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METABOLISM OF 4(5)-(3,3-DIMETHYL-1-TRIAZENO)-IMIDAZOLE-5(4)-CARBOXAMIDE TO 4(5)-AMINO-IMIDAZOLE-5(4)-CARBOXAMIDE IN MAN*

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Abstract—Administration of 4(5)-(3,3-dimethyl-1-triazeno)imidazole-5(4)-carboxamide (DIC), a structural analogue of 4(5)-aminoimidazole-5(4)-carboxamide (AIC), i.v. or p.o., to patients with cancer was followed by increased urinary AIC excretion. After a single i.v. administration of 4·5 mg/kg of DIC, AIC excretion in the 24-hr period after DIC administration was 20 per cent of the dose of DIC given. When the same dose was given p.o., about 16·5 per cent of the dose of DIC was excreted as AIC within 24 hr. One patient given DIC p.o. at a dose of 1·5 mg/kg every 6 hr for four consecutive doses excreted about 13·6 per cent of each dose as AIC in each 6-hr period. Two patients were given DIC-2-^{14}C p.o. Ion-exchange chromatography of plasma samples resulted in the detection of a peak of radioactivity in the column eluate fraction containing AIC. After ion-exchange chromatography of urine samples, 20·9 and 8·7 per cent, respectively, of the radioactivity appeared in the AIC fractions. ^{14}C-AIC was isolated from urine and characterized by paper-chromatographic behavior, ultraviolet fluorescence and color reaction after diazotization and coupling identical to that of authentic AIC, and was recrystallized to constant specific activity. The specific activity of the urinary ^{14}C-AIC from one patent was 78 per cent of that of the DIC-2-^{14}C given. These data suggest that in man most of the increased urinary AIC excretion observed after DIC administration is the direct result of metabolism of DIC. The mechanism(s) of action of DIC as an antitumor agent in man is unknown.

THE ANTINEOPLASTIC agent 4(5)-(3,3-dimethyl-1-triazeno)imidazole-5(4)-carboxamide (DIC) is a structural analogue of 4(5)-aminoimidazole-5(4)-carboxamide (AIC),[1-3] and has shown significant antitumor activity in mice[3, 4] and in man during preliminary clinical trials.[5, 6] Clinical pharmacological studies of DIC in man were reported.[6, 7]

The ribotide of AIC occupies a central position in the *de novo* purine synthetic pathway.[8] A structural analogue such as DIC could be a potential antimetabolite of the *de novo* purine synthetic pathway. Increased urinary excretion of AIC was observed after administration of amethopterin to rats[9, 10] and to patients with leukemia.[11] The more pronounced elevation of urinary AIC reported after the administration of DIC to man[12, 13] suggested, as in the case of amethopterin, possible interference with AIC metabolism and the *de novo* purine synthetic pathway. An alternative conclusion from these data was that the increased urinary AIC observed

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after administration of DIC arose from the metabolism of DIC. *N*-Demethylation of DIC was shown to occur in rats and man.[14] The product of *N*-demethylation, 4(5)-(3-monomethyl-1-triazeno) imidazole-5(4)-carboxamide (MIC), is unstable[15] and should decompose spontaneously to yield AIC and a possible methylating intermediate.[15–17] The identification of AIC as a metabolite of DIC by the use of ring-labeled DIC-2-^{14}C is the subject of this report.

MATERIALS AND METHODS

DIC was provided by the Clinical Branch, Collaborative Research, National Cancer Institute, U.S. Public Health Service. DIC-2-^{14}C (15·478 mc/m-mole) was provided by Monsanto Research Corp. (Dayton, Ohio) under contract from the Cancer Chemotherapy National Service Center, National Cancer Institute. AIC (hydrochloride), chromatographically pure, was purchased from Sigma Chemical Co., St. Louis. Dowex 50W (H^+) (12% cross-linkage, 200–400 mesh) was prepared as previously described.[18]

