The Physiological Disposition of the Carcinostatic Imidazole-4 (or 5)-carboxamide, 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno] (NSC 82196) (Imidazole Mustard) in Mice and Dogs

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SUMMARY

Studies with imidazole mustard (NSC 82196)-^14^C were performed to elucidate the in vitro stability of this compound and its physiological disposition, absorption, excretion, and metabolism in mice and dogs, after p.o. and parenteral administration. In vitro at pH 1, the drug decomposes to an ionic transformation product in the dark at 37°C; ultraviolet light-catalyzed decomposition, however, primarily yields 2-azahypoxanthine. In vivo studies show that there is no particular organ localization of radioactivity in mice after 24 hr. Plasma half-time in dogs is 2 hr after i.v. administration, but penetration of the blood-brain barrier is negligible. Gastrointestinal absorption after p.o. administration is poor and erratic. The primary route of excretion of absorbed drug is renal in both species, with greater than 60% excreted in urine by dogs within 6 hr after i.v. administration. Drug metabolism studies indicate that the drug is not excreted as intact NSC 82196, but that approximately 60% of urinary radioactivity is present as 2-azahypoxanthine. The precise mechanism of action of the drug remains unknown, it is possible that at least some of its antitumor activity rests on in vivo conversion to 5-diazo-imidazole-4-carboxamide and nor nitrogen mustard with resultant antimetabolite and possibly alkylating activity.

INTRODUCTION

Imidazole-4(or 5)-carboxamide, 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno] (imidazole mustard) (NSC 82196) is 1 of a series of triazeno-imidazole carboxamide derivatives in general (12), and NSC 82196 in particular (10, 11), have been found to be unstable in the presence of light. Ultraviolet spectral analysis was performed on NSC 82196, its ionic "transformation product" (1, 10), and 2-azahypoxanthine in 0.1 N HCl. Extinction coefficient of the latter could not be determined because of insufficient amounts of compound. As a test of the stability of NSC 82196, aliquots of a solution of the drug (10 µg/ml) were stored at different temperatures in the presence and absence of light for varying intervals of time, and then analyzed with a modification of the method described by Loo and Strasswender (6). Two ml of NSC 82196 and 0.1 ml of 0.2% Bratton-Marshall [N-(1-naphthyl)-ethylenediamine dihydrochloride] reagent (Fisher Scientific Co., Fair Lawn, N. J.) were combined in a silica cuvet, mixed, and exposed to longwave ultraviolet light (Wood's lamp) at 5 cm for 15 min. Samples were analyzed at 520 µm on a Beckman DB-G spectrophotometer. Ultraviolet spectral analysis was done on duplicate aliquots. All subsequent studies were performed in a darkened room.

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Biological fluids obtained from in vivo animal experiments were carefully handled to avoid excess exposure to light prior to and during thin-layer chromatography. Biological fluids obtained from in vivo animal experiments were carefully handled to avoid excess exposure to light prior to and during thin-layer chromatography.

**Purity of NSC 82196-14C.** NSC 82196-14C, with a specific activity of 9,614 mCl/mM, was synthesized and obtained labeled on the 2nd carbon of the imidazole ring from the Monsanto Research Corporation, Dayton, Ohio (Chart 1). A portion of this material was dissolved in 0.1 N HCl, and varying concentrations of this solution were initially chromatographed on a Silica Gel G thin-layer plates (Eastern Chromagram Sheet 6060) in a solvent system of isomyl alcohol:acetic acid:water (4:1:1) for 3 hr in foil-covered chromatography tanks at room temperature. An additional solvent system consisting of ethyl alcohol:ethyl acetate (2:1) was used subsequently in further radiopurity studies. One- and 2-dimensional chromatograms were prepared with both systems. Plates were dried and examined under shortwave ultraviolet light. Quantitative measurement of the various components of the dose solution was accomplished by dividing the path described on the chromatographic plate into 1-cm squares, with each square being added to 15 ml of toluene:phosphor solution. Radioactivity in each square was compared with an appropriate reference spot. For determination of the chemical purity of the drug, 1 mg of NSC 82196-14C was weighed on a Cahn electrobalance. A solution of 10 µg/ml was tested with Bratton-Marshall reagent and the absorbance was read at 520 µm. This was compared with a standard curve for the product of the Bratton-Marshall reaction with NSC 82196.

