Preliminary Clinical Trial and the Physiologic Disposition of 4(5)-(3,3-Dimethyl-1-triazeno)imidazole-5(4)-carboxamide in Man

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SUMMARY

4(5)-(3,3-Dimethyl-1-triazeno) imidazole-5(4)-carboxamide (DIC, NSC 45388) was administered i.v. to 15 patients with malignant melanoma and to 17 patients with other tumors at a dosage of 4.5 mg/kg/day for 10 days. In the melanoma group, 5 patients responded to therapy. No responses were seen in the 17 patients with other tumors. Toxicity was manifested by nausea and vomiting, leukopenia, and thrombocytopenia.

The plasma levels and urinary excretion patterns of DIC, administered to patients with cancer, were determined by a revised colorimetric assay. Rapid i.v. injection of 4.5 mg/kg of DIC resulted in initial plasma concentrations of 8.8 ± 1.8 (S.D.) μg/ml which fell to zero within 6 hr. About 30% of the dose was excreted in the urine as DIC 0–6 hr after administration. Plasma levels following multiple daily doses of i.v. therapy varied with dosage, and the amount excreted in the urine 0–6 hr after each dose was about 30%. Intravenous infusion of 4.5 mg/kg of DIC over a 24-hr period produced no detectable plasma levels, while infusion of the same dose over an 8-hr period resulted in a plasma level of nearly 1.0 μg/ml after 7 hr. In both cases, urinary excretion during the infusion period was about 30% of the dose. Following a single oral dose of 4.5 mg/kg, plasma DIC levels were highest [2.4 ± 0.9 (S.D.) μg/ml] 30 min after administration, and fell to zero within 6 hr. Peak plasma DIC levels following multiple daily oral doses of 1.5 mg/kg every 6 hr varied with each dose, but fell to zero before the next dose was given. Following either oral dosage schedule, 14–23% of the dose was excreted into the urine 0–6 hr after administration. The renal clearance of DIC exceeded the glomerular filtration rate. These data suggest that high and sustained plasma levels of DIC can be achieved by either i.v. or oral, multiple daily dose therapy, and that these may be worthy of consideration for clinical trial of this drug.

INTRODUCTION

In 1960, Shealy et al. (13) reported the synthesis of 5-diazoimidazole-4-carboxamide from 5-aminimidazole-4-carboxamide, and its potential antitumor activity. Because of its instability in aqueous solution, disubstituted and monosubstituted triazenoimidazole-4(5)-carboxamide derivatives were synthesized from the parent compound (3, 10, 11). Many of these derivatives showed antitumor activity against mouse neoplasms (3, 10), especially 5(4)-(3,3-dimethyl-1-triazeno)imidazole-4(5)-carboxamide (DIC, NSC 45388) (12) (Chart 1). DIC is presently undergoing clinical trial (6, 8). Preliminary trials with DIC seemed to indicate high activity in patients with malignant melanoma, and, hence, this study attempted to include as many cases of malignant melanoma as possible.

Recently, Loo et al. (5) reported a method of assay for dialkyltriazenoimidazoles in plasma and urine, as well as pharmacologic studies of DIC (4). DIC and other dialkyltriazenoimidazoles were converted to the diazonium salt 5-diazoimidazole-4-carboxamide by exposure to ultraviolet light. This salt, in the presence of the aromatic amine N-(1-naphthyl)-ethylene diamine [NEDA, Bratton-Marshall Reagent (1)], formed an azo dye. In the presence of excess NEDA, the color generated in ultraviolet light could be quantitated. If NEDA was not present, intramolecular coupling occurred with the formation of 2-azahypoxanthine (imidazo-[4,5-d]-v-triazin-4(3H)-one), a colorless and unreactive product. This method was found to be unsuitable for plasma assay due to high and inconsistent blank values. Therefore, a modification of the
method of Loo and Stasswender (5) for plasma DIC assay was devised. This method was then utilized to investigate plasma DIC levels and urinary excretion patterns observed following i.v. and oral administration of both single and multiple daily doses to human patients with cancer. Slow i.v. infusion of DIC and renal clearance also were studied. The method of plasma assay and the subsequent data from these clinical studies are presented in this report.

