Protective Effects of Thymidine, 5-Aminoimidazolecarboxamide, and Riboflavin against Fetal Abnormalities Produced in Rats by 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide

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SUMMARY

Single doses of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DIC), 200 to 1000 or 25 to 1000 mg/kg, injected i.p. into rats on the 11th or 12th day of pregnancy, respectively, produced dose-related malformations of the mandible, palate, brain, and limbs of fetuses examined on the 21st day of gestation. Doses of 200 to 1000 mg/kg injected on Days 9 or 10 were lethal but not teratogenic. Similarly, 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide and dimethylnitrosoamine injected on Day 12 at dosages of 100 to 800 and 5 to 40 mg/kg, respectively, were lethal but not teratogenic. The maternal toxicity of dimethylnitrosoamine was about 6.5 times greater than that of 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide and 10 times greater than that of DIC, while its lethal effect on the fetus was 10 and 20 times greater than that of 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide and DIC, respectively.

Injections i.p. (on a mg/kg basis of maternal body weight) of thymidine (25 to 1000 mg), 5-aminoimidazolecarboxamide (AIC; 50 to 800 mg), riboflavin (10 to 20 mg), and DL-cysteine (200 to 400 mg) into 12th-day pregnant rats were neither toxic nor teratogenic. When these doses were given simultaneously (0 time) with 400 mg of DIC per kg, varying degrees of protection (increased number of normal fetuses at Day 21) against DIC-induced teratogenicity were observed. Thus 50 to 1000 mg of thymidine per kg gave from 54 to 78% protection, and 200 and 400 mg of AIC per kg gave from 9 to 26% protection. Combinations of riboflavin and cysteine (doses of 20 and 400 and of 10 and 200 mg/kg, respectively) provided from 2.7 to 4.9% (p < 0.001) protection, while 800 mg of AIC or 400 mg of cysteine per kg alone with DIC did not protect.

When thymidine (200 mg/kg) was given at varying time intervals (15 to 30 min) before or after DIC treatment (400 mg/kg), its protectivity declined with pre- or posttreatment time. On the other hand, pretreatment (30 min) with AIC (400 mg/kg) provided protection equivalent to that seen when the same amount of this compound was given at 0 time.

INTRODUCTION

A series of triazenoimidazole compounds, the structural analogs of AIC in which the amino group has been replaced by various monoalkyl and dialkyltriazeno groups, were synthesized and their antineoplastic effect on experimental rodent tumors was evaluated (36, 40).

In humans, the analog DIC was found to be effective against malignant melanomas (6, 12, 27). The pharmacological action of DIC in man (26, 44), its metabolism in man (42, 45), and its carcinogenicity in rats (43) have been reported. The growth-inhibitory effect of DIC on rat and mouse hepatomas (23, 25, 34) and microorganisms (30, 33, 38, 52) can be reversed by the administration of riboflavin, DL-cysteine, and aminoimidazolecarboxamide ribotide.

The objectives of this study are to determine the effect of DIC on rat fetal development when the compound is administered to pregnant rats at various stages of gestation and to evaluate the possibility of modifying or preventing the effect of DIC on fetal development by the administration of a variety of compounds (thymidine, AIC, cysteine, and riboflavin). Effects in the 12th-day pregnant rat of 2 compounds structurally related to DIC [namely, BIC, which is active against human (7, 48) and experimental neoplasms (39), and DMN, which is a mouse carcinogen (28)] were also tested.

MATERIALS AND METHODS

Chemicals

DIC (NSC 45388) and BIC (NSC 82196) were supplied by the Drug Research and Development Branch, Cancer Research Institute of Rehabilitation Medicine, New York University Medical Center, New York, New York 10016.

Received December 20, 1972; accepted June 5, 1973.
Treatment Division, National Cancer Institute, Bethesda, Md. DMN, thymidine, riboflavin, and cysteine were purchased from Sigma Chemical Co., St. Louis, Mo.

Injection Solution

All compounds were suspended in 0.5% carboxymethylcellulose and were prepared fresh on each injection day.

Animals

CFN Wistar female rats (10 weeks old) were placed overnight with males. The next morning vaginal smears were examined for the presence of sperm; if sperm were present, that day was regarded as Day 0 of pregnancy. The animals were maintained on Purina chow, with free access to water. The rats were given the compounds according to the following schedules.