**Radioactive Determination.** The Packard automatic Tri-Carb liquid scintillation spectrometer, Model 4322, was used in all radioactive determinations. Urines, CSF, and dose solutions were counted directly as 0.1-ml aqueous samples in 18 ml of 30% absolute methanol in toluene (v/v) with 3 g/liter of POPOP (4). Radioactivity in plasma, tissues, and feces was determined by the method of Mahin and Lofberg (7). Tissue slices of 50 to 100 mg, or whole organs when the wet weight did not exceed 100 mg, or 0.2 ml plasma, were digested and decolorized in a mixture of 0.2 ml perchloric acid (60%) and 0.2 ml hydrogen peroxide (30%). Intestines with contents or intestinal contents alone (obtained by distilled water washing) were homogenized with a Potter-Elvehjem homogenizer with a round glass pestle, and 0.1- or 0.2-ml aliquots were prepared for liquid scintillation counting. A Waring Blender was used to homogenize the residual carcass. Feces were likewise homogenized in approximately 5 parts distilled water. Samples prepared in the methanol-toluene systems were counted with an absolute efficiency of 58% and the “tissue” system was counted at 41%. Quenching in samples was determined with the automatic external standardization with a standard quench curve previously determined for each counting system.

**Excretion and Distribution of Radioactivity.** Doses for animal studies were prepared in reduced light within 5 min of administration. The radioactive compound was dissolved in a small amount of 0.1 N HCl, and bulk powdered nonradioactive NSC 82196 suspended in 2% carboxymethyl cellulose ether (sodium salt) (Matheson, Coleman and Bell, East Rutherford, N. J.) was added to the dissolved radioactive compound. The mixture was continually stirred and shaken to assure even suspension in subsequent aliquots. An aliquot of this suspension was used for determination of radioactivity in the dose and chromatography. The remainder was administered to mice either p.o., by oral-gastric cannula, or by i.p. injection, and to a dog in a separate study through an oral-gastric tube. Intravenous studies in dogs were accomplished with a newly developed freeze-dried prepara tion (kindly supplied by Dr. Joseph E. Gallelli, Pharmaceutical Development Branch, NIH) which was readily soluble in 0.9% NaCl solution. This nonradioactive preparation was 95% pure at time of reconstitution.

Male CDF1 mice received single doses of 1.1 to 18 µCi (mean, 4.3 µCi) of NSC 82196 p.o. (0.5 ml fluid volume) or i.p. (0.2 ml fluid volume). Five hundred mg/kg approximates the optimal i.p., single injection, antitumor dose in murine L1210 leukemia, while 1300 mg/kg p.o. is approximately 40% of the optimal single p.o. dose (5). For urinary and fecal excretion studies, individual mice were housed in glass metabolism cages for periods up to 4 days. Water was provided ad libitum and food was given to all animals studied for longer than 6 hr. Feces were collected by the anal cup technique described by Oliverio and Davidson (8). Urine samples were examined by chromatography in duplicate at 2, 6 and 24 hr in all animals carried for at least 24 hr (Mice D to H and K to M). Radiochromatography of urine specimens after 24 hr was not useful due to low levels of radioactivity in the urine at these later intervals. After

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2The abbreviations used are: CSF, cerebrospinal fluid; TCA, trichloroacetic acid.
excretion studies were completed, mice were killed by cervical dislocation. Tissue and organs were immediately removed, weighed, and processed for liquid scintillation counting.

Female mongrel dogs (15 to 18 kg) under pentobarbital anesthesia received 25 to 60 μCi of drug either i.v. or p.o., at a dose of 10 mg/kg. Urines were collected during the subsequent 6 hr via an indwelling urinary catheter. Blood specimens were collected via an indwelling polyethylene catheter in a femoral artery. CSF was sampled from an indwelling spinal needle at the basal cistern. Duplicate samples of CSF and plasma were chromatographed at 1, 5, 15, and 30 min; chromatography of dog urine was performed on samples of the 6-hr urines only.

Identification of NSC 82196 and Metabolites in Biological Fluids. Urine samples of mice and dogs were assayed by thin-layer chromatography with Silica Gel G or alumina (Eastern Chromagram Sheet 6062) plates and with isoamyl alcohol:glacial acetic acid:water (4:1:1), or butanol:glacial acetic acid:water (4:1:1). Duplicate posttreatment urine, CSF, and plasma specimens were compared with known solutions of intact NSC 82196, NSC 112970 [ionic transformation product or NSC 82196 (Fig. 1)], 2-azahypoxanthine, and nor nitrogen mustard (all supplied by Cancer Chemotherapy National Service Center, Bethesda, Md.). Identification of these compounds on silica gel plates with the isoamyl alcohol solvent system was accomplished as follows: intact NSC 82196 (RF 0.7), by the development of a purple color with 0.2% aqueous Bratton-Marshall reagent and analyzed by thin-layer chromatography in this system did not reveal any contaminant had a peak at 237 mζ, which corresponds to the transformation product. NSC 82196-' 4C of greater purity was confirmed, since at the time of these subsequent experiments a supply of 2-azahypoxanthine could not be obtained.

