a murine liver homogenate. Thus future molecular modifications of **3** should bear in mind approaches to reduce or minimize unwanted side effects.

1. Introduction

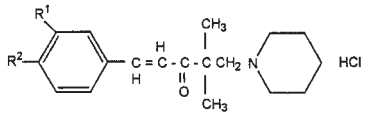
A number of studies from these laboratories have been undertaken pertaining to the syntheses of a variety of candidate anticancer and cytotoxic Mannich bases (β-aminoketones). The bioactivities of many of these compounds have been summarized in a review article [1]. The reasons for pursuing these compounds as potential antineoplastic agents included the following considerations. First, Mannich bases having at least one hydrogen atom on the carbon atom adjacent to the carbonyl group have the facility to undergo deamination leading to the corresponding α,β-unsaturated ketones [2, 3]. This latter cluster of compounds has a marked affinity for thiols in contrast to amino groups [4, 5]. Since thiols are absent in nucleic acids, cytotoxic α,β-unsaturated ketones may be free from the problems of carcinogenicity and mutagenicity which are found in a number of anticancer drugs [6]. The observation that the Mannich bases **1** did not display mutagenicity in the Ames test [7] supported this viewpoint. Second, one of the sites of action of a number of Mannich bases is the mitochondria [8, 9]. Various Mannich bases caused a greater inhibition of respiration in mitochondria isolated from malignant hepatic cells than from the same organelles obtained from normal liver tissue [7]. This selectivity in a biocemical action of certain Mannich bases may translate into compounds displaying preferential toxicity for cancer cells rather than the corresponding normal tissue. Third, a series of Mannich bases **2** were free from cross resistance to melphalan-resistant and adriamycin-resistant tumour cell lines [10] suggesting that their modes of action are different from certain established anticancer drugs. Hence they may find utility in treating drug-resistant neoplasms.

A Mannich base which is currently of interest is 2-dimethylaminomethyl-1-phenyl-2-propen-1-one hydrochloride (**3**) due to its cytotoxic and anticancer properties [11]. Compound **3** displayed potencies comparable to melphalan when evaluated against murine P388 and L1210 cells *in vitro*. Against a panel of human tumour cell lines from



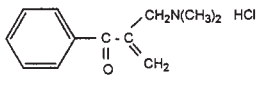
1

$R^1 = R^2 = R^3 = H, Cl ; R^4 = H, (CH_2)_4CH_3$



2

$R^1 = R^2 = H, Cl, Br, CH_3, OCH_3$



3

different neoplastic diseases, **3** was approximately four times as potent as melphalan and demonstrated a selective toxicity towards leukemic cells. The *in vivo* results were also encouraging since this Mannich based reduced the sizes of human colon and non-small cell lung tumour xenografts passaged in athymic mice. Furthermore, **3** virtually tripled the life spans of mice with Ehrlich ascites carcinoma which were increased yet further by formulating **3** in niosomes [11].

In view of the potential uses of Mannich bases in cancer chemotherapy in general, and also the establishing of **3** as a candidate antineoplastic agent in particular, the decision was made to determine whether **3** caused adverse effects. The results from such an investigation would provide direction in pursuing development of **3** and related compounds.

2. Investigations and results

Four different approaches were used in the evaluation of whether **3** possessed toxic properties; namely, first, an examination of the serum of mice treated with this Mannich base, second a study of possible damage to certain body organs of mice, third whether **3** caused chromosome aberrations *in vivo* and finally an *in vitro* test for mutagenicity was utilized.

The first experiment involved the administration of multiple doses of 2 mg/kg of **3** to mice. This quantity of the Mannich base was used since this dose led to an enormous increase in the lengths of lives of mice with Ehrlich ascites carcinoma [11]. The sera of treated and control animals were collected and various biochemical tests undertaken. These data are presented in Table 1. The results indicate that under the experimental conditions chosen, **3** does not cause marked liver damage since the concentrations of alanine transaminase (ALT) and aspartate transaminase (AST) were normal in treated mice. Small elevations in the concentrations of total cholesterol and triglycerides were noted in mice receiving **3** revealing some interference with lipid metabolism. The creatinine and urea concentrations increased slightly in the treated animals which may have been due to renal dysfunction or possibly **3** caused dehydration. The Mannich base **3** exerted no statistically significant effect on haematopoiesis since the red and white blood cell counts were not altered in the treated animals. The protein concentrations in sera are elevated in a number of pathological conditions including dehydration, inflammation and food deprivation; however **3** did not appear to exert any of these effects since the protein concentrations were normal in treated mice.