MATERIALS AND METHODS

Materials. DIC was provided by the Clinical Branch, Collaborative Research, National Cancer Institute, USPHS. Clinical Trial Group. Patients selected for study had disseminated cancer and had one or more measurable lesions. There was histologic proof for each diagnosis. Patients were acceptable for study only if hepatic, renal, and hematopoietic functions were within normal limits, and no chemotherapy had been administered to them for at least 4 weeks prior to initiation of DIC treatment. DIC was given by single i.v. injection at a dose of 4.5 mg/kg/day for 10 consecutive days. A positive response to therapy was based on objective tumor regression of at least 50% of the sum of 2 perpendicular diameters of the original size of the lesions, provided no deterioration in the patient’s condition occurred and no new lesions appeared. Regression had to be maintained for a minimum of 12 weeks in order to be considered as a therapeutic response.

Drug Assay. Urinary excretion of DIC was measured by the method of Loo and Stasswender (5). This method was reported as specific for dialkytriazene compounds (5). A trace amount of an unknown diazimidazole moiety was detected in the urine of dogs following DIC administration (4). Prochlorperazine was reported to interfere with the urinary DIC assay (4); however, in the present assay no significant interference was noted when known amounts of prochlorperazine were added to urine samples in excess of that which might appear in human urine.

The plasma assay (5) was modified and employed as follows. The collected blood sample was immediately centrifuged in an International Clinical Centrifuge Model CL at 3200 rpm for 10 min. The plasma drawn off was cooled on ice (0–4°C) for 10 min in the dark. Two ml of cold 20% sulfosalicylic acid were added to 3 ml of the cooled plasma. The mixture was centrifuged in an International portable refrigerated centrifuge, Model Pr-1 at 15,000–16,000 rpm and 4°C for 10 min. To 3 ml of supernate was added 0.15 ml of 0.2% NEDA. After exposure to ultraviolet light for 30 min at a fixed distance of 2 cm from the light source, the absorbance at 540 mµ was measured against 20% sulfosalicylic acid with a Bausch and Lomb Spectronic 20. All assays were performed in duplicate.

Standard curves were prepared for each patient prior to drug therapy by adding known amounts of DIC to pretreatment plasma samples and assaying for DIC according to the procedure described above.

Subjects for Pharmacologic Study. Patients selected for study were those accepted for Phase I clinical trial with DIC. All had disseminated cancer, but their condition was good. Hepatic, renal, and hematopoietic functions were within normal limits. During study, the patients’ clinical conditions remained stable. The only other medication given during the study was prochlorperazine (Compazine, Smith, Kline & French) for nausea and vomiting.

Drug Administration. Single i.v. injections of DIC were given at a dose of 4.5 mg/kg of body weight to 6 patients. Multiple daily doses, 0.5 mg/kg and 1.1 mg/kg, were given i.v. every 6 hr for 4 successive doses. For continuous i.v. infusion, 4.5 mg/kg of DIC were dissolved in lactated Ringer’s solution and administered in a volume of 1000 ml over a 24-hr period in one patient and over an 8-hr period in another patient.

Two patients given daily injections of 4.5 mg/kg of DIC for 8–10 days were observed for 0, 30 min, and 6-hr plasma DIC levels and for total 24-hr urinary excretion.

Four patients with malignant melanoma received DIC orally at a dose of 4.5 mg/kg of body weight. The drug was dissolved in water (10 mg/ml), added to 250 ml of orange juice, and given after the patients underwent an 8-hr fast from solids only. Two patients were given the oral dose on Day 1 and the same dose i.v. on Day 2. The other 2 patients received DIC in the reverse order. Thus, both routes of administration were studied in these 4 patients. One other patient received an oral dose of 1.5 mg/kg every 6 hr for 4 successive doses.

