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DON, CONV AND DONV—III. PHARMACOLOGIC AND TOXICOLOGIC STUDIES*

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Abstract—The pharmacologic, toxicologic and oncolytic properties of the ketoamino acids DON (L-DON; 6-diazo-5-oxo-L-norleucine), CONV (L-CONV; 5-chloro-4-oxo-L-norvaline; 2-amino-5-chlorolevulinic acid) and DONV (L-DONV; 5-diazo-4-oxo-L-norvaline) were examined. DON was found to be the most active therapeutic agent of the three drugs against leukemia 1210 and also the most potent cytocidal agent against KB tumor cells in culture. The acute LD_{50} values of the agents were dissimilar: CONV was the most toxic drug of the three after single intraperitoneal injections, and DONV the least toxic. Only DON showed evidence of prominent cumulative toxicity. In studies with isolated cells of leukemia 5178Y rendered resistant to L-asparaginase (L5178Y/AR), all three agents appeared to compete both with L-asparagine and with L-glutamine for transport into the cell. DONV competed most effectively with L-glutamine and CONV most effectively with L-asparagine. In mice, all three drugs were cleared from the plasma and excreted into the urine at a rapid rate. None was bound to the proteins of mouse plasma. After an intraperitoneal injection of 100 mg/kg, the concentration of DONV in the pancreas was approximately ten times that of CONV or DON; after comparable intravenous injections, only DONV could be identified in this tissue. Although the metabolism of all three ketoamino acids was found to be minor in degree, evidence is presented that they can be degraded *in vitro* by organ homogenates and also that purified enzymes can catalyze their transamination. In addition, DON was a good substrate for renal γ-glutamyl transferase (EC 2.3.2.2). In the case of DONV, some conversion to CO_2 by isolated tumor cells also was observed. From these and previous studies it is concluded that, of these analogs of L-glutamine and L-asparagine, DON is the most "L-glutamine-like" agent of the three, DONV the most "L-asparagine-like," while CONV has important attributes of both amino acids.

In companion papers [1, 2] it was reported that DON (L-DON; 6-diazo-5-oxo-L-norleucine), CONV (L-CONV; 5-chloro-4-oxo-L-norvaline; 2-amino-5-chlorolevulinic acid) and DONV (L-DONV; 5-diazo-4-oxo-L-norvaline) were all effective inhibitors of L-asparagine synthetase (glutamine hydrolyzing, EC 6.3.5.4) *in vitro*, principally when L-glutamine was utilized as substrate. *In vivo*, DON and CONV produced significant inhibition of L-asparagine synthetase of leukemia 5178Y rendered resistant to L-asparaginase by repeated subcurative doses of the enzyme (L5178Y/AR) and of the analogous enzyme from mouse pancreas irrespective of the parenteral route by which the drugs were administered. DONV was significantly efficacious only by the intravenous route and inhibited the synthesis of L-asparagine only in the pancreas. Furthermore, with DONV, the inhibition produced was exceedingly transitory when compared to the span of action of DON and CONV.

In an effort to understand the basis for the discrepancy between their potency *in vivo* and *in vitro*, additional studies were undertaken to determine the fate and distribution of these ketoamino acids in the intact organism. Moreover, in order to characterize further their pharmacological similarities and differences, the drugs have been compared in a number of other systems *in vivo*. Thus, the oncolytic and toxicologic properties of DON, CONV and DONV have been examined, along with the mechanisms responsible for their transport into the intact tumor cell.

MATERIALS AND METHODS

The reagents and techniques used for the present work are the same as those used in the first and second papers of this series [1, 2], with the following additions.

Therapeutic studies. Measurements of the cytocidal [3], tumoricidal [3] and toxicologic [4] properties of DON, CONV and DONV were carried out according to the published protocols of the Division of Cancer Treatment of the National Cancer Institute, National Institutes of Health, Bethesda, Md.

Amino acid analysis. Mouse brain, liver, kidney and pancreas were frozen immediately after removal and homogenized in 5% perchloric acid or 0.1 M HCl. After neutralization with KOH or NaOH and centrifugation at 12,000 *g* for 3 min, the extracts were chromatographed on the long column of the Jeolco amino

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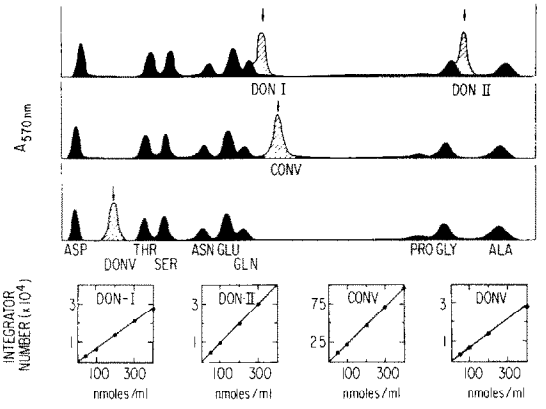

Fig. 1. Elution pattern of DON, CONV and DONV on the long column of the amino acid analyzer using lithium citrate buffers. Analyses were carried out according to the procedures outlined in Materials and Methods. Integration numbers are the arbitrary units representing the area under the photometric curve for the respective amino acid. The two breakdown products of DON (DON I and II) are probably HON (6-hydroxy-5-oxo-L-norleucine) and CON (6-chloro-5-oxo-L-norleucine), although exact assignments of structure or of elution positions have not been made (personal communication: Dr. K. V. Rao, University of Florida, Gainsville, Fla.). It should be stressed that the technique as used does not distinguish between the native drugs and enzymatic or spontaneously hydrolyzed products *in vivo*.

acid analyzer using the lithium citrate system of Benson *et al.* [5]. Plasma was separated from blood taken from the ophthalmic venous plexus, diluted 1:5 (v/v) with 0.1 M HCl, centrifuged at 105,000 *g* for 30 min, and chromatographed in the same manner. Using this system, the three ketoamino acids could be successfully resolved from the normal acidic amino acids (Fig. 1). It should be pointed out, however, that this technique does not distinguish between the native drug and the enzymatic or spontaneously hydrolyzed products *in vivo*.