Teratogenic Studies

Eighty-seven rats were given single i.p. injections of DIC at doses ranging from 50 to 1000 mg/kg on Days 9 to 12 of gestation, while 34 animals received single injections of BIC (100 to 800 mg/kg) or DMN (5 to 20 mg/kg) on Day 12 of gestation. Thirteen control animals were given appropriate amounts of 0.5% carboxymethylcellulose.

Protection Studies

On Day 12 of gestation, a total of 145 rats were treated according to the following schedule.

Group 1. Twenty-nine rats were given DIC (400 mg/kg) and thymidine (25 to 1000 mg/kg) simultaneously (0 time); 31 animals received thymidine (200 mg/kg), 15 to 60 min before or after the administration of DIC (400 mg/kg).

Group 2. Fifty-five animals were given single injections of DIC (400 mg/kg) combined with other compounds as follows: (a) AIC (50 to 800 mg/kg) at 0 time (17 rats) or AIC (400 mg/kg) 30 min before DIC treatment (6 rats); (b) riboflavin (20 mg/kg) at 0 time (6 rats); (c) combinations of riboflavin (20 mg/kg) and cysteine (400 mg/kg) (8 rats) or riboflavin (10 mg/kg) and cysteine (200 mg/kg) (8 rats) at 0 time; (d) cysteine (400 mg/kg) at 0 time (6 rats) or 30 min before DIC (4 rats). Corresponding control animals received either (a) AIC (50 to 800 mg/kg) (5 rats); (b) riboflavin (20 mg/kg) (4 rats); (c) riboflavin (20 mg/kg) and cysteine (400 mg/kg) or riboflavin (10 mg/kg) and cysteine (200 mg/kg) (7 rats); or (d) cysteine (400 mg/kg) (4 rats). Dosages were calculated on the basis of mg/kg of maternal body weight.

On the 21st day of gestation, the pregnant animals were given a general anesthesia (ether) and the fetuses were removed, weighed, and examined for gross malformations. A selected number from each litter were fixed in 95% ethanol for subsequent staining in alizarin S for skeletal examination (14). The number of dead and resorbed fetuses and implantation sites were also recorded.

RESULTS

Comparative Effects of Administration of DIC, BIC, and DMN on the 12th Day of Gestation

Maternal Toxicity. The effects of single i.p. injections of DIC, BIC, and DMN in pregnant rats are shown in Table 1. DMN was the most toxic compound; single injections of 40 mg/kg were fatal to 100% of the adults. On a mmole/kg basis, the LD₉₀ of DMN was 6.5 times greater than that of BIC and 10 times greater than that of DIC. Examination of the uterine horns of the dead pregnant rats showed total resorptions at implantation sites and/or hemorrhagic, macerated embryos in which gross malformations could not be identified.

Fetal Mortality. Of the 3 compounds, DMN was also most toxic to the fetus, with an estimated LD₁₀₀ of >0.27 mmole/kg, which is more than one-tenth that of BIC (>2.86 mmoles/kg) and one-twentieth that of DIC (>5.49 mmoles/kg). At lower doses (100 to 400 mg/kg), the toxic effects of DIC and BIC were comparable (0 to 10%) but, at 600 mg/kg, BIC produced a higher rate of fetal death and resorption (13.7%).

Fetal Weight. All 3 compounds significantly reduced fetal body weight at the doses tested. The mean weight of control litters was 5.53 ± 0.50 g. By comparison, fetuses from litters treated with DMN (5 to 20 mg/kg) weighed from 4.2 to 4.7 ± 0.2 g (p < 0.01), while those treated with BIC (100 to 600 mg/kg) and DIC (50 to 1000 mg/kg) weighed from 2.7 to 3.8 ± 0.43 g (p < 0.001) and 1.6 to 4.4 ± 0.07 to 0.3 g (p < 0.001), respectively.

Among the 3 compounds, only DIC produced malformations in the fetus.

The Effect of DIC Administered at Different Stages of Gestation

The effects of DIC in fetal rats following maternal injections on Days 9 to 12 of gestation are shown in Chart 1.

Fetal Mortality. A single dose of DIC, 800 mg/kg, which destroyed fetuses totally on Day 9, was only partially lethal on Day 10 (13% resorptions) and did not exceed the controls (0 to 10%) on Days 11 and 12. A significantly higher incidence of resorptions was observed when 1000 mg/kg were given on Days 10 (39%; p < 0.001) and 12 (15%; p < 0.001) than when the compound was given on Day 11 (11%; p < 0.2).