The second phase of the study consisted in examining the following tissues in the autopsies of four treated male mice, namely liver, heart, kidney, brain, intestine, stomach, lung and spleen; in addition, the testes were examined in two mice. No pathological symptoms were noted in heart, kidney, brain, spleen and testes. In the four other tissues, the following effects were noted (number of animals out of 4 displaying toxicity), namely mild inflammation of the lungs (2), moderate inflammation of the lungs (1), mild congestion of the lungs (2), mild congestion of the liver (3), mild cellular atypia of the liver (1), moderate inflammation of the liver (1), mild inflammation of the intestine (1) and mild inflammation of the stomach (1).

The third evaluation of **3** used a micronuclei assay which was undertaken in order to determine whether chromosomal damage occurred in both polychromatic erythrocytes, which are immature erythrocytes, as well as normochromatic erythrocytes which have the normal quantity of haemoglobin. These data are presented in Table 2 which reveal that the Mannich base **3** caused chromosomal damage.

Finally the mutagenic potential of **3** was examined using seven auxotrophic *Salmonella typhimurium* Ames assay strains [12–15] as well as two *Escherichia coli* RK mutatest strains [16, 17]. No mutagenicity was shown with the seven Ames assay strains in the absence of the S9 rat liver homogenate. On the other hand, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) produced strong mutagenic responses in TA100 (0.01–1.0 μg) and TA1535 (0.1–1.0 μg) while the IRC-191 control caused mutagenesis in TA97A cells (10 μg). A concentration of 500 μg of **3** per assay plate killed all cells in treatments with the added S9 extract (data not shown). As indicated in Table 3, the

Table 2: Effect of compound 3 on micronuclei induction in murine bone marrow[a]

Biochemical parameter	Treated ± SE	Control ± SE
Number of micronuclei per 1000 polychromatic erythrocytes (PE)	3.5 ± 0.02[b]	2.4 ± 0.08
Number of micronuclei per 1000 normochromatic erythrocytes (NE)	0.8 ± 0.01[b]	0.2 ± 0.01
PE/NE ratio	1.02 ± 0.3	1.02 ± 0.01

[a] Mice were administered 2 mg/kg of **3** by the intraperitoneal route
[b] $P < 0.0001$ using the student 't'test

Table 1: Determination of various biochemical parameters in mice after treatment with 3

Biochemical parameter	Mean value for male mice ± SD n = 5		Mean value for female mice ± SD n = 5		Mean value for male and female mice ± SD	
	Treated	Control	Treated	Control	Treated	Control
Alanine transaminase (IU)	82.60 ± 17.69	74.60 ± 10.3	88.2 ± 21.3	78.8 ± 19.9	85.4 ± 18.7	76.7 ± 15.1
Aspartate transaminase (IU)	177.4 ± 16.5	171.6 ± 18.9	194.0 ± 29.0	178.0 ± 19.7	185.7 ± 23.9	174.8 ± 18.5
Total cholesterol (mg/dl)	135.8 ± 1.6[a]	120.4 ± 6.4	138.2 ± 7.8	126.8 ± 8.4	137.0 ± 5.5[a]	123.6 ± 7.8
Triglycerides (mg/dl)	107.8 ± 5.7	99.0 ± 8.4	105.8 ± 5.0	98.6 ± 7.8	106.8 ± 5.2[b]	98.8 ± 7.6
HDL-Cholesterol (mg/dl)	52.20 ± 2.68	49.80 ± 3.42	53.00 ± 4.64	50.80 ± 3.49	52.60 ± 3.60	50.30 ± 3.30
Creatinine (mg/dl)	0.52 ± 0.05	0.46 ± 0.09	0.50 ± 0.07	0.44 ± 0.06	0.51 ± 0.06[b]	0.45 ± 0.07
Urea (mg/dl)	57.20 ± 7.43	53.8 ± 3.9	58.00 ± 5.92[b]	50.00 ± 3.39	57.6 ± 6.35[b]	51.9 ± 3.99
Red blood cells (10^6/μl)	9.24 ± 2.38	8.20 ± 1.12	8.40 ± 1.28	9.03 ± 1.69	8.82 ± 1.85	8.62 ± 1.42
White blood cells (10^6/l)	4.88 ± 1.68	5.93 ± 1.06	4.19 ± 1.12	4.80 ± 1.22	4.54 ± 1.40	5.37 ± 1.23
Protein (μg/mg tissue)	31.84 ± 10.72	33.3 ± 10.2	32.96 ± 9.22	38.88 ± 11.14	32.4 ± 9.45	36.1 ± 10.5