Microbiological assays. A logarithmic-ratio microbiological assay has been developed for the estimation of concentrations of DON, CONV and DONV in the body fluids and tissues of mice. More than 100 species of micro-organisms were tested for sensitivity to each of the three compounds. A strain derived from *Escherichia coli B* (ATCC 11303), resistant to 1 mg/ml of 6-mercaptopurine (*E. coli* B/6-MP), was selected as the asssay organism for CONV; a strain derived from *E. coli*, resistant to 1 mg/ml actinobolin-C (*E. coli*/ACB-C), was selected as the assay organism for DON; and a *Candida sp.* (Mt. Vernon strain 512), resistant to 1 mg/ml methyl-glyoxal-bis-guanyl hydrazone (NSC-32946), was selected as the assay organism for DONV.

Bacterial cultures were maintained on agar slants of a glucose-salts medium [6] supplemented with 1 mg/ml of either 6-mercaptopurine or actinobolin-C. The yeast was maintained on Sabouraud dextrose agar slants (Difco). For the preparation of seeded agar plates (100 × 15 mm, No. D1906, Lab-Tek Products, Naperville, Ill.), stationary cultures of the micro-organisms were grown for 16–18 hr at 37° in the above-mentioned media. Cells from these cultures were collected and washed three times by centrifugation in sterile, 0.85% (w/v) NaCl, resuspended in saline and adjusted to 20 per cent light transmittance (660 nm) in a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). The suspension was diluted 1:10 (v/v) in saline; 1 ml of this suspension was added to 1 liter of cooled glucose-salts agar medium and 6 ml of the mixture was dispensed into the previously described plates.

A stock solution of 1250 μg/ml of each ketoamino acid was prepared in sterile saline. A 1:100 dilution (v/v) of DON, a 1:10 (v/v) dilution of CONV, or a 1:2 (v/v) dilution of DONV was made in saline, heparinized mouse blood, plasma, or 20% (w/v) organ homogenate, and an aliquot of 0.08 ml of each concentration was pipetted onto filter paper discs (1.27 cm in diameter, No. 840-E, Schleicher & Schuell Co., Keene, N.H.). Empirically selected control solutions of 0.04 μg DON/disc, 1 μg CONV/disc and 10 μg DONV/disc also were prepared in saline directly from the stock solution. All plates were prepared in triplicate. Each individual plate contained a maximum of three discs—two discs contained, individually, either "standard curve" solutions of different concentrations or an experimental sample. The third disc contained an empirically selected concentration, previously described, which allowed for correction of plate-to-plate variation in zone sizes. After incubation of the assay plates at 30° for 16–18 hr, the resulting zones of inhibition on the triplicate plates were measured and corrected [7]. The resulting standard curves are shown in Fig. 2.

Fate and distribution. Swiss or BDF_1 mice of both sexes, 18–22 g, were used for tissue distribution studies. Blood samples were obtained by cardiac puncture, and urine samples were collected from the bladder. Tissues were removed immediately after sacrifice and blotted well with absorbent tissue. All tissues were prepared for assay by homogenization in 1 ml saline (microbiological assay) or 0.1 M HCl (automatic amino acid analysis). Individual tissues were pooled before assay. Blood and urine were assayed individually.

The effect of a variety of metabolites on inhibition of the respective sensitive micro-organisms by these ketoamino acids was determined by incorporating the individual metabolites in the inoculated medium. Graded concentrations of DON, CONV or DONV were pipetted onto filter paper discs which were placed on agar plates. After incubation at 30° for 16–18 hr, the diameters of the zones of inhibition were measured. Change of mean zone diameters on metabolite-containing plates as compared to replicate control plates (no added metabolite in the medium) was taken as the criterion of metabolite modification of the inhibitory effect of the agent.

Cellular uptake studies. Ascitic fluid was removed from mice bearing 6- to 8-day-old intraperitoneal implants of leukemia 5178Y/AR. The cells were harvested by centrifugation at 1000 *g* for 10 min and washed twice with Hank's balanced salt solution (HBSS), and then resuspended in HBSS to obtain 4×10^8 cells/ml of suspension.