Fetal Body Weight. One of the conspicuous effects of DIC was the significant reduction of body weight of fetuses examined at Day 21 of gestation. This occurred at all dosage levels on all 4 days of treatment and was dose related. The greatest weight loss occurred when the compound was given on Day 12 (Chart 2).

Gross and Skeletal Malformations. DIC was teratogenic only when administered on Day 11 or 12 of gestation. The principal types of malformations produced on both days included polydactyly; syndactyly; and clubbing of the fore-
Table 1

Lethal effects produced by single i.p. injections of DIC, BIC, and DMN in the 12th-day pregnant rat

The rats were autopsied on the 21st day of gestation.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>DIC</th>
<th>BIC</th>
<th>DMN</th>
<th>DIC</th>
<th>BIC</th>
<th>DMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–10</td>
<td>6/0</td>
<td>6/0</td>
<td>N*</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>5/0</td>
<td>5/0</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>2/2</td>
<td>2/2</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>10/0</td>
<td>10/0</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100–200</td>
<td>6/0</td>
<td>6/0</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>6/0</td>
<td>6/0</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>6/0</td>
<td>6/0</td>
<td>N</td>
<td>13.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>7/0</td>
<td>7/0</td>
<td>N</td>
<td>15.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>8/4</td>
<td>8/4</td>
<td>N</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Estimated maternal LD$_{10}$

(mg/kg) 1000 1000* <40
(mmoles/kg) 5.49 3.58* <0.54

Estimated fetal LD$_{100}$

>1000 >800 >20

* Died 6 days postinjection.
* N, normal (within the control range of 0 to 10%).
* Value is for nonpregnant female rats weighing 150 to 200 g.

Chart 1. Lethal and teratogenic effects of single i.p. injections of DIC into pregnant rats on various days of gestation; autopsied on Day 21. One-half of the treated animals died by Day 6 when given 1000 mg/kg on Day 11 or 12 of gestation.

Chart 2. Effect of DIC on fetal size. Each bar represents fetuses from 4 to 6 pregnant rats. Numbers in parentheses, S.D. All differences were significant (p < 0.001).

alizarin-stained skeletons are illustrated in Fig. 2. The skeletal defects included incompletely ossified skull and pelvic bones, crooked fused ribs and cervical vertebrae, scrambled sternbrae, and indented malformed scapula. In malpositioned and phocomelic limbs, the long limb bones were curved, reduced, or absent.

Since 400 mg/kg was the DIC dose that produced the widest range of malformations and was not lethal to the
Protective Effects of Thymidine

The results of the interaction of single injections of thymidine and DIC at 0 time are summarized in Table 3. Thymidine, given alone in single doses ranging from 25 to 1000 mg/kg was neither lethal nor teratogenic to pregnant rats. When these doses were given along with DIC (400 mg/kg), no fetal lethality was produced. With the exception of 25-mg doses of thymidine, all others (50 to 1000 mg/kg) gave variable but statistically significant degrees of protection against DIC-induced malformations (54.3 to

Table 2
Gross malformations produced by single i.p. injections of DIC given to 11th- or 12th-day pregnant rats

<table>
<thead>
<tr>
<th>Specific malformation</th>
<th>Day 8</th>
<th>Day 10</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deformed forelimb</td>
<td>43.5</td>
<td>25</td>
<td>95.7</td>
</tr>
<tr>
<td>Deformed hindlimb</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ectro- and/or syndactylous forepaw</td>
<td>70</td>
<td>85.5</td>
<td>70</td>
</tr>
<tr>
<td>Ectro- and/or syndactylous hindpaw</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Short, kinked tail</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cleft palate</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Micrognathia</td>
<td>85</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>Open eyes</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Encephalocele</td>
<td>53.3</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

% of malformations after DIC treatment

Table 3
Protective effects of simultaneous injections (0 time) of varying amounts of thymidine against abnormalities produced by a single injection of DIC (400 mg/kg) in the 12th-day pregnant rat