Sample Collections. Urine samples were collected in brown bottles and assayed immediately upon collection. For urine collection beyond 6 hr, 5–10 ml of 1N HCl were added, and the urine was kept refrigerated.

Venous blood samples were obtained aseptically by venipuncture in ethylenediaminetetraacetate vacutainer tubes (Becton-Dickenson) at the times indicated after single i.v. injection. An indwelling 16-gauge Deseret Angiocath (C. R. Bard, Inc.) was used for patients given i.v. infusions or multiple daily doses, and the blood was placed in EDTA tubes.

Renal Clearance. Renal clearances were calculated from the data obtained from single i.v. injections using the plasma concentration at the midpoint of each time interval. Renal clearances of DIC and inulin were determined simultaneously on a single patient. The dose of DIC was 4.5 mg/kg of body weight given in 1000 ml of lactated Ringer’s solution infused at a rate of 300 ml/hr. Inulin was given in a loading dose of 3800 mg, and the plasma level was maintained by infusing 42 mg/min. Plasma and urinary inulin were measured by the method described by Roe et al. (9).

RESULTS

Clinical Trial. Five of 15 patients with disseminated or recurrent malignant melanoma had objective responses to DIC therapy (Table 1). Three of the 5 patients had complete disappearance of all radiographic and visible or palpable evidence of disease for 9, 9, and 19+ months respectively. In the group of 17 patients with other malignancies, no responses were obtained. Hematopoietic and gastrointestinal toxicity was observed. Nausea and vomiting were most frequent on the first 3 days of therapy. Leukopenia and thrombocytopenia occurred as early as the 5th day of therapy and as late as the 3rd week after therapy was initiated. The average time for recovery from bone marrow toxicity was 17 days after initiation of therapy. The average duration of toxicity was 1
week, and all patients recovered within a 2-week period. There were no significant changes in laboratory tests related to hepatic or renal function.

**Drug Stability.** Using the colorimetric assay method of Loo and Stasswender (5), the stability of DIC in lactated Ringer's solution was measured at various time periods (Chart 2). When kept in the dark, there was no detectable loss of drug over a 24-hr period. This observation was confirmed by the ultraviolet spectra of DIC in lactated Ringer's solution (Chart 3) which demonstrated marked spectral alteration only after exposure to light.

**Plasma Assay.** The plasma assay using sulfosalicylic acid offered better reproducibility and increased sensitivity than the trichloroacetic acid (TCA) method (5). When 6 N H₂SO₄-TCA (5) solution was used, the intensity of color formed decreased with time after exposure to ultraviolet light; when sulfosalicylic acid was employed, the color observed remained stable for a period of 30 min (Chart 4). Reproducible standard curves and accurate plasma levels were difficult to obtain employing the 6 N H₂SO₄-TCA method (5).

### Table 1

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of patients</th>
<th>Responses</th>
<th>Duration of response (months)</th>
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<td>Malignant melanoma</td>
<td>15</td>
<td>5</td>
<td>5+, 6+, 9, 9, 19+</td>
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<tr>
<td>Sarcoma</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cancer of colon</td>
<td>1</td>
<td>0</td>
<td></td>
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<tr>
<td>Cancer of breast</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cancer of lung</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cancer of salivary gland</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td>0</td>
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</table>

Summary of results of preliminary clinical trial with 4(5)-3,3-dimethyl-1-triazeno)imidazole-5(4)-carboxamide.

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**Chart 2.** Amount of 5(or 4)-(3,3-dimethyl-1-triazeno)imidazole-4(or 5)-carboxamide (DIC) in solution remaining with time when kept in the dark or exposed to light measured by the method of Loo and Stasswender (5).