[a] Significantly different from the control value ($p < 0.001$)
[b] Significantly different from the control value ($p \leq 0.05$)

Table 3: Effect of 3 on Salmonella typhimurium TA100 in the absence and presence of S9 mix

Concentration of **3** (μg/assay plate)	Number of colony forming units	
	S9 mix absent ± SE	S9 mix present ± SE
0	676 ± 75	474 ± 19
0.005	–	491 ± 1
0.05	–	638 ± 34
0.5	–	533 ± 49
5	526 ± 23	645 ± 35
50	55 ± 11	1337 ± 286

TA100 strain revealed mutagenesis as demonstrated by the increase in revertants with increasing concentrations of **3**. For example, a concentration of 50 μg of **3** in the presence of the S9 extract significantly increased the appearance of mutants (*his* revertants), while parallel treatments with **3** in the absence of S9 showed more than a tenfold drop in revertants due to a loss of cell viability.

All of the treatments using **3** were highly toxic to the RK assay strains yielding cell viabilities of less than 10^{-5} with no mutants produced due to the high toxicity of this Mannich base.

3. Discussion

The potential of 2-dimethylaminomethyl-1-phenyl-2-propen-1-one hydrochloride (**3**) as a candidate cancer chemotherapeutic agent was outlined in the introductory section of this report, namely (i) its *in vitro* and *in vivo* potencies, (ii) the novelty of both its structure and putative mode of action and (iii) the likelihood of **3** demonstrating preferential toxicity to neoplasms in contrast to the corresponding normal cells. The question posited in the light of the biodata generated using **3** is whether development of this compound and/or its analogues should be pursued. In addition, if pursuit be considered a viable option, the possibility of vigilance in certain areas may be necessary to be considered. The Mannich base **3** was designed as a thiol alkylator. Hence, in theory at least, it should be devoid of some of the side effects of various alkylating agents used in cancer chemotherapy which include DNA damage [6], induction of acute myelogenous leukemia [18], birth defects [19] and adverse effects to the reproductive systems [20, 21].

The positive aspects of this compound which emerged from this assessment of its possible toxicity are as follows. First, the employment of the same dose of **3** which had demonstrated marked anticancer activity *in vivo* did not cause any mortalities after a total of 30 daily doses were administered. Second, there were no severe adverse effects on hepatic function as revealed by the concentrations of ALT and AST in serum. Third, while a number of anticancer drugs affect haematopoiesis [22], the Mannich base **3** did not lead to changes in the number of red and white blood cells. In addition, no pathological effects were noted in five of nine body organs in treated mice.

On the other hand, a number of toxic effects of **3** were detected. Chromosomal damage was observed in a micronuclei test and metabolism of **3** produced one or more mutagens as revealed in the Ames test. Second, first pass metabolism takes place in the liver and lungs [23] and inflammation in both organs was detected by necroscopy of treated animals. Finally, changes in lipid metabolism as well as creatinine and urea concentrations were observed.

The conclusion to be drawn from this study is that while **3** is an important prototypic cytotoxic and anticancer agent, molecular modifications should take place not only with a view to increasing potency but to eliminating, or at least reducing, some of the side effects. For example, prodrug formation enabling the gradual release of **3** may permit retention of the cytotoxic and anticancer properties while amelioration of the unwanted toxic symptoms occurs. In addition, the use of certain cytoprotective agents such as mesna, which has been coadministered with cyclophosphamide in order to reduce the side effects but not the anticancer activity [24] should be considered. Hence the formation of thiol adducts of **3** and congeners should be considered which may permit a retro-Michael reaction to occur *in vivo* liberating an antineoplastic agent plus a thiol. Finally the insertion of electron-releasing groups into the aryl ring of **3** should reduce the electrophilicity of the terminal olefinic carbon atom of **3** thereby reducing chemical reactivity and possibly toxicity.