<table>
<thead>
<tr>
<th>DIC, 400 mg/kg, plus</th>
<th>25 mg/kg</th>
<th>1000 mg/kg</th>
<th>25 mg/kg</th>
<th>50 mg/kg</th>
<th>200 mg/kg</th>
<th>400 mg/kg</th>
<th>800 mg/kg</th>
<th>1000 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats treated</td>
<td>7</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>No. of fetuses examined</td>
<td>69</td>
<td>102</td>
<td>47</td>
<td>49</td>
<td>45</td>
<td>58</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>N*</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Abnormal fetuses (%)</td>
<td>95.7</td>
<td>0</td>
<td>100</td>
<td>27.6</td>
<td>17</td>
<td>30.5</td>
<td>41.4</td>
<td>33.3</td>
</tr>
</tbody>
</table>

% of specific malformations

| Cleft palate         | 19.3     | 0        | 18.9     | 0        | 0         | 0         | 0         | 0          |
| Micrognathia         | 66.6     | 0        | 78.5     | 0        | 0         | 0         | 74.9      | 100        |
| Deformed forelimb    | 100      | 0        | 73       | 87.5     | 53.7      | 100       | 91.7      | 90         |
| Deformed hindlimb    | 92.3     | 0        | 83.8     | 72.5     | 36.2      | 100       | 91.7      | 90         |
| Syndactylous forepaw| 100      | 0        | 86.5     | 100      | 63        | 100       | 91.1      | 100        |
| Ectro- and/or syndactylous hindpaw | 89.3 | 0 | 78.5 | 50 | 62 | 100 | 67.1 | 60 |
| Open eyes            | 27.3     | 0        | 12.1     | 6.2      | 0         | 0         | 0         | 0          |
| Short tail           | 75.7     | 0        | 21.6     | 0        | 0         | 0         | 0         | 0          |

* TdR, thymidine.

* N, normal (within the control range of 0 to 10%).
78.7% normal fetuses; all $p$ values, <0.001). The maximal protective dose of thymidine was 200 mg/kg (78.7% normal fetuses). A decreased incidence of all individual abnormalities accompanied all protective doses of thymidine; the tail, palate, and eyes received greater protection than the limbs and paws.

The average weight of fetuses from protected litters, with the exception of those given thymidine (1000 mg/kg), was in the same range as DIC-treated fetuses (mean weight, $3.0 \pm 0.2$ g) and less than thymidine-treated fetuses (mean weight, $5.1 \pm 0.2$ g). In other words, thymidine protection against malformation did not significantly influence the growth-retardation effect of DIC.

In the timed experiments in which thymidine (200 mg/kg) was given 15 and 30 min before DIC (400 mg/kg) 40 and 21% (respectively) of the fetuses were protected at Day 21. The protective effect was approximately the same when the treatment was reversed and thymidine was given after DIC (Table 4). When the interval between thymidine and DIC administration was extended to 60 min, no protection was seen in either group. When protection did occur, there was a time-related decrease in frequency of all malformations, with maximal protection afforded to the jaw, eye, palate, and tail.

### Protective Effects of AIC

The results of interactions of single doses of AIC given 30 min prior to or simultaneously with DIC (400 mg/kg) in 12th-day pregnant rats are summarized in Table 5. Injections of AIC alone at 50 to 800 mg/kg did not produce any fetal mortality or teratogenesis, and when these doses were given simultaneously with DIC, varying and significant protection was observed with all doses ($p < 0.001$), except 50 and 800 mg/kg. The maximal number of normal survivors was obtained with 400 mg/kg. When protection occurred, the incidence of all abnormalities except those of the palate was reduced. Although AIC (400 mg/kg) given 30 min before DIC provided protection by increasing the number of normal fetuses that survived to Day 21 of gestation, it did not lower the incidence of individual malformations.

### Protective Effects of Riboflavin

The effects of single injections of riboflavin (20 mg/kg) and DIC (400 mg/kg) into 12th-day pregnant rats at 0 time are shown in Table 6. Riboflavin given alone did not produce any adverse effects in the maternal or fetal rat and, when given along with DIC, it significantly increased the number of fetuses surviving to the 21st day (4.9% normal fetuses; $p < 0.001$) and reduced the incidence of some malformations (limbs, paws, and tail).

### Effects of Cysteine

Cysteine is a relatively nontoxic compound. Although adult nonpregnant rats tolerate as much as 1000 mg/kg without adverse effects (5), it has been shown that pregnant rats are considerably more sensitive to drugs (9, 10) than are nonpregnant animals (31, 32, 46). For this reason, the dose of 400 mg of cysteine per kg was arbitrarily selected for protective activity against DIC teratogenesis in this study. However, this dose of cysteine not only proved to be lethal to fetuses; all $p$ values, <0.001). The maximal protective dose of thymidine was 200 mg/kg (78.7% normal fetuses). A decreased incidence of all individual abnormalities accompanied all protective doses of thymidine; the tail, palate, and eyes received greater protection than the limbs and paws.