Patients selected for study were those accepted for Phase I and Phase II clinical cancer chemotherapy trials with DIC.[6] DIC was given i.v. at a dose of 4·5 mg/kg of body weight to three patients for 10 days. Three patients were studied after a single i.v. injection of DIC. One of these patients was given the same dose orally on Day 1 and the i.v. dose on Day 2, and one received DIC in the reverse order. One other patient received an oral dose of 1·5 mg/kg every 6 hr for 4 consecutive doses. Two patients were given a single dose of DIC-2-^{14}C orally. One male patient, G.H., received 151·03 μc with 302 mg of DIC, and respiratory $^{14}CO_2$ was collected.[14] He had been receiving 300 mg of DIC i.v. daily for the 4 days preceding the oral dose. One female patient, R.C., received 205·75 μc with 202·7 mg of DIC after two consecutive daily doses of 210 mg of DIC i.v. Blood samples were obtained at 15 min, 30 min, 1 hr and hourly for 6 hr after drug administration.[6] Pre- and post-treatment total 24-hr urine collections were obtained from all patients.[6]

Urinary DIC was measured by the colorimetric method of Loo and Stasswender.[19] Plasma DIC assay was performed as described by Skibba *et al.*[6] The determination of plasma and urinary AIC was described.[20] All radioactive samples were counted as previously described.[21]

Labeled AIC was isolated from the plasma and urine of patients given DIC-2-^{14}C by column chromatography with Dowex 50W (H^+) resin. The 2·4 N HCl eluate from a Dowex 50W (H^+) column which contained the AIC sample used for colorimetric analysis[20] was taken to dryness. This residue was applied in a volume of 3 to 5 ml of 0·1 N HCl to a Dowex 50W (H^+) column (1·2 cm O.D. $\times$ 15 cm) and chromatographed using an exponential gradient of 0 to 2·4 N HCl. The initial water reservoir volume was 1000 cc. To obtain complete separation from other substances, the AIC peak was rechromatographed on a column of the same size using a reservoir of 450 cc of water and a gradient of 0·5 N to 2·4 N HCl. The column effluent was monitored at wavelengths 260 mμ and 324 mμ using a Beckman DB-G spectrophotometer with a DB Programmer. The wavelength of 324 mμ was employed to monitor the DIC in the effluent. Fractions of 10 ml were collected, and 1-ml aliquots of even numbered fractions were taken to dryness and counted. The remainder of the eluate corresponding to individual peaks was pooled, taken to dryness, and redissolved in 0·5 ml of 0·1 N HCl for paper chromatography and recrystallization to constant specific activity.

The AIC peak was identified by paper chromatography and its ultraviolet spectrum as described.[20] The ascending solvent system of Mason and Berg with ammonia was used.[22] The spots identified under u.v. light were also cut out and counted.[21]

Recrystallization of AIC was accomplished by dissolving the AIC in 10 ml of hot 95% ethanol with 50 mg of cold carrier added. The solution was cooled on ice and 7 ml of cold ethyl acetate added. Then ether was added until crystallization was completed (25–30 ml).

RESULTS

Urinary AIC excretion after DIC administration. Elevated urinary AIC levels were observed after either i.v. or p.o. administration of DIC. The 24-hr urinary excretion of AIC which followed the administration of single, consecutive daily injections of DIC to three patients is shown in Table 1. The average normal daily excretion of AIC by this method was 0·91 mg,[20] and was similar to that reported by others employing different methods of AIC analysis.[23–25] The pretreatment values are within the normal range. After DIC administration, AIC excretion was markedly elevated, but returned

TABLE 1. DAILY 4(5)-AMINOIMIDAZOLE-5(4)-CARBOXAMIDE EXCRETIONS FROM THREE PATIENTS GIVEN 4(5)-(3,3-DIMETHYL-1-TRIAZENO)IMIDAZOLE-5(4)-CARBOXAMIDE I.V. DAILY AT A DOSE OF 4·5 mg/kg

Patient:	1		2		3	
Day	DIC I.v. (mg/day)	AIC Excreted (mg/day)	DIC I.v. (mg/day)	AIC Excreted (mg/day)	DIC I.v. (mg/day)	AIC Excreted (mg/day)
0	0	2·7	0	0·3	0	0·8
1	435	33·4	300	48·0	286	43·1
2	435	34·4	300	55·0	286	38·8
3	435	35·0	300	50·5	286	44·5
4	435	27·8	300	55·3	286	47·3
5	435	15·8	300	38·6	286	41·2
6	435	8·0	300	49·3	286	36·8
7	435	29·1	300	49·4	286	46·3
8	435	37·0	300	47·0	286	45·0
9	435	63·9	300	49·2		
17	0	2·3	0	1·9	0	2·7

TABLE 2. URINARY 4(5)-AMINOIMIDAZOLE-5(4)-CARBOXAMIDE EXCRETION BY THREE PATIENTS GIVEN A SINGLE I.V. DOSE OF 4(5)-(3,3-DIMETHYL-1-TRIAZENO)IMIDAZOLE-5(4)CARBOXAMIDE 4·5 mg/kg AND TWO PATIENTS GIVEN THE SAME DOSE P.O.