**Chart 3.** Ultraviolet spectra of 5(or 4)-(3,3-dimethyl-1-triazeno)imidazole-4(or 5)-carboxamide (DIC) in lactated Ringer's solution (pH 6.5) showing spectral alteration on exposure to light. The curves marked 0 and 24 hr were obtained with the solution kept in the dark. Curves 1, 2, and 4 represent time after exposure to light.

**Chart 4.** Stability of the 5(or 4)-(3,3-dimethyl-1-triazeno)imidazole-4(or 5)-carboxamide (DIC) azo dye after exposure to ultraviolet light when measured in 6 N H₂SO₄-trichloroacetic acid (5) or sulfosalicylic acid.
Standard curves determined by both methods are illustrated in Chart 5. Employing the 6 N H$_2$SO$_4$-TCA method (5), there was a high and inconsistent plasma blank as shown in the blocked areas of Chart 5. The plasma-interfering substances were reduced markedly by employment of sulfosalicylic acid. The large variation of plasma blank values found with the 6 N H$_2$SO$_4$-TCA method (5) limited the sensitivity of the assay at low plasma levels as well as its reproducibility. The sensitivity of plasma analysis was increased when sulfosalicylic acid was used because of greater color development per unit of added DIC.

**Single Intravenous Injection.** Measurement of plasma DIC levels following a single i.v. injection in 6 patients is presented in Chart 6. Initial concentrations were 8.8 ± 1.8 (S.D.) µg/ml and fell to zero within 6 hr. One patient had a detectable plasma level longer than 6 hr after injection. In Chart 7, the data of Chart 6 were plotted as a logarithmic function of plasma concentrations. After 1 hr there was an exponential disappearance of DIC from the plasma with a half-life of approximately 75 min.

The urinary DIC excretion 0–24 hr after a single dose was 32 ± 4% (S.D.) of the total dose of 4.5 mg/kg (Chart 8), with almost all of the drug excreted in the first 6 hr after injection. Small amounts were excreted after 6 hr when the plasma DIC levels were zero.

**Intravenous Multiple Daily Dose.** Two patients received multiple daily dose therapy i.v. Results from Patient G. D., given 0.5 mg/kg of DIC every 6 hr for 4 consecutive doses, are illustrated in Chart 9. The highest plasma level achieved 5 min after the first injection was 0.8 µg/ml of plasma. Plasma levels

Plasma DIC levels approached 1.0 µg/ml near the end of the infusion period. Total urinary excretion was 25% of the dose. Excretion continued for 4 hr after cessation of the infusions.

Daily Intravenous Therapy. Results from 2 patients receiving daily injections of DIC are presented in Chart 12. There was considerable fluctuation in the daily urinary DIC excretion. However, plasma levels were more consistent 30 min after injection and were always zero by 6 hr and remained so at 24 hr after DIC administration.

Single Oral Dose. Plasma DIC levels achieved after a single oral dose of 4.5 mg/kg are shown in Chart 13. The highest

Chart 10. Plasoma 5(5,4)-(3,3-dimethyl-1-triazeno)imidazole-4(or 5)-carboxamide (DIC) levels and urinary excretion which followed when Patient K. N. was given DIC i.v. every 6 hr. Cumulative urinary DIC excretion represents % recovery of each single dose.

Chart 11. Plasma 5(5,4)-(3,3-dimethyl-1-triazeno)imidazole-4(or 5)-carboxamide (DIC) levels during an 8-hr and 24-hr i.v. infusion. Cumulative urinary DIC excretion represents % recovery of the total dose infused up to that time.
plasma DIC level reached after oral administration was $2.4 \pm 0.9$ (S.D.) $\mu g/ml$ at 30 min. One patient had a detectable plasma level longer than 6 hr. A semilogarithmic plot of the results of oral therapy (Chart 14) shows that after 1 hr the plasma level of DIC decreased exponentially with a half-life of 66 min.