A 500-μl aliquot of the cell suspension containing 2×10^8 cells was dispensed into an Eppendorf 1500-μl test tube, and gassed with 95% O_2–5% CO_2. At time zero, L-asparagine, L-glutamine or DON was

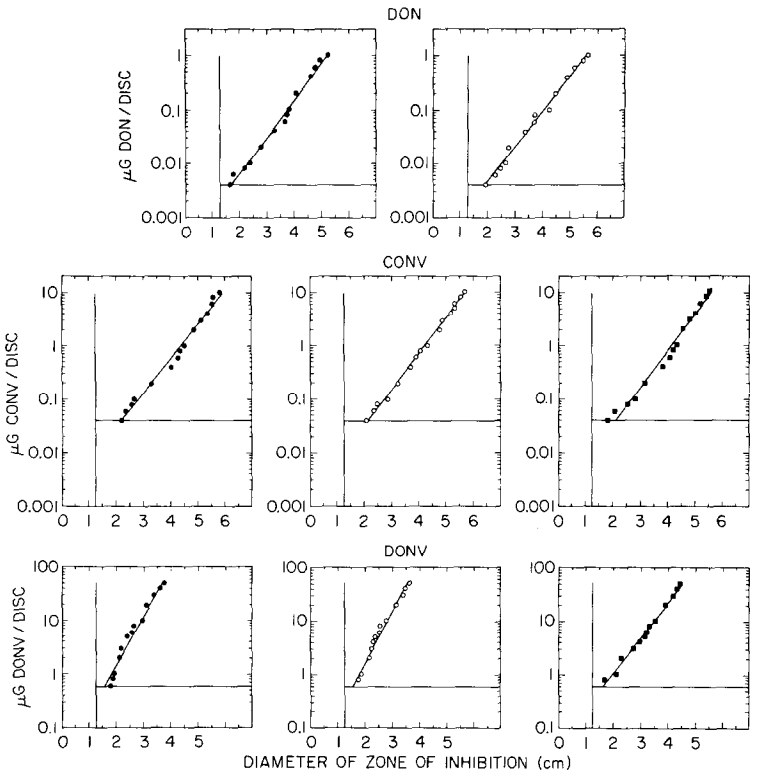

Fig. 2. Standard curves for the microbiological assay of DON, CONV and DONV. Microbiological assays were conducted according to the methodology presented in Materials and Methods. Key: (●) drug in saline, (○) drug in mouse blood, and (■) drug in mouse serum.

added to a final concentration of 1 mM. After 1 min, 0.3180 μmole L[5-^{14}C]DONV (sp. act. 1.7248 mCi/m-mole) was added; 50-μl aliquots were taken at 20-sec intervals, added to 1.5 ml of ice-cold HBSS, and centrifuged at 12,000 *g* in the Eppendorf Zentrifuge for approximately 20 sec. The pellet was washed once with ice-cold HBSS and frozen immediately on dry ice. The entire washing procedure lasted approximately 1 min. Each pellet then was suspended in 50 μl of 40% NaOH and digested at 95° for 20 min. The entire vessel was immersed in scintillation fluid and counted at 86 per cent efficiency in the Beckman LS-230 scintillation spectrometer.

To another 500-μl aliquot containing 2×10^8 cells, treated as described earlier, the drug (DON, CONV or DONV) was added at time zero, to a final concentration of 1 mM. After 1 min, 1 μCi L[U-^{14}C]asparagine (sp. act.: 151 mCi/m-mole) or L[U-^{14}C]glutamine (sp. act.: 220 mCi/m-mole) was added; samples were taken and processed as described earlier.

Measurement of decomposition of DON, CONV and DONV in vitro. Freshly excised mouse brain, liver, kidney and pancreas were homogenized 1:3 (w/v) in 0.05 M Tris–HCl buffer, pH 7.6, containing 1 mM dithiothreitol and 0.5 mM EDTA. The homogenates then were dialyzed against a large excess of the homogenization buffer for 16 hr at 4°. To duplicate 100-μl aliquots of these homogenates, DON, CONV or DONV was added to a final concentration of 1 mM; another set of duplicate organ samples received the drugs plus α-ketoglutaric acid at a final concentration of 1 mM, and a third set of vessels received the drugs plus nicotinamide adenine dinucleotide (NAD) at a final concentration of 1 mM. After 30 min at 37°, the reactions were terminated by the addition of 1 ml of 50% (v/v) ethanol, and the concentrations of DON and DONV estimated spectrophotometrically [8] in a small aliquot of the supernatant fluid from this step. CONV was measured on the amino acid analyzer. Zero time recoveries of DON, CONV and DONV from biological samples were greater than 80 per cent.

Transamination. Determination of the ability of DON, CONV and DONV to serve as substrates for purified L-glutamate oxaloacetate transaminase was carried out essentially as described earlier [9] using [1-^{14}C]α-ketoglutaric acid as the ketoacid in the transamination.

Determination of the ability of DON, CONV and DONV to support transamination of L-glutamine by crude murine hepatic enzymes was carried out radiometrically: in a final volume of 20 μl were admixed 0.25 μCi L[U-^{14}C]glutamine, 20 nmoles of either DON, CONV or DONV or 5 μl of 0.1 M Tris–HCl buffer, pH 8.4. To initiate the reaction, 5 μl of the 12,000 *g* (3 min) supernatant from a 1:3 (w/v) homogenate of BDF_1 mouse liver was added. After 30 min at 37°, the reaction was terminated by the addition of 20 μl of 0.01 M NaOH. Then 50 μl of 1% (v/v) H_2O_2 in 1 M HCl was added to the incubation mixture, and 5 μl of 40% (w/v) KOH was deposited on the underside of the lid of the reaction vessel, which then was closed in order to trap [^{14}C]O_2 arising from the oxidative decarboxylation of α-ketoglutaramic acid [10].