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Table 5
Protective effects of single injections of AIC (50 to 800 mg/kg) against DIC (400 mg/kg) in the 12th-day pregnant rat when the 2 compounds were given simultaneously (0 time) or when AIC (400 mg/kg) was given 30 min before DIC

The rats were autopsied on the 21st day of gestation.

<table>
<thead>
<tr>
<th>DIC, 400 mg/kg, plus AIC, 400 mg/kg</th>
<th>AIC, 50 mg/kg at 0 time</th>
<th>AIC, 200 mg/kg at 0 time</th>
<th>AIC, 400 mg/kg at 0 time</th>
<th>AIC, 800 mg/kg at 0 time</th>
<th>AIC, 400 mg/kg at 30 min after DIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats treated</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Maternal lethality (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No. of fetuses examined</td>
<td>69</td>
<td>41</td>
<td>42</td>
<td>51</td>
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<td>Fetal effects</td>
<td></td>
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<tr>
<td>Fetal mortality (%)</td>
<td>N*</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>50*</td>
</tr>
<tr>
<td>Abnormal fetuses (%)</td>
<td>95.7</td>
<td>0</td>
<td>96.3</td>
<td>86.5</td>
<td>69.3</td>
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% of specific malformations

<p>| | | | | | |</p>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Cleft palate</td>
<td>19.3</td>
<td>0</td>
<td>21</td>
<td>41</td>
<td>25.5</td>
</tr>
<tr>
<td>Micrognathia</td>
<td>66.6</td>
<td>0</td>
<td>52</td>
<td>46</td>
<td>44.6</td>
</tr>
<tr>
<td>Deformed forelimb</td>
<td>96.5</td>
<td>0</td>
<td>84.4</td>
<td>71</td>
<td>74</td>
</tr>
<tr>
<td>Deformed hindlimb</td>
<td>92.3</td>
<td>0</td>
<td>76</td>
<td>68</td>
<td>61.6</td>
</tr>
<tr>
<td>Syndactylous forepaw</td>
<td>98.5</td>
<td>0</td>
<td>90</td>
<td>85.2</td>
<td>76</td>
</tr>
<tr>
<td>Ectrodactylus hindpaw</td>
<td>89.3</td>
<td>0</td>
<td>63</td>
<td>59.2</td>
<td>58</td>
</tr>
<tr>
<td>Open eye</td>
<td>27.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10.5</td>
</tr>
<tr>
<td>Short, kinked tail</td>
<td>75.7</td>
<td>0</td>
<td>51.1</td>
<td>43</td>
<td>44.5</td>
</tr>
</tbody>
</table>

* Died 5 days postinjection.

N, normal (within the control range of 0 to 10%).

One rat was completely resorbed.

to a significant number (25% died) of the mothers, when given alone or in combination with DIC (at 0 and 30 min prior to DIC), but it also potentiated fetal mortality and failed to protect against DIC teratogenesis (Table 6). Lower doses of cysteine were not tested for protective effect.

Effects of Riboflavin and Cysteine

The effects of single doses of DIC (400 mg/kg) given with riboflavin (10 or 20 mg/kg) and cysteine (200 or 400 mg/kg) are shown in Table 6. Maternal death occurred in the control group given riboflavin (20 mg/kg) and cysteine (400 mg/kg) (25% mortality) and in the 2 experimental groups given the protective agents and DIC (25 and 37% mortality). The higher riboflavin-cysteine dosage combination produced fetal death in the control (13.6% mortality) as well as in the experimental group (14.5% mortality). Both riboflavin-cysteine dose combinations afforded significant protection (2.7 and 4.9% normal fetuses; \( p < 0.001 \)) against DIC malformations. This protection was approximately that given by riboflavin alone (4.8% normal fetuses; \( p < 0.001 \)). The combination also decreased the frequency of malformations (except palate and jaw) more than did riboflavin alone.

DISCUSSION

In rats, both DIC and BIC are metabolized rapidly. Field et al. (16) found only 10% of the intact compounds in the blood immediately after a single i.v. injection; they found 2% at 15 min and none at 3 hr. The high percentage of fetal mortality encountered when this compound was given on Days 9 and 10 (800 or 1000 mg/kg) may reflect general cytotoxicity that is incompatible with life, while the array of malformations (open eye, microcephaly, micrognathia, and limb defects) found after treatment on Day 11 or 12 may be related to impaired differentiation at strategic stages of organogenesis.