I.v.	Dose (mg)	Milligram AIC/24 hr	Per cent DIC as AIC*	Per cent DIC excreted
1	306	40·7	14·9	22·2
2	376	54·5	16·2	23·3
3	300	76·8	28·6	23·0
Oral				
1	306	48·9	17·9	14·8
2	376	51·1	15·2	19·1

* Per cent DIC as AIC is the estimated percentage of the dose of DIC recovered as AIC. Total AIC (hydrochloride) in mg was converted to a corresponding amount of DIC and the percentage of the total DIC dose computed.

to normal 1 wk after cessation of therapy. Table 2 shows the 24-hr urinary excretion of DIC and AIC which followed the administration of single i.v. or p.o. doses of DIC. These values were expressed as a percentage of the dose of DIC given. If the AIC measured was derived from DIC, then for a 24-hr period after i.v. administration, an average of 19·9 per cent of the drug given was recovered as AIC; and after p.o. administration an average of 16·6 per cent of the dose of DIC was recovered as AIC. About 13·6 per cent of the dose of DIC was excreted as AIC when DIC was given p.o. every 6 hr (Table 3). The total amount of AIC excreted per time period indicated in Tables 1 to 4 was not corrected for AIC of endogenous origin.

TABLE 3. URINARY 4(5)-AMINOIMIDAZOLE-5(4)CARBOXAMIDE EXCRETED AFTER ORAL ADMINISTRATION OF 100 mg OF 4(5)-(3,3-DIMETHYL-1-TRIAZENO)IMIDAZOLE-5(4)CARBOXAMIDE EVERY 6 hr FOR FOUR CONSECUTIVE PERIODS

Time period	Milligram AIC	Per cent DIC as AIC*	Per cent DIC excreted
0– 6 hr	12·3	13·8	17·0
6–12 hr	11·2	12·6	19·1
12–18 hr	13·3	14·9	23·1
18–24 hr	11·5	12·9	20·2
Total	48·3		
Average		13·6	19·8

* Per cent DIC as AIC has same meaning as in Table 2.

TABLE 4. OBSERVATIONS ON THE DISTRIBUTION AND METABOLISM OF 4(5)-(3,3-DIMETHYL-1-TRIAZENO)IMIDAZOLE-5(4)CARBOXAMIDE-2-^{14}C DURING THE FIRST 6-hr PERIOD AFTER ORAL ADMINISTRATION TO TWO PATIENTS

Samples	Patient G.H.	Patient R.C.
Total urinary ^{14}C	45·7%*	24·6%
Urinary DIC	21·4%	13·4%
Urinary AIC	41·4 mg	15·9 mg
Per cent DIC as AIC†	15·3%	8·7%
Per cent radioactivity in AIC eluate‡	20·9%	8·7%
Emesis	0·2%	
$^{14}CO_2$	0·1%	

* Per cent (%) refers to percentage of total dose of DIC given.
† Per cent DIC as AIC has same meaning as in Table 2.
‡ Per cent radioactivity in the AIC eluate expresses the percentage of total activity of the given dose which appeared in the 2·4 N HCl eluate which was assayed for AIC colorimetrically.