RESULTS AND DISCUSSION

In Vitro Stability of NSC 82196

In vivo stability of NSC 82196-' 4C was found to have a radiopurity of 81%. On thin-layer chromatography of this preparation, in the isoamyl alcohol:acetic acid:water system, 2 distinct spots were seen (at RF 0.70 and 0.15) with a streak connecting the 2. Thirty to 40% of the radioactivity was located at the spot identified as intact NSC 82196 (RF 0.7), with most of the remaining radioactivity being found as transformation product (RF 0.15). Two-dimensional chromatography in this system indicated that the intact drug appeared to be decomposing on the thin-layer plate. Subsequently, the ethyl alcohol:ethyl acetate system (2:1) proved more useful in the radiopurity studies. Radiochromatography in this system revealed 2 distinct spots; 81% of the radioactivity resided at the spot with the RF value of NSC 82196 and 19% resided at the RF value of the ionic transformation product. Two-dimensional chromatograms in this system did not reveal any decomposition of NSC 82196 on the thin-layer plate. The quantitative Bratton-Marshall reaction showed the drug to be decomposed at 4°, 12% at 23° and 89% at 37°. Seventy percent of the drug remained intact at 4° at 72 hr. Drug decomposition in the presence of light at 37° for 3 hr resulted in greater than 99% decomposition in sunlight and longwave ultraviolet light, but only 39% decomposition after exposure to fluorescent lighting. Ultraviolet spectra of the solutions exposed to sunlight or ultraviolet light were similar to, but not identical with, 2-azahypoxanthine. Peaks appeared at 277 and 249 mζ instead of 277 and 249, which were the peaks obtained with pure 2-azahypoxanthine at pH 1. Downward shift of the 249 mζ peak could be explained by some degree of summation with the ionic transformation product, which peaks at 237 mζ, but this could not be confirmed, since at the time of these subsequent experiments a supply of 2-azahypoxanthine could not be obtained.

Purity of NSC 82196-' 4C

NSC 82196-' 4C was found to have a radiopurity of 81%. On thin-layer chromatography of this preparation, in the isoamyl alcohol:acetic acid:water system, 2 distinct spots were seen (at RF 0.70 and 0.15) with a streak connecting the 2. Thirty to 40% of the radioactivity was located at the spot identified as intact NSC 82196 (RF 0.7), with most of the remaining radioactivity being found as transformation product (RF 0.15). Two-dimensional chromatography in this system indicated that the intact drug appeared to be decomposing on the thin-layer plate. Subsequently, the ethyl alcohol:ethyl acetate system (2:1) proved more useful in the radiopurity studies. Radiochromatography in this system revealed 2 distinct spots; 81% of the radioactivity resided at the spot with the RF value of NSC 82196 and 19% resided at the RF value of the ionic transformation product. Two-dimensional chromatograms in this system did not reveal any decomposition of NSC 82196 on the thin-layer plate. The quantitative Bratton-Marshall reaction showed the drug to be 92% chemically pure. The ultraviolet spectrum of another aliquot of this specimen showed that the primary contaminant had a peak at 237 mζ, which corresponds to the transformation product. NSC 82196-' 4C of greater purity was not available.

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Distribution and Excretion

Mice. Table 1 shows the distribution of $^{14}$C in the organs and tissues of mice at various time intervals following p.o. and i.p. administration of NSC 82196. When the radioactivity in the various organs was expressed as tissue-specific activity, no significant organ localization was noted. It can be seen that after p.o. administration in mice the bulk of radioactivity could be accounted for within the first 24 hr in the urine, feces, and gastrointestinal tract.

After i.p. administration, urinary excretion was rapid (16.2% in 2 hr and 61.5% in 24 hr). A 24-hr fecal excretion of 8.4% suggests some degree of biliary excretion. The striking accumulation found at 2 hr in peritoneal cavity and residual carcass probably reflects incomplete absorption from the peritoneal cavity. The apparent concentration in the seminal vesicles at 2 hr (2.5%) represents drug attached to the peritoneal covering of this organ, which was visibly stained yellow at the time the animal was sacrificed.