In culture, L1210 leukemia cells are 2 times more sensitive to BIC than to DIC (50). If fetal death and resorption indicate drug sensitivity, then the 12th-day fetal rat is 2 and 20 times more sensitive to BIC and DMN, respectively, than to DIC (estimated fetal LD\(_{100}\)).

During normal prenatal development of organs, there is a proportional increase in weight, net protein, and total DNA (15, 51). Extraneous interference during critical stages of fetal morphogenesis results in reduction of organ weight and decreased protein synthesis (51). DIC inhibits protein synthesis \textit{in vitro} in L1210 leukemia (41) and in \textit{Escherichia coli} (33). In this study, it produced significant fetal growth retardation which could be the result of decreased protein synthesis.

The ability of exogenous thymidine to counteract inhibition induced by antitumor agents (24, 35, 37) and its protective role against teratogenesis produced by fluoropyrimidines in rats (8) and mice (13) have been reported. In this study, thymidine (50 to 1000 mg/kg), injected simultaneously (0 min) with DIC (400 mg/kg) into the 12th-day pregnant rat, protected the fetus against DIC malfor-
mations by significantly increasing the number of normal fetuses and reducing the incidence of all (at 50 and 200 mg/kg) or some (at 400, 800, and 1000 mg/kg) malformations at Day 21 of gestation. The fact that thymidine (200 mg/kg) gave maximal protection (78.7% normal fetus) intermediate between 200 and 400 mg/kg (Table 2; 41.7% normal fetuses), while the effect seen at 30 min approximates the effect that would have occurred with a DIC dose intermediate between 200 and 400 mg/kg (Table 2; 71.6% normal fetuses). These data indicate that DIC teratogenicity is linearly related to dose. They also indicate that about 58% of the effect of DIC on fetal rats is completed by 15 min and that about 77% is completed within 30 min. Absence of protection thereafter (60 min) indicates that the teratogenic effect of DIC is completed by this time.

The protective activity of thymidine also declined when it was given at various times before DIC treatment. In these experiments, some protection was seen at 15 and 30 min (Table 4; 40.2 and 21.2% normal fetuses, respectively), but none was seen at 60 min. This decrease in protective activity of thymidine with time preceding the administration of DIC might be related to its rate of metabolism in the maternal rat and is consistent with the report of Kriss and Révész (24), who found less than 2% of a 2-µCi i.v. dose (0.22 µmole) of labeled thymidine in the blood of rats 1 hr after injection.

At least 3 mechanisms of action of DIC have been proposed. It may act as a DNA inhibitor (17, 33, 38, 50, 52) affecting purine synthesis (38). Its AIC metabolite, which is a carrier of diazomethane (43), may act as an alkylator (22), and the ability of glutathione, homocysteine, and cysteine to reverse its inhibition in bacteria (39, 52) indicates the possibility of an SH-interaction.

In tumors (2), regenerating rat liver (19), and mammalian cells in culture (47), labeled exogenous nucleotides are incorporated into nucleic acids. Thymidine kinase plays an important role in controlling DNA synthesis in regenerating liver (3) and in tissues of fetal and neonatal rats (4) by controlling the synthesis of dTTP. Thymidine stimulates mitotic activity in mouse tissues (18) and elevates thymidine kinase specificity in the rat (21). The mechanism by which DIC produces teratogenesis in the rat is not clear, but the fact that thymidine prevents this suggests that DIC may act, in part at least, by interfering with embryonic DNA synthesis and limiting the amount of thymidine available.

Cysteine reverses the growth-inhibitory effects of DIC in Bacillus subtilis (38) and protects rats against toxicity produced by alkylating agents (11). In this study, cysteine given alone or along with DIC produced maternal death.