Administration of DIC-2-^{14}C. The data obtained from observations made in the first 6-hr period after drug administration are summarized in Table 4. G.H. vomited 2 hr after drug administration, and no significant amount of the dose of DIC-2-^{14}C could be recovered in the emesis. The amount of activity recovered as $^{14}CO_2$ from G.H. was negligible. R.C. had no emesis and no detectable plasma AIC levels. Plasma AIC in G.H. was 1·8 μg/ml 30 min after drug administration and undetectable within 6 hr

(Fig. 1). The plasma AIC levels were parallel to those of plasma DIC (Fig. 1). The plasma DIC levels recorded for both patients G.H. and R.C. were similar to those reported previously.[6]

While the AIC and DIC excreted in the first 6-hr urine samples accounted for the majority of the total DIC-2-^{14}C given to both patients, 2·5 to 6 per cent of the ^{14}C was excreted as unidentified metabolite(s). Small amounts of AIC continued to be excreted in the urine 6 to 24 hr after drug administration. When AIC is expressed as a percentage of DIC given, then 1·0 per cent was recovered from G.H. and 2·2 per cent from R.C. Less than 1 per cent of intact drug was recovered from the urine of both

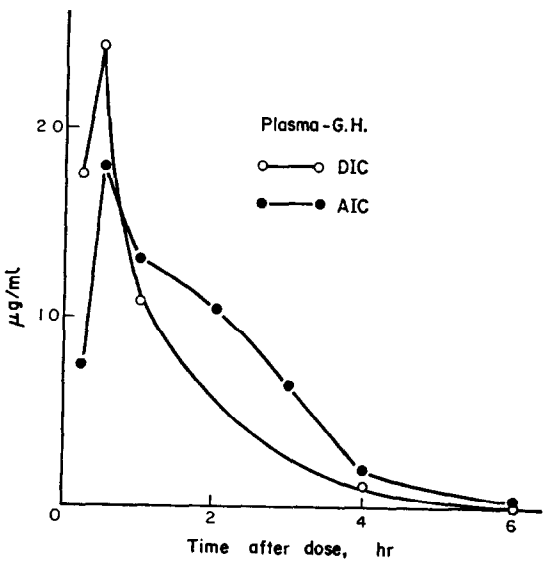

FIG. 1. Plasma AIC and DIC levels which followed the administration of 302 mg of DIC orally to patient G. H.

patients in the same time period. The urinary excretion of unidentified ^{14}C-metabolite(s) 6 to 24 hr after administration of DIC-2-^{14}C was 3·8 and 6·8 per cent of the total dose given to G.H. and R.C. respectively. Recovery of the total ^{14}C given was incomplete at the end of 24 hr. This may be explained by the incomplete gastrointestinal absorption of this orally given drug, by continued excretion of AIC-2-^{14}C or other metabolites after 24 hr, or by diversion of AIC-2-^{14}C into the *de novo* purine synthetic pathway, with subsequent delay in the complete excretion of the label.

Figures 2 to 4 show the distribution of radioactivity in the eluates from the column chromatography of the AIC-containing fractions of plasma and urine samples from patient R.C. Although plasma AIC levels never were detectable by colorimetric measurement,[20] the 8 plasma samples assayed were pooled and chromatographed with 100 μg of cold AIC, and a peak of radioactivity corresponding to AIC was located. Because of the appearance of a small peak of absorption at 324 mμ coincident with the 260 mμ peak of AIC, the pooled fractions were rechromatographed as described in Methods. A distinct AIC peak then appeared (Fig. 4). This unidentified peak of absorption at 324 mμ, which was not DIC, did disappear if the original 2·4 N HCl column

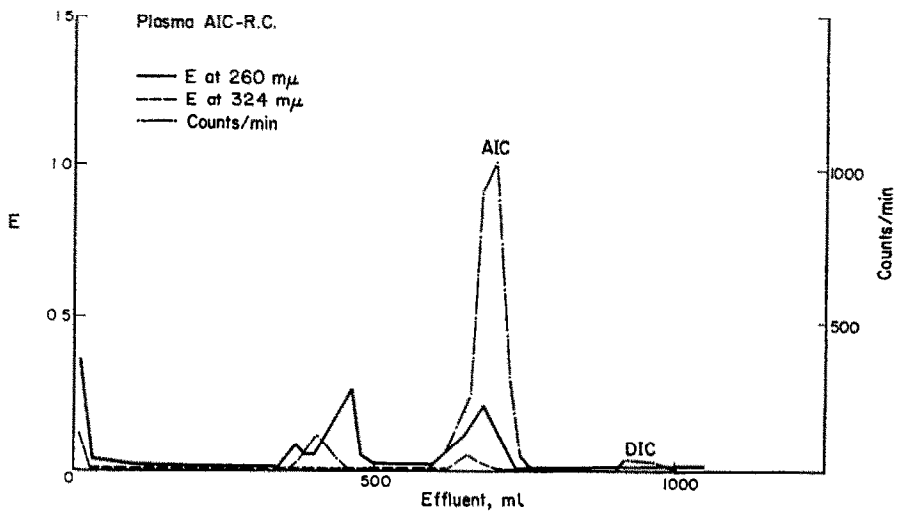

FIG. 2. Distribution of radioactivity in the AIC-containing sample from the plasma of patient R.C. Chromatography was carried out by gradient elution from 0 to 2·4 N HCl on a Dowex 50W (H^+) column.