The percentage of excretion of $^{14}$C by mice following single p.o. and i.p. doses of NSC 82196 is shown in Table 2. After p.o. administration, intestinal absorption appears erratic; 33.3 to 61.4% of p.o. administered radioactivity can be found in the feces over a 96-hr period, with the bulk of the remaining radioactivity being excreted in the urine.

The effect of dose on rate of excretion of NSC 82196 was examined after p.o. administration (Mouse D compared with Mice E to H) and after p.o. administration (Mouse K compared with Mice L and M). Rate of excretion in urine and feces after drug administration by either route was not significantly altered by the doses tested.

Dogs. Urinary excretion of radioactivity within 6 hr after i.v. administration in 2 dogs was 62 and 65%, but was only 9% after p.o. administration in a 3rd. Six % of the administered radioactivity in this animal was aspirated from the stomach by oral-gastric suction at the conclusion of the experiment. Plasma half-time after i.v. administration was approximately 2 hr (Chart 2). Entry into the CSF was negligible after both i.v. and p.o. administration.

Absorption and Excretion of $^{14}$C after p.o. Administration of in Vitro Metabolites of NSC 82196

Thin-layer radiochromatography and qualitative testing with Bratton-Marshall reagent showed that the solution of NSC 82196 exposed to longwave ultraviolet light for 2 weeks contained 35% NSC 112970-$^{14}$C, 49% 2-azahypoxanthine-$^{14}$C, and other unidentified radioactive metabolites, but no intact NSC 82196. Administration p.o. of this mixture to 1 mouse resulted in recovery of 43% of the administered radioactivity from the urine and 45% from feces. The radioactivity excreted in the urine was divided between the 2 major products in the same proportion as the administered dose; 45% was present as 2-azahypoxanthine-$^{14}$C and 34% was present as transformation product. These data suggest that 2-azahypoxanthine and transformation product are incompletely absorbed from the gastrointestinal tract, but, when absorbed, are at least in part excreted unchanged in the urine.

Identification of Metabolites in Body Fluids

Immediate qualitative and quantitative testing of urine with Bratton-Marshall reagent, in both mice and dogs, revealed the absence of Bratton-Marshall-positive material. Since intact drug did not appear in the urines tested, it remained possible

| Organ distribution of radioactivity (percentage of dose) in mice following administration of NSC 82196 |
|---|---|---|---|---|---|---|
| Time after drug administration | 2 hr | 2 hr | 2 hr | 24 hr | 24 hr | 2 hr | 24 hr |
| Organ | Mouse A | Mouse B | Mouse C | Mouse E | Mouse F | Mouse J | Mouse L |
| Liver | 1.4 | 0.8 | 0.8 | 0.3 | 0.4 | 1.6 | 0.4 |
| Kidney | 1.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.6 | 0.1 |
| Spleen | <0.1 | <0.1 | <0.1 | 0.1 | 0.1 | 0.1 | <0.1 |
| Lungs | <0.1 | <0.1 | <0.1 | 0.1 | 0.1 | 0.2 | <0.1 |
| Heart | <0.1 | <0.1 | <0.1 | <0.1 | 0.1 | 0.2 | <0.1 |
| Bladder | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 | 0.2 | <0.1 |
| Brain | <0.1 | <0.1 | <0.1 | <0.1 | 0.1 | 0.1 | <0.1 |
| Gonads | 1.0 | <0.1 | <0.1 | <0.1 | 0.1 | 0.1 | <0.1 |
| Seminal vesicles | 2.5 | 0.3 | 2.5 | 0.3 | 2.5 | 0.3 |
| Body cavities | 27.0 | 1.1 | 27.0 | 1.1 | 27.0 | 1.1 |
| Feces | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| Urine | 9.5 | 5.5 | 9.0 | 24.5 | 29.9 | 16.2 | 61.5+ |
| Carcass | 2.4 | 1.4 | 18.8 | 0.5 | 0.8 | 17.9 | 2.5 |
| Stomach with contents | 12.3 | 11.8 | 4.8 | 13.3 | 2.9 | 0.5 | 0.2 |
| Intestines with contents | 63.3 | 63.0 | 64.6 | 6.6 | 2.6 | 8.6 | 0.2 |
| Total | 91.3 | 82.7 | 98.3 | 84.9 | 85.2 | 78.4 | 74.6+ |

aDose, 1300 mg/kg p.o.
bDose, 2600 mg/kg i.p.
cError precluded determination of total urinary radioactivity.
transformation product. It could not be determined whether the transformation product recovered in the urine was the result of biochemical transformation or whether it was derived from NSC 82196 converted by simple chemical change within the gastrointestinal tract or peritoneal cavity. Since the purity of the radioactive drug and, presumably, the dose administered was approximately 80% NSC 82196-'\(^4\)C, it is unlikely that absorption and excretion of the 20% of the dose as transformation product could alone account for the large recovery of this product in the urine. This point may prove difficult to resolve because of the instability of NSC 82196 under physiological conditions.