Measurement of γ-glutamyltransferase (EC 2.3.2.2). The kidneys of ten adult Balb/C mice were homogenized in 20 vol. (w/v) of 0.1 M Tris–HCl buffer, pH 8.4, and the resultant homogenate was centrifuged at 20,000 *g* for 30 min. The supernatant was discarded and the pellet washed twice with 10 vol. of the homogenization buffer. The supernatant was discarded, and the pellet solubilized in 3 vol. of 0.5% sodium deoxycholate in 0.1 M Tris–HCl buffer, pH 8.4. After 12 hr at 4°, the extract was centrifuged at 20,000 *g* and the supernatant used as the source of γ-glutamyltransferase (EC 2.3.2.2). For analysis, 1 ml of 1 mM substrate (L-glutamine, DON, CONV, DONV or L-asparagine) in 0.1 M Tris–HCl buffer, pH 8.4 was mixed with 100 μl of 1 mM γ-glutamyl nitroanilide in 0.5 M Tris–HCl buffer, pH 9.0, or with 100 μl of 0.05 M reduced glutathione. To initiate the reaction, 5–20 μl of crude enzyme was added. When γ-glutamyl nitroanilide was the substrate, the increase in absorbance at 400 nm was monitored as a function of time; when glutathione was the substrate, the generation of product was monitored by paper electrophoresis on Whatman 3MM paper at 3000 V, in 0.1 M sodium phosphate buffer, pH 6.8, for 1 hr. Spots were identified by ultraviolet absorbance and then by ninhydrin positivity.

Measurement of protein binding. Neutralized solutions of DON, CONV, DONV or water were added to 500 μl of fresh, heparinized mouse plasma to achieve a final concentration of 5 mM, and the solutions were diafiltered through Amicon Centriflo cones (Amicon Corp., Lexington, Mass.) for 40 min, at 4° and 1000 *g*. The concentrations of DON and DONV in the filtrate were measured spectrophotometrically at 274 nm. CONV was measured on the amino acid analyzer as described above. In every case, the concentration of DON, CONV and DONV in both the filtrate and retentate also was estimated by measurement of the inhibition of L-asparagine synthetase.

Metabolism of L[5-^{14}C]*DONV.* L5178Y/AR cells were collected from mice with ascites and washed as described earlier. One ml of packed, washed tumor cells in HBSS was dispensed into plastic bottles equipped with center wells [11]; to each was added 0.3180 μmoles L[5-^{14}C]DONV (sp. act. of 1.7248 mCi/m-mole) and 1 ml HBSS. To the center well of the bottle was added 200 μl of 40% (w/v) KOH. The closed vessels were incubated at 37° with gentle shaking for various time periods; the reaction was stopped by injecting 0.2 ml of 2 M HCl. The [^{14}C]O_2 so liberated was trapped in the KOH, and radioactivity measured by scintillation spectrometry as described.

Measurement of the uptake of L-asparagine, L-glutamine, DON, CONV, and DONV by mouse erythrocytes. Swiss mice anesthetized with pentobarbital were exsanguinated from the right ventricle with heparinized syringes. To 2-ml aliquots of the pooled whole blood were added L[U-^{14}C]asparagine (1 μCi), L[U-^{14}C]glutamine (1 μCi), and DON, CONV or DONV, each to a final concentration of 6×10^{-4} M. After 30 min at 37°, a 500-μl aliquot of whole blood from each experimental set was taken and acidified at once by the addition of 50 μl of 60% perchloric acid (PCA). The remainder of the blood was centrifuged at 12,000 *g* for 3 min and 500-μl aliquots of the plasma also were acidified with 50 μl of 60% PCA. Then, 1 ml of 0.1 M potassium phosphate buffer, pH 6.7 was added to each sample in order to precipitate the excess perchloric acid. After centrifugation at 12,000 *g* for 3 min, the clear supernatants were analyzed either radiometrically (in the case of L-asparagine and L-glutamine) and/or by automatic column chromatography on the Jeolco amino acid analyzer.

Incorporation of L[5-^{14}C]*DONV into L5178Y/AR cell proteins.* A washed tumor cell suspension (1 ml) was incubated for various time periods up to 1 hr with 6.36×10^{-4} M L[5-^{14}C]DONV; the reaction was stopped by the addition of 0.1 vol. of 50% (w/v) trichloroacetic acid (TCA). The cell pellet was washed twice with 1 ml of 5% (w/v) TCA and divided into two parts; one part was digested with 50 μl of 40% (w/v) NaOH, and the radioactivity of the resultant solution estimated by scintillation spectrometry. The other part was digested for 24 hr at 37° with 0.1% (w/v) pronase in 0.1 M Tris–HCl buffer, pH 7.4, containing 0.001 M $CaCl_2$. An aliquot of this digest was chromatographed on the Jeolco amino acid analyzer.

RESULTS

Therapeutic studies. As a first step in the further characterization of the properties of DON, CONV and DONV, their cytocidal activity was examined 3 days after drug addition to tissue cultures of KB cells, an epithelial line originating from a human epidermoid carcinoma of the nasopharynx. The results of this study indicate that DON and CONV were more potent than DONV. The ED_{50} expressed in terms of μg/ml for each of the materials averaged: 0.32 for DON, 2.09 for CONV, and 130 for DONV.

When the oncolytic activity of these drugs was examined in leukemia 1210, either in the presence or absence of L-asparaginase, DON proved to be the most effective chemotherapeutic agent. The results of these studies, presented in Table 1, also indicate that only DON exhibited prominent schedule-dependent antineoplastic activity. When given in combination with L-asparaginase, none of the three ketoamino acids exhibited significant synergism against leukemia 1210.

Also evident from the data given in Table 1 is the observation that the maximally effective doses of these three drugs are dramatically different. In addition, the doses of each of the three agents producing lethal toxicity to the host are notably diverse. To characterize this toxicity more meaningfully, LD_{50} values of DON, CONV and DONV were determined in Swiss mice with the results shown in Table 2.

It can be seen that CONV is the most toxic of the three drugs in single-dose studies, followed in order by DON and DONV. When the dosing is extended for five daily injections, DON emerges as the most toxic of the three. Indeed, the 5-day LD_{50} for DON is 400 times lower than its single-dose LD_{50}. On the other hand, neither CONV nor DONV showed any strong evidence of comparable cumulative toxicity.

In a limited histopathologic examination of the tissues of mice succumbing to an LD_{50} dose of DON, CONV or DONV, some further toxicologic differences among the agents were uncovered. Thus, DON inflicted maximal damage to the intestinal mucosa

Table 1. Optimum effect of DON, CONV and DONV on the murine intraperitoneal L1210 leukemia survival model *in vivo**

Drug	Drug regimen	Maximum increased life span (%)	Dose (mg/kg/injection) Optimum	Range tested
DON	Single dose, day 1	36	50	100–25
(NSC-7365)	Q4D days 1, 5 and 9	81	12.5	200–1.56
	Q2D days 1 and 3	116	5.0	15 0.62
	QD × 5 or more	118	0.4	0.6–0.1
	Single dose, day 1 100 I.U. L-asparaginase 1 hr later	44	50	100 25
DONV	Single dose, day 1	12	800	1600–400
(NSC-117,613)	Q4D days 1, 5 and 9	8	1024	2048–256
	QD × 5 or more	64	800	800–50
	Single dose, day 1 100 I.U. L-asparaginase 1 hr later	11	800	1600–400
CONV	Single dose, day 1	22	40	40–10
(NSC-124,412)	Q4D days 1, 5 and 9	40	25	50–6.25
	QD × 5 or more	38	37.5	75–9.4
	Single dose, day 1 100 I.U. L-asparaginase 1 hr later	36	40	40–10
L-Asparaginase	Single dose, day 1	5	100 I.U./mouse	100 I.U./mouse
(NSC-109,229)	Q4D days 1, 5 and 9	3	16 and 8 I.U./mouse	16 and 8 I.U./mouse
	QD 1–9	16	1600 I.U./mouse	1600–50 I.U./mouse

* Groups of 10 BDF_1 mice were injected intraperitoneally with 10^5 L1210 cells. Twenty-four hr after tumor implantation, DON, DONV, CONV or L-asparaginase was administered intraperitoneally at several dose levels to randomly selected groups of the tumored animals, by the drug regimens indicated. The median survival time of each test group was compared to the median survival time of the tumored untreated control group to determine the increased life span percentage of each test.

Table 2. Lethality studies with DON, CONV and DONV in mice*

Drug	LD_{50} (mg/kg)	LD_{50} (mg/m^2)	LT_{50} (days)	Gross signs and principal histopathologic lesions
Single dose (i.p.)				
DON	220	660	3	Colitis, hepatic steatosis
CONV	65	190	3	Peritonitis, pancreatitis, necrosis of islets of Langerhans
DONV	1000	3000	1	Convulsions
Five daily doses (i.p.)				
DON	0.5	1.5	8	Colitis
CONV	45	135	8	Peritonitis, pancreatitis, necrosis of islets of Langerhans
DONV	700	2100	6	Convulsions
Single dose (i.v.)				
DON	200	600	5	Colitis, hepatic steatosis
CONV	120	360	1	Sedation and collapse
DONV	850	2550	1	Acute convulsions, "flag tails"

* Male Swiss mice weighing 25 g were divided into groups of ten and given geometrically spaced doses of the drugs by the routes and schedules listed. For histopathology, moribund animals were sacrificed and complete necropsies performed; LT_{50} indicates the day on which 50 per cent of the recipients died.

and liver, organs long known to be the principal targets of its toxicity both in lower animals and man [12]. DONV, on the other hand, spared the bowel and produced only mild hepatic pathology, while CONV, given intraperitoneally, caused a florid necrotic peritonitis with ileitis, and superficial damage to every organ in the abdominal cavity. The pancreas was hardest hit, and in some recipients the necrotic pancreatitis was extreme, involving even the islets of Langerhans.

Fate and distribution. To determine whether these patterns of toxicity reflected differential excretion of DON, CONV and DONV, and to gain insight into the distinctive inhibitory potency of these three drugs *in vivo* versus L-asparagine synthetase, attempts were made to monitor their fate and distribution in mice.

Using the lithium citrate buffer system of Benson *et al.* [5], and where appropriate a treatment step with mineral acid for the purpose of totally cleaving the diazo function, the three agents cold be successfully resolved from the normal acidic amino acids on an automatic amino acid analyzer. Although the ninhydrin chromogens formed with DON, CONV and DONV were comparatively feeble, in selected cases, this chromatographic system proved to be suitable for monitoring the half-lives of the drugs in plasma.

Using amino acid analysis, then, it was possible to measure the rate at which these three compounds are removed from the blood stream, as well as from the two principal sites of L-asparagine synthesis (pancreas and tumor) after an intravenous dose of 100 mg/kg. The results of these analyses are presented

Table 3. Clearance and uptake of DON, CONV and DONV by tumor-bearing (L5178Y/AR) and non-tumor-bearing BDF_1 mice*

Drug	Organ	Concn of drug (nmoles/g or nmoles/ml) 3 min	6 min
DON	Tumor	0	0
	Pancreas	0	0
	Plasma a†	1850	1150
	Plasma b‡	1100	880
CONV	Tumor	0	0
	Pancreas	0	0
	Plasma a†	200	0
	Plasma b‡	310	180
DONV	Tumor	1140	880
	Pancreas		1600
	Plasma a†	5800	3000
	Plasma b‡	2750	1400

* L5178Y/AR-bearing or non-tumor-bearing BDF_1 mice were injected intravenously with 100 mg/kg of DON, CONV or DONV. At the times indicated, a blood sample was collected for plasma assay, then the mice were killed by cervical dislocation, the tumor and pancreas collected, frozen and homogenized in 9 vol. (w/v) of 0.1 M HCl, then centrifuged at 12,000 *g* for 9 min. One-ml aliquots of the supernatants were neutralized with NaOH, centrifuged at 12,000 *g* for 9 min and analyzed by automatic amino acid analysis for DON, CONV and DONV as described in Materials and Methods.

† Normal mice.

‡ Tumor-bearing mice.

in Table 3 and warrant the conclusion that the approximate plasma half-life of all three drugs is very short, ranging from 5 to 8 min in the case of DON, to approximately 3 min in the case of CONV and DONV.

In view of the rapid removal of DON, CONV and DONV from the plasma of the mouse, attempts were made to examine the rate and degree of entry of these amino acids into mouse erythrocytes. Even after a 30-min incubation of erthrocytes with 1 μCi L[U-^{14}C]-glutamine, 1 μCi L[U-^{14}C]asparagine and 0.6 mM of DON, CONV or DONV, less than 10 per cent of the added agents could be accounted for within the erythrocyte. In addition, when diafiltration was used to determine whether DON, CONV or DONV were bound to the proteins of mouse plasma, less than 5 per cent of the amounts added could be identified in the retentate. Thus, neither protein binding nor entry into the red cell is responsible for the disappearance of these ketoamino acids.

Microbiological assays. The problem of the tissue disposition of the ketoamino acids could not be explored in all cases with the amino acid analyzer. Therefore, a microbiological assay was devised. Table 4 presents the antibacterial action of DON, CONV and DONV versus a panel of micro-organisms with known patterns of resistance to a large number of drugs. It can be concluded that DONV is devoid of antibacterial activity versus *E. coli*, whereas DON inhibits a great many of the strains of this organism. Interestingly, CONV, whose carbon skeleton is identi-

Table 4. Antimicrobial activity of DON, CONV and DONV*

Organism	Zone diameter (cm) DON	CONV	DONV
E. coli ATCC 9637	5.05	2.0	0
/AZA (NSC-742)	0	0	0
/DON (NSC-7365)	0	0	0
/ACB-C (31083)	5.65	3.55	0
/MTX (NSC-740)	5.3	2.4	0
/DAP (NSC-743)	2.85	0	0
/FUDR (NSC-27640)	4.85	3.05	0
/PEN, STREP	4.6	2.85	0
/MeCCNU (NSC-95441)	5.5	2.5	0
E. coli B96/CCNU (NSC-79037)	NT†	3.6	0
E. coli B96 ATCC 13473	4.15	0	0
/MTX (NSC-740)	0	3.9	0
/PEN	0	0	0
/DON (NSC-7365)	0	0	0
/MeCCNU (NSC-95441)	3.4	3.45	0
/BCNU (NSC-409,962)	NT	4.0	0
E. coli B ATCC 11303	5.1	3.3	0
/MTX (NSC-740)	4.1	3.85	0
/AZA (NSC-742)	0	0	0
/DON (NSC-7365)	4.7	3.35	0
E. coli ATCC 9637/CCNU (NSC-79037)	NT	2.35	0
E. coli B (Hill)	4.8	2.85	0
/BCNU (NSC-409,962)	5.3	2.8	0
E. coli ATCC 9637/BCNU (NSC-409,962)	NT	2.3	0
Candida sp. Mt. Vernon strain 512	2.5	2.15	1.7

* Antimicrobial activity was tested with the methodology given in Materials and Methods. The abbreviations used are: / (slash) = resistant to: AZA: azaserine; DON; 6-diazo-5-oxo-L-norvaline; ACB-C: actinobolin C; MTX: methotrexate; DAP: daraprim (pyrimethamine); FUDR: deoxyriboside of fluorouracil; PEN STREP: penicillin-streptomycin; CCNU: cyclohexylchloroethyl nitrosourea; BCNU: bischloroethyl nitrosourea; MeCCNU: methyl CCNU.

† NT = not tested.

Table 5. Antagonism by selected metabolites of the antimicrobial activity of DON, CONV and DONV*

Metabolite	Concn (μg/ml)	Zones of inhibition (cm) with: DON (0.3 μg/disc)	CONV (0.3 μg/disc)	DONV (3 μg/disc)
None		3.97	3.36	1.85
L-Asparagine	100	4.00	3.80	1.70
L-Glutamine	100	3.40	2.43	1.75
L-Histidine HCl	100	2.80	3.87	1.8
L-Threonine	100	4.22	2.10	1.9
Adenine	10	2.77	3.6	2.95
Adenosine	10	2.70	3.95	1.95
Adenylic acid	10	2.40	3.95	2.05
Guanine	10	2.40	4.15	1.60
Guanosine	10	0	4.12	2.15
Guanylic acid	10	0	2.2	2.2
Inosine	10	0	3.52	2.1
Inosinic acid	10	2.50	2.37	1.95
L-α-Aminobutyric acid	100	3.6.	2.12	2.25
Glutathione	100	4.07	0	1.6
DL-Homocysteine	100	5.07	0	1.85
DL-Isoleucine	100	4.33	1.80	1.75
L-Leucine	100	3.73	0	1.85
DL-Methionine	100	3.17	0	1.9
p-Aminobenzoic acid	10	4.57	0	1.75
DL-Serine	100	3.47	1.92	1.6
L-Valine	100	4.13	0	1.75
α-Ketoglutaric acid	100	4.23	4.03	1.6
L-Proline	100	4.17	2.55	0
L-Aspartic acid	100	3.9	3.25	0

* Incubations were conducted as described in Materials and Methods under "Microbiological assays." Results are presented as average diameters (cm) of zones of inhibition surrounding a disc 1.27 cm in diameter. Each plate was tested in triplicate.

cal to that of DONV, is an active antibacterial agent.

Moreover the finding that certain strains sensitive to CONV are resistant to DON and vice versa suggests that these two drugs are unlikely to be acting by identical mechanisms.

Parenthetically, it should be pointed out that several intermediary metabolites were able to reverse the antimicrobial activity of these ketoamino acids. Thus, guanosine, guanylic acid, inosine and to a lesser degree, guanine, adenine, adenosine, adenylic acid, inosinic acid and L-histidine counteracted inhibition by DON (Table 5). DL-Homocysteine, DL-methionine, L-valine, glutathione, L-leucine, *p*-aminobenzoic acid, and to a lesser degree, L-aminobutyric acid, DL-isoleucine, DL-serine and L-threonine reversed inhibition by CONV. L-Aspartic acid and L-proline reversed inhibition of the test organism by DONV whereas adenine increased the sensitivity of the culture to inhibition by this agent. Noteworthy was the failure of L-asparagine to reverse the action of any of these antimetabolites against the organisms chosen. L-Glutamine did counteract the inhibitory action of CONV and DON, but this effect was minor in degree.

Despite the presumption that many of the counteragents reported in Table 5 occur in the tissues of mice, it is relevant to point out here that microbiological standard curves for DON, CONV and DONV carried out in 1:4 (w/v) saline homogenates of representative organs were not significantly different from comparable curves conducted in saline alone.

Figure 3 represents the average concentrations of DON, CONV and DONV detected in the body fluids and organs of BDF_1 mice given a single intraperitoneal dose of DON (10 mg/kg), CONV or DONV (100 mg/kg), using the microbiological assay. Maximum concentrations of all three drugs were reached rapidly in blood and urine and declined to undetectable levels within 1 hr after administration. The calculated half-life for DON, CONV and DONV was 19.5, 1.9 and 7.0 min respectively. Peak levels corresponding to 0.5 mM DON, 0.15 mM CONV and 63.6 mM DONV were reached in the pancreas. In the case of DON and CONV, the level of drug in the pancreas declined rapidly, while in the case of DONV extraordinarily high levels persisted up to 1 hr after administration. Tissue distribution studies revealed some accumulation of DON in the lung, liver, spleen and kidney, while no measurable levels were detected in the brain. No CONV or DONV was detected in erythrocytes, serum, brain, kidney, liver, lung or spleen at any time after drug administration. Biliary excretion, while not shown, was minimal in all cases. It should be noted that the concentration of DONV in the pancreas is high irrespective of the technique used to measure it, but that microbiological assay yielded a result over thirty times higher than chromatographic analysis. Metabolism of DONV to a material with augmented antimicrobial activity may explain this discrepancy.

Cell-uptake studies. To examine further the rates at which these ketoamino acids penetrate the intracel-

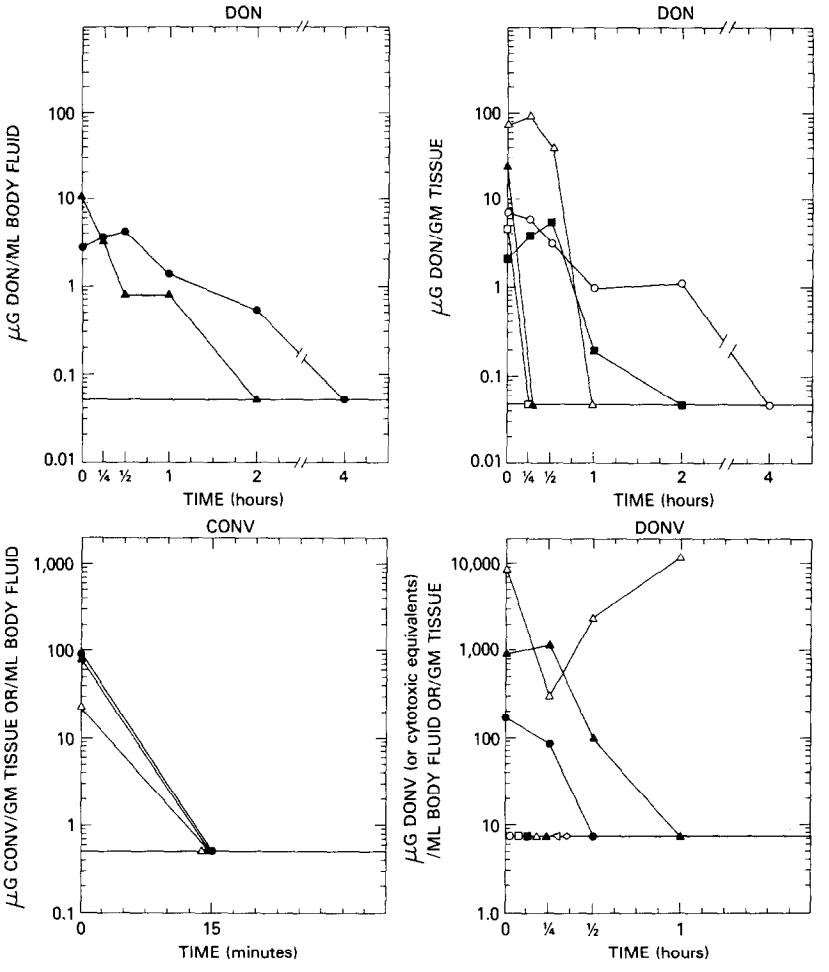

Fig. 3. Microbiological measurements of the concentration of DON, CONV and DONV in the blood and tissues of mice after single intraperitoneal doses of the drugs. Top left panel: (▲) urine and (●) blood; top right panel: (△) pancreas, (▲) spleen, (□) kidney, (■) lung and (○) liver; bottom left panel: (●) blood, (▲) urine and (△) pancreas; and bottom right panel: (△) pancreas, (▲) urine, (●) blood. Microbiological assays were conducted according to the methodology presented in Materials and Methods.

lular space, uptake experiments with intact cells were undertaken. Twenty min after the addition of DON, CONV or DONV at a final concentration of 5 mM, L5178Y/AR cells had achieved an intracellular concentration of 13.2, 6.7 and 6.7 mM respectively.

To define the transport system responsible for this uptake, the degree to which each of the three amino acids interfered with the cellular uptake of L[U-^{14}C]-asparagine or L[U-^{14}C]glutamine was investigated next. From the data in Table 6, it can be appreciated that CONV and DONV impeded the interiorization of L-glutamine most effectively, but that each of the three amino acids impeded the uptake both of L-asparagine and of L-glutamine to a prominent degree.

Advantage also was taken of the availability of L[5-^{14}C]DONV, for studies of the extent to which L-asparagine, L-glutamine and DON would antagonize the uptake of the radioactive diazoketone (Fig. 4). In these studies, L-glutamine was the strongest inhibitor of the transport of L[5-^{14}C]DONV.

Studies on the uptake of L[5-^{14}C]DONV by L5178Y/AR cells at 37° and at 0° indicated that hypothermia inhibited the transport of this agent by 95 per cent. Thus, the interiorization of DONV (and presumably of CONV and DON) is likely to be an active process.

Orlowski and Meister [13] have speculated recently that the entry of amino acids into cells is mediated by a series of enzymatic reactions which they have labeled the "γ-glutamyl cycle." The first enzyme in this cycle is γ-glutamyl transferase (γ-glutamyl transpeptidase), a membrane-bound protein catalyzing the transfer of the L-glutamyl residue of glutathione to an amino acid. In the light of the transport studies summarized above, we have examined the ability of this enzyme to utilize DON, CONV and DONV as substrates. The results of this study, presented in Table 7, indicate that DON is the best substrate of the three and that CONV is virtually inert. For purposes of comparison, the utilization of L-asparagine and L-glutamine by this enzyme also was examined. While L-glutamine is linked to L-glutamate at a rapid rate, L-asparagine proved to be a comparatively poor substrate. Despite such enzymologic data, it should be stressed that the relative velocity of transport of these two amides into intact tumor cells (see footnote Table 6) was not radically dissimilar.

Table 6. Effect of DON, CONV and DONV on the uptake of L[U-^{14}C]asparagine and L[U-^{14}C]glutamine into L5178Y/AR cells *in vitro**

Inhibitor	Concn ($\times 10^{-5}$ M)	Per cent inhibition of uptake of: L[U-^{14}C] asparagine	L[U-^{14}C] glutamine
DON	5	23	25
	10	37	34
	100	49	46
CONV	5	0	0
	10	38	31
	100	35	54
DONV	5	0	8
	10	0	23
	100	28	6

* L5178Y/AR cells were prepared and treated as described in Materials and Methods under "Cellular uptake studies." Five hundred μl L5178Y/AR cell suspension (2×10^8 cells) was incubated at 37° with DON, CONV or DONV at the concentrations listed for 1 min, after which 1 μCi L[U-^{14}C]glutamine was added. Samples then were processed as described in Materials and Methods. The uninhibited rates of transport of L-asparagine and L-glutamine in these studies were 0.095 and 0.135 nmole/min/10^6 cells respectively.

To verify that DON was, in fact, a substrate for γ-glutamyl transferase, attempts were made to identify the product of the enzymatic reaction between this diazoketone and glutathione. Electrophoresis of typical reaction mixtures revealed the presence of new ninhydrin-positive and ultraviolet-absorbing spots, one of which is presumed to be γ-glutamyl-L-DON (Fig. 5). This product could not be obtained either in the absence of enzyme or in the presence of heat-inactivated enzyme.

Attempts also were made to determine whether DON, CONV or DONV were subject to other biotransformations. When extracts of most of the principal organs of the mouse were incubated with these three ketoamino acids, either in the presence or absence of NAD or of α-ketoglutaric acid (added as putative cofactors), only negligible losses of the drugs could be demonstrated by spectrophotometric techniques. However, when microbiological and radiometric methodologies were used, it became possible to demonstrate that one or more of the agents was subject to metabolism.

Thus, homogenates of lung, spleen, kidney and testis proved to be active in the decomposition of DON as adjudged by microbiological assay while