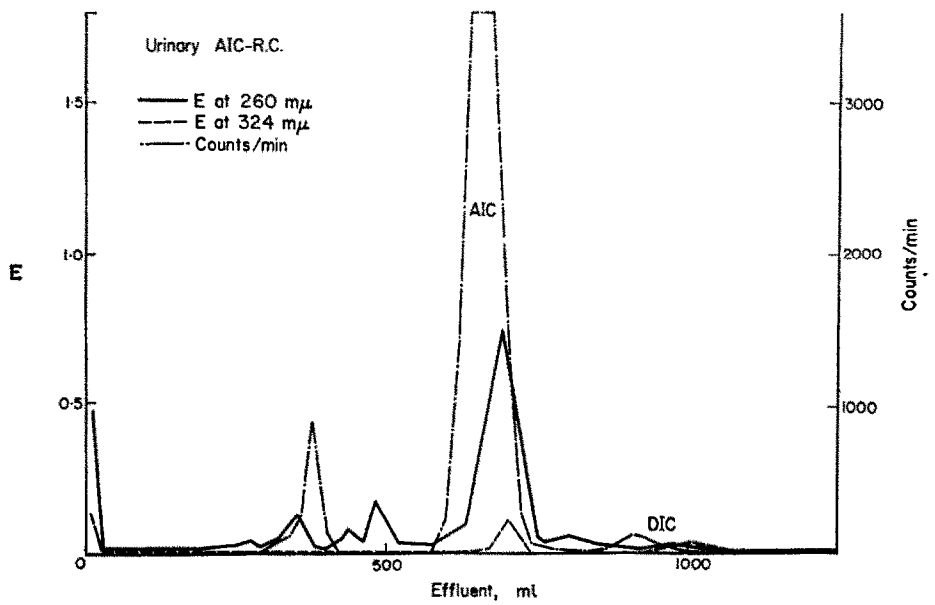

FIG. 3. Distribution of radioactivity in the AIC-containing sample from the urine of patient R.C. The sample was chromatographed as in Fig. 2.

eluate for the AIC assay was allowed to stand overnight at room temperature. There was a second small peak of radioactivity detected and represented in Figs. 2 and 3 located at about 375 ml on the abscissa. This peak could represent a second metabolite of DIC or could be the secondary decomposition product of DIC, 2-azahypoxanthine (imidazo-[4,5-*d*]-*v*-triazin-4-(3H)-one).[1, 2] Under the conditions of these experiments, small amounts of DIC may dissociate to form dimethylamine and the diazonium salt of AIC.[2] 2-Azahypoxanthine is formed spontaneously from the intramolecular coupling of this diazonium salt.[1] Similar chromatographic results were obtained from the plasma and urine of patient G.H.