In dogs, low levels of radioactivity in CSF precluded meaningful chromatographic analysis. No identifiable Bratton-Marshall-positive material could be found in whole plasma. In precipitated plasma, the nonspecific color reaction between TCA and Bratton-Marshall reagent described by those authors (2) was noted, but no purple color connoting the presence of NSC 82196 was observed after exposure to longwave ultraviolet light. Only negligible amounts of radioactivity was found in the TCA precipitate. Greater than 75% of the radioactivity in both plasma specimens was found as ionic transformation product 1 minute after i.v. drug administration.

As in mice, the primary compound recovered in dog urine was transformation product (60%), but an additional 25% was present in 1 animal at an RF of 0.20; this substance was seen in significant quantities in mouse urine. This substance was seen in the dose dilution of this animal and may well represent an in vitro decomposition product that was excreted unchanged in this animal.

The finding of rapid and extensive excretion of transformation product is of practical significance, since this

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Table 2

<table>
<thead>
<tr>
<th>Route</th>
<th>Excretion of radioactivity (cumulative percentage of dose) by mice following administration of NSC 82196</th>
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<tbody>
<tr>
<td></td>
<td>p.o.</td>
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<tr>
<td></td>
<td>1300 (mg/kg)</td>
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<tr>
<td>Mouse</td>
<td>Urine (hr)</td>
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<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>3.0</td>
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<td>2</td>
<td>9.5</td>
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<td>6</td>
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<td>72</td>
<td>41.3</td>
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<tr>
<td>96</td>
<td>45.0</td>
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<tr>
<td>Feces (hr)</td>
<td>&lt;0.1</td>
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</tbody>
</table>

* aN.D., not determined  
* bError precluded determination.
compound is inactive in animal tumor systems (10). This would imply that the active moiety of NSC 82196 is either an unidentified intermediate, active in small quantities, or that the drug or its active metabolites performs its biological function with little structural change, thus enabling it to retain its ability to reform the ionic transformation product. Since both NSC 45388 and NSC 82196 form the same highly reactive diazo compound, 5-diazoimidazole-4-carboxamide, and all of these compounds are active in murine L1210 leukemia, it remains possible that this diazene is the active moiety for both compounds, as suggested by Shealy et al. (13, 14). The liberation of nor nitrogen mustard, a possible metabolite (Chart 3), might account for the alkylating activity of the drug in microbial systems (9).

CONCLUSIONS

Major in vitro decomposition products of imidazole-4(or 5)-carboxamide, 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno] (NSC 82196) are an ionic transformation product (at body temperature in the absence of light) and 2-azahypoxanthine (after exposure to ultraviolet light).

Physiological disposition studies after p.o. and i.p. administration of NSC 82196-14C to mice indicate that there is no significant organ localization within 96 hr. Plasma half-time in dogs is approximately 2 hr and penetration of the blood-brain barrier is poor. The primary route of excretion in mice and dogs is renal, with the bulk of urinary radioactivity being present as ionic transformation product, with little or no intact NSC 82196 being recovered. Gastrointestinal absorption is both poor and erratic. To date, the only formulation for clinical use is an oral one because of the instability of the drug and its insolubility in physiological solutions. It might be anticipated that optimal results will not be achieved clinically until a stable and reliable parenteral formulation is achieved. Similar problems were encountered with a related compound, NSC 45388, before intravenous formulation was accomplished.

The precise mechanism of action of NSC 82196 remains to be elucidated, but it is possible that the activity of the drug rests on the in vivo conversion of a small fraction of the administered dose to 5-diazoimidazole-4-carboxamide and nor nitrogen mustard with resultant antimetabolite and possible alkylating activity. It remains possible, however, that there is an unidentified active intermediate in the major pathway from NSC 82196 to the formation of the inactive ionic transformation product which accounts for the activity of the drug.

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REFERENCES


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