4. Experimental

4.1. Synthesis of the compound

The preparation of **3** has been described previously [25].

4.2. Toxicity evaluation in mice

The toxicity study was undertaken using six to eight week old Swiss albino mice (25–30 g) which were obtained from the Central Animal House, Kasturba Medical College, Manipal, India. The animals had free access to food and water.

A dose of 2 mg/kg of **3** was injected daily by the intraperitoneal route into each of five male mice and also five female mice for 30 days. The control group of animals comprising five male and five female mice received injections of distilled water in the same manner as the treated animals. All mice were observed for the appearance of any physiological, behavioural and lethal effects caused by the injections. Twenty-four hours after the last injections, i.e., on day 31, blood samples from each mouse were collected separately into non-heparinized tubes and allowed to coagulate. The serum was separated by centrifugation and the alanine transaminase, aspartic transaminase, total cholesterol, triglycerides, HDL-cholesterol, creatinine and urea contents were determined using a Hitachi 911 autoanalyzer. The number of red and white blood cells were obtained from blood collected in sterile heparinized tubes by a literature procedure [26]. The total protein content in a portion of hepatic tissue was obtained by a published methodology [27]. Portions of the liver, lung, heart, spleen, testis, intestine, stomach, brain and kidneys were placed in Bovin's fixative and processed for histological examination. The slides were stained with haematoxylin and eosin and examined under a low power microscope for any gross pathological changes. The micronuclei assay was undertaken by a previously reported method [28] using six treated mice and six control animals of either gender.

4.3. Short-term bacterial toxicity and mutagenesis assays

The assays employed used various Ames' strains of *Salmonella typhimurium* and were undertaken by literature procedures [12–15]. In the absence of the S9 liver extract, the Mannich base **3** as well as the reference compounds were applied as 10 μL spots to Vogel-Bonner minimal medium (1.5% agar and 2.0% glucose) plates that had been overlaid with TA1535, TA1538, TA97A, TA98, TA100, TA102 or TA104 cells (0.1 mL) mixed with 2 mL of top agar (molten agar 0.6% containing 0.5% sodium chloride, 0.1 μM histidine and 0.1 μM biotin). Compound **3** or MNNG were dissolved in sterile deionized water containing dimethylsulphoxide (DMSO, 10% v/v) to produce solutions containing 5, 0.5, 0.05, 0.005 and 0.0005 μg/10 μL of **3** and 1.0, 0.1 and 0.01 μg/10 μL of MNNG. In addition, IRC-191 (9-chloro-3-(2-chloroethylamino)propylamino-8-methoxyquinoline) was dissolved in 100 mM acetate butter pH 5.0 containing DMSO (5% v/v) to give a concentration of 10 μg/10 μL. In the presence of the S9 extract, the Ames' strains TA97A, TA98, TA100, TA102 and TA104 were assayed using the same and higher (500 and 50 μg) concentrations of **3** vide supra except that the compound was dissolved in 100 mM acetate buffer. The assay was carried out using 0.1 mL of cells, 0.5 mL of S9 mix, 10 μL of solution and 2 mL of the top agar and were conducted in duplicate except that when the TA100 strain was used, the control, 5 and 50 μg experiments were performed in quadruplicate.

The RK assay was carried out using *Escherichia coli* RK mutatest strains Y832 and B457 using literature procedures [16, 17]. In this case, the cells

were mixed for 10 min with 2000, 200 and 50 µg of **3** in a solution containing 0.01 M sodium chloride in 0.01 M tris(hydroxymethyl)aminomethane, pH 7.4 in 10% DMSO.