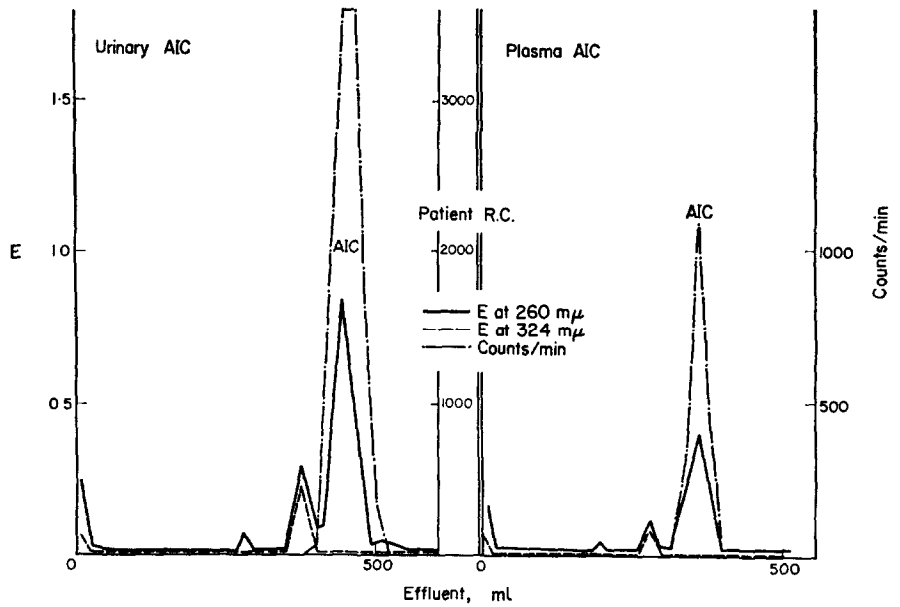

FIG. 4. Rechromatography of the AIC peaks from the plasma and urine samples of patient R.C. Chromatography was carried out by gradient elution from 0·5 to 2·4 N HCl on a Dowex 50W (H^+) column.

Each peak labeled as AIC in Fig. 4, as well as the AIC peaks from the plasma and urine of G.H., contained a compound that demonstrated paper-chromatographic behavior, ultraviolet fluorescence and color reaction after diazotiazation and coupling[20] identical to that of authentic AIC. This compound was the only diazotizable amine that could be detected in this chromatographic fraction.[20] In addition, the AIC contained in the peaks demonstrated in Fig. 4 and obtained from the urine of G.H. was recrystallized to constant specific activity. No ^{14}C-labeled AIC could be isolated from plasma or urine after addition of DIC-2-^{14}C to normal samples. Thus, the ^{14}C-labeled AIC isolated does not appear to be an artifact of isolation.

The AIC peak from the urine of R.C. was rechromatographed twice on a Dowex 50W (H^+) column, quantitated colorimetrically,[20] and the radioactivity counted. The specific activity of the AIC obtained by these methods was 78 per cent of that of the DIC-2-^{14}C given.

DISCUSSION

The elevated urinary AIC levels observed after the administration of DIC to patients in this study were considerably higher than those reported by Housholder and Loo.[13] This probably reflects differences in methodology, i.e. solvent extraction[24] compared with ion-exchange chromatography.[20] However, the primary concern in both studies was the genesis of AIC. The results presented (Figs. 2–4) lend strong support to our proposal that most of the elevated urinary AIC observed after DIC administration was the consequence of *N*-demethylation and subsequent *in vivo* alteration of DIC. The evidence is strengthened further by the close comparison of the percentage of radioactivity of the total dose of DIC-2-^{14}C given which appeared in the urinary AIC-containing fraction with the total percentage of DIC estimated colorimetrically[20] as AIC (Table 4), and by the estimated high specific activity (78 per cent of the DIC-2-^{14}C given) of the urinary AIC isolated from R.C. Previous studies on the *N*-demethylation of DIC in man given ^{14}C-methyl-DIC p.o. showed that 21·4 per cent of the radioactivity given was expired as $^{14}CO_2$ within 6 hr.[14] One other patient (unpublished results) expired 16 per cent of the radioactivity as $^{14}CO_2$ within 6 hr when given ^{14}C-methyl-DIC. These results with ^{14}C-methyl-DIC indicate the extent of *N*-demethylation of DIC which occurred in man *in vivo*. There was close quantitative agreement between the amount of $^{14}CO_2$ recovered and the amount of AIC present in the urine after the administration of ^{14}C-methyl-DIC and DIC-2-^{14}C respectively.

The mechanism for the antitumour activity of DIC remains unknown. DIC was synthesized because of the instability of the parent compound, 4(5)-diazoimidazole-5(4)-carboxamide (Diazo-ICA), which was shown to have antitumor activity.[1, 2] DIC was proposed to act as a latent form of Diazo-ICA.[3] Studies by Hano *et al.*[26] and Yamamato[27] showed that an active form of DIC and other triazeno compounds for tumor inhibition *in vitro*[26] and antibacterial activity[27] was Diazo-ICA. The monomethyl derivative, MIC, has shown antitumor activity in mice.[15] Metabolic activation of DIC would then be a probable requirement for its activity.

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