Chart 15 shows the cumulative % of dose appearing in the urine following i.v. administration compared with that following oral administration. The total 24-hr excretion following the oral dose was about 4% less than that following the i.v. dose, but was not statistically significant ($0.3 \geq P \geq 0.2$). One patient excreted the same amount of DIC following the oral and i.v. dose. The total excretion for these 4 patients was 10–14% lower than that observed for the 6 patients presented in Chart 8.

**Oral Multiple Daily Dose.** Patient C. M. received 1.5 mg/kg of DIC orally every 6 hr for 4 doses (Chart 16). Peak plasma levels occurred at different times after each dose. The highest level, 1.0 $\mu g/ml$, occurred after the third dose was given. Plasma levels returned to nearly zero 6 hr after a dose was given. Urinary DIC excretion was about 20% of the dose.

**Renal Clearance.** Calculated renal clearances following single i.v. injections of DIC are shown in Table 2. The clearance values were higher than the expected inulin clearance of 120 ml/min. When the inulin and DIC clearance were determined simultaneously following i.v. infusion, the excretion of DIC exceeded the inulin clearance. During the infusion period, DIC plasma levels slowly rose from 1.1 $\mu g/ml$ at 60 min to 1.6 $\mu g/ml$ at 180 min. Inulin plasma levels fell from 0.50 $mg/ml$ at 60 min to 0.25 $mg/ml$ at 180 min. While the inulin clearance was below that expected in a 40-year-old man, the DIC clearance was decidedly in a range indicating net tubular secretion of the drug. These results are in agreement with clearance studies using the dog (7).

URINARY DIC EXCRETION

4 PATIENTS

45 MG/KG BODY WEIGHT

MEAN ± S.D

10

TIME (HOURS)

20

Chart 15. The cumulative 5(or 4)-(3,3-dimethyl-1-triazeno)-imidazole-4(or 5)-carboxamide (DIC) urinary excretion following a single oral and i.v. dose. The data represents the mean excretion ± S.D. from 4 patients.

Table 2

<table>
<thead>
<tr>
<th>No. of patients studied</th>
<th>Period, duration (min)</th>
<th>DIC clearance (ml/min ± S.D.)</th>
<th>Inulin clearance (ml/min ± S.D.)</th>
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<tbody>
<tr>
<td>Following single i.v. injection</td>
<td>5 30</td>
<td>293 ± 100</td>
<td></td>
</tr>
<tr>
<td>5 30</td>
<td>190 ± 49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 120</td>
<td>181 ± 55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>221</td>
<td></td>
</tr>
<tr>
<td>Following 3 hr i.v. infusion</td>
<td>1 60</td>
<td>205</td>
<td>95</td>
</tr>
<tr>
<td>1 30</td>
<td>247</td>
<td>109</td>
<td></td>
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<tr>
<td>1 30</td>
<td>234</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>1 30</td>
<td>239</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>231 ± 18</td>
<td>99 ± 7</td>
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</table>

Renal clearance of 4(5)-(3,3-dimethyl-1-triazeno)imidazole-5(4)-carboxamide (DIC).

DISCUSSION

Bratton and Marshall (1) suggested two reasons why the sulfosalicylic acid method of plasma DIC assay may be superior to the 6 N H₂SO₄-TCA method of Loo and Stasswender (5). Although Bratton and Marshall used TCA as protein precipitant, they noted that a reaction does occur between TCA and NEDA when exposed to light. Their blank on human blood was negligible, but the assay for 5-dialkyltriazenoimidazole-4-carboxamide requires activation of the reaction solution by exposure to ultraviolet light. This accounts for the high plasma blanks obtained when 6 N H₂SO₄ and TCA are employed (5) (Chart 5).

Secondly, Bratton and Marshall (1) diazotized and coupled sulfanilamide in a pH range of 1—2, and further stated that the stability of azo dye formed was not affected by pH changes from 1—2 for a period of 1 hr or more. This observation applies to the determination of DIC in plasma, especially since the TCA method included 6 N H₂SO₄ as the TCA solvent. Strong acid not only affects the intensity of color of the azo dye formed but also the stability of the color formed with time (Chart 4).