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References

1 Dimmock, J. R.; Kumar, P.: Curr. Med. Chem. **4**, 1 (1997)
2 Dimmock, J. R.; Shyam, K.; Hamon, N. W.; Logan, B. M.; Raghavan, S. K.; Harwood, D. J.; Smith, P. J.: J. Pharm. Sci. **72**, 887 (1983)
3 Gordon, P. M.; Johnston, J. D.; English, A. R.: in Hobby, G. L. (Ed.): Antimicrobial Agents and Chemotherapy, p. 165, American Society for Microbiology, Bethesda 1965
4 Dimmock, J. R.; Raghaven, S. K.; Logan, B. M.; Bigam, G. E.: Eur. J. Med. Chem. **18**, 248 (1983)
5 Baluja, G.; Municio, A. M.; Vega, S.: Chem. and Ind., 2053 (1964)
6 Benvenuto, J. A.; Connor, T. H.; Monteith, D. K., Laidlaw, J. L.; Adams, S. C.; Matney, T. S.; Theiss, J. C.: J. Pharm. Sci. **82**, 988 (1993)
7 Dimmock, J. R.; Hamon, N. W.; Chow, E. W. K.; Kirkpatrick, D. L.; Smith, L. M.; Prior, M. G.: Can. J. Pharm. Sci. **15**, 84 (1980)
8 Khachatourians, G. G.; Holmlund, P. K.; Dimmock, J. R.: J. Pharm. Sci. **73**, 803 (1984)
9 Hamon, N. W.; Kirkpatrick, D. L.; Chow, E. W. K.; Dimmock, J. R.: J. Pharm. Sci. **71**, 25 (1982)
10 Dimmock, J. R.; Kumar, P.; Quail, J. W.; Pugazhenthi, U.; Yang, J.; Chen, M.; Reid, R. S.; Allen, T. M.; Kao, G. Y.; Cole, S. P. C.; Batist, G.; Balzarini, J.; De Clercq, E.: Eur. J. Med. Chem. **30**, 209 (1995)
11 Dimmock, J. R.; Vashishtha, S. C.; Patil, S. A.; Udupa, N.; Dinesh, S. B.; Devi, P. U.; Kamath, R.: Pharmazie **53**, 702 (1998)
12 Ames, B. N.; McCann, J.; Vamaski, E.: Mutat. Res. **31**, 347 (1975)
13 Maron, D. M.; Ames, B. N.: Mutat. Res. **113**, 173 (1983)
14 Ames, B. N.; Durston, W. E.; Yamasaki, E.; Lee, F. D.: Proc. Natl. Acad. Sci. USA **70**, 2281 (1973)
15 Gee, P.; Maron, D. M.; Ames, B. N.: Proc. Natl. Acad. Sci. USA **91**, 11606 (1994)
16 Hayes, S.: Toxicol. Assess. **3**, 287 (1988)
17 Hayes, S.; Gordon, A.; Sadowski, I.; Hayes, C.: Mutat. Res. **130**, 97 (1984)
18 Bloom, J. C.; Brandt, J. T.; in: Klaason, C. D. (Ed.): Casarett and Doull's Toxicology, 6. Ed., p. 402, McGraw-Hill Companies, Inc., New York 2001
19 Warkany, J.; in: Wilson, J. G.; Warkany, J. (Eds.): Teratology: Principles and Techniques, p. 1, University of Chicago Press, Chicago 1965
20 Lee. J. P.: Am. J. Ind. Med. **4**, 135 (1983)
21 Pederson, R. A.; Brandriff, B.; in: Generoso, W. M.; Shelby, M. D.; DeSerres, F. J. (Eds.): DNA Repair and Mutagenesis in Eukaryontes, p. 389, Plenum Press, New York 1980
22 Bloom, J. C.; Brandt, J. T.; in: Klaasen, C. D. (Ed.): Casarett and Doull's Toxicology, 6. Ed., p. 393, McGraw-Hill Companies, Inc., New York 2001
23 Thomas, G.: Medicinal Chemistry: An Introduction, p. 201, John Wiley and Sons, Ltd., Chichester 2000
24 Thurston, D. E.; Lobo, S. G. M. J.; in: Smith, H. J. (Ed.): Smith and Williams' Introduction to the Principles of Drug Design and Action, 3. Ed., p. 351, Harwood Academic Publishers, Amsterdam 1998
25 Dimmock, J. R.; Patil, S. A.; Leek, D. M.; Warrington, R. C.; Fang, W. D.: Eur. J. Med. Chem. **22**, 545 (1987)
26 Wintrobe, M. M.: Clinical Haematology, p. 391, Lea and Febiger, Philadelphia 1961
27 Lowry, O. H.; Rosenbrough, N. J.; Farr, A. I.; Randall, R. J.: J. Biol. Chem. **193**, 265 (1951)
28 Schmid, W.: Mutat. Res. **31**, 9 (1975)