**DIC Teratogenesis**

**Table 6**

*Effects of single injections of protective agents riboflavin and cysteine against DIC (400 mg/kg) in the 12th-day pregnant rat when the compounds were given simultaneously (0 time) or when cysteine (400 mg/kg) was given 30 min before DIC.*

<table>
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<tr>
<th>Treatment</th>
<th>DIC, 400 mg/kg, plus Riboflavin, 20 mg/kg</th>
<th>Riboflavin, 20 mg/kg</th>
<th>Cysteine, 400 mg/kg, at 0 time</th>
<th>Cysteine, 400 mg/kg, at 30 min before DIC</th>
<th>Riboflavin, 20 mg/kg</th>
</tr>
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<tbody>
<tr>
<td>No. of rats treated</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>4</td>
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<tr>
<td>Maternal lethality (%)</td>
<td>0</td>
<td>0</td>
<td>25*</td>
<td>0</td>
<td>25*</td>
</tr>
<tr>
<td>No. of fetuses examined</td>
<td>69</td>
<td>29</td>
<td>30</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>Fetal effects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal mortality (%)</td>
<td>N*</td>
<td>N</td>
<td>13.1</td>
<td>N</td>
<td>13.6</td>
</tr>
<tr>
<td>Abnormal fetuses (%)</td>
<td>95.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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</table>

% of specific malformations

- Cleft palate 19.3
- Micrognathia 66.6
- Deformed forelimb 96.5
- Deformed hindlimb 92.5
- Syndactyous forepaw 98.5
- Ectro- and/or syndactyous hindpaw 89.3
- Open eye 27.3
- Short, kinked tail 75.7

* Died 5 days postinjection.
* N, normal (within the control range of 0 to 10%).

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(25 to 37%) and did not protect the fetus against DIC teratogenesis. The reason for maternal lethality could be that the pregnant rat is more sensitive to cysteine than is the nonpregnant rat (5). The failure of cysteine to protect against fatal malformations may be related to the fact that 400 mg/kg was not enough to maintain tissue concentration sufficient to effectively counteract the effect of DIC on the fetus.

Aminoimidazolecarboxamide ribotide, the key compound in the purine biosynthetic pathway, reverses the growth-inhibitory effects of DIC in microorganisms (30). In rats, AIC (200 and 400 mg/kg) protected against DIC teratogenesis by significantly increasing the number of normal fetuses surviving to the 21st day of gestation (9.2 and 26.4%, respectively) and by decreasing the incidence of all malformations except that of the palate.

Since DIC is a structural analog of AIC (40), it could be a potential antimetabolite of the de novo purine biosynthetic pathway. The protective effect of AIC in fetal rats may imply an involvement of DIC in purine metabolism, possibly through competition for the same enzyme site.

The importance of riboflavin nucleotides in biological oxidations and their enzymatic pathway in the synthesis from riboflavin has been documented (1). Riboflavin reverses DIC effects in microorganisms (33), inhibits the growth of rodent hepatomas (23, 25, 34) but not of adenoscarcinomas and mammary tumors (20, 29), and increases in vitro cell proliferation in normal chick fibroblasts but not in mouse tumor cells (49). In fetal rats, riboflavin provided only limited protection against DIC teratogenesis. In lieu of its extensive participation in oxidation-reduction reactions in tissues and its variable effects on biological systems, it is conceivable that the riboflavin protection observed in fetal rats might result from complex intercellular reactions involving accelerated metabolism.

This study indicates the need to recognize that DIC (or its metabolite) traverses the placenta readily, causing severe growth retardation and severe malformations in the fetus. The results of the protection experiments suggest that DIC may act by interfering with macromolecular synthetic processes at multiple points during fetal development.

ACKNOWLEDGMENTS

I am grateful to Dr. Chester A. Swinyard, Professor of Rehabilitation Medicine at this Institute, for many helpful suggestions during the preparation of this manuscript and Dr. H. B. Wood, Jr., Director of the Drug Research and Development Branch, Cancer Treatment Division of the National Cancer Institute, Bethesda, Md., for providing compounds NSC 45388 and NSC 82196. The technical help of J. Koechel and P. Muck is appreciated.

REFERENCES


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Fig. 1. Representative fetuses from pregnant rats treated with single i.p. injections of DIC on Day 12 and autopsied on Day 21 of gestation. a, control; b, c, d, and e, fetuses from rats given 200, 400, 800, and 1000 mg/kg, respectively.

Fig. 2. Fetal skeleton at Day 21 of gestation stained with alizarin S. a, control; b, c, d, and e, fetuses from rats given DIC, 200, 400, 800, and 1000 mg/kg, respectively, on Day 12 of gestation.
Protective Effects of Thymidine, 5-Aminoimidazolecarboxamide, and Riboflavin against Fetal Abnormalities Produced in Rats by 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide

Shakuntala Chaube


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