The present study was undertaken to aid in determining the best mode of administration for DIC as well as the most advantageous dosage schedule. Clearly, DIC is rapidly excreted into the urine through net tubular secretion. The plasma is cleared of DIC within 6 hr after a single oral or i.v. dose, with small amounts of drug being excreted for 2—4 hr thereafter. There is no accumulation of DIC in the blood after 8—10 days of daily single injections of 4.5 mg/kg (Chart 12). By dividing that daily dose into 4 equal i.v. doses given at 6-hr intervals, the time of detectable blood levels per day can be prolonged from 4—6 hr to 14—16 hr. To accomplish this same time of exposure by i.v. infusion would require an infusion rate of 0.3—0.5 mg/kg/hr for 14—16 hr (Chart 11).

Patient K. N. (Chart 10) received 1.1 mg/kg i.v. every 6 hr for 10 consecutive days without any significant hematopoietic or gastrointestinal toxicity. There was also no detectable plasma DIC after each 24-hr period. However, oral administration is more advantageous if acceptable blood levels can be attained. The plasma levels following oral DIC administration were 2.4 ± 0.9 (S.D.) µg/ml after 30 min and 2.0 ± 0.8 (S.D.) µg/ml after 60 min (Chart 13). In the same patients given the same dose i.v., plasma DIC levels were 3.3 ± 1.3 (S.D.) µg/ml after 30 min and 2.2 ± 1.0 (S.D.) µg/ml after 60 min. Thus, within 1 hr the plasma DIC levels following either i.v. or oral administration are within the same range. Employing these observations, multiple daily oral dose therapy of DIC was given to Patient C. M. (Chart 16). Within 1 hr, the plasma levels were in the same range as the levels which were seen 60 min after i.v. injection of 1.1 mg/kg every 6 hr to Patient K. N. (Chart 10).

The patients in this study experienced several side effects following the administration of DIC. The most frequent were nausea and vomiting 2—3 hr following a given dose. One patient did not vomit. Nausea and vomiting were generally limited to the first dose of daily therapy, or to the first and second doses in the case of multiple daily dose therapy. These occurred after both oral and i.v. administration. Transient flushing
Plasma and Urinary DIC Levels

occurred immediately and within a few minutes following an i.v. injection in about one-third of the patients. During the injection on two occasions the patient complained of a metallic taste in the mouth. Flushing and emesis may be explained on the basis of the observations of Hano et al. (2). They noted the vasodepressant properties of the 4-dialkyltriazenoimidazole-5-carboxamides and depression of the electroencephalogram of rabbits given these drugs i.v. Both effects were augmented with an increasing length of the alkyl side chain. However, 4-diazoimidazole-5-carboxamide had no vasodepressant activity, but caused the greatest amount of electroencephalogram depression. Thus the flushing may be due to this vasodepressant activity, and emesis may be due to the effect of DIC or a metabolite on the central nervous system.

The preliminary clinical trial with DIC showed it to be a potentially effective compound in the treatment of malignant melanoma. DIC was well tolerated at the employed dosage schedule. No deaths were observed in patients receiving this drug. Although the results for tumors other than melanoma were negative, clinical trials with DIC in other tumor types should be continued in order to confirm these observations.

The data presented in this report suggest that i.v. or oral multiple daily dose therapy with DIC merits a clinical trial in order to determine whether or not more sustained plasma DIC levels can increase its clinical efficacy. Multiple daily dose therapy could be utilized by the oral route if DIC were dissolved prior to administration.

ACKNOWLEDGMENTS

The expert assistance of Mrs. E. Myers in drug administration and sample collection is gratefully acknowledged. We thank Mrs. C. Schlotthauer and Miss S. Wagner for assistance with the preparation of the charts and the manuscript.

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NOVEMBER 1969 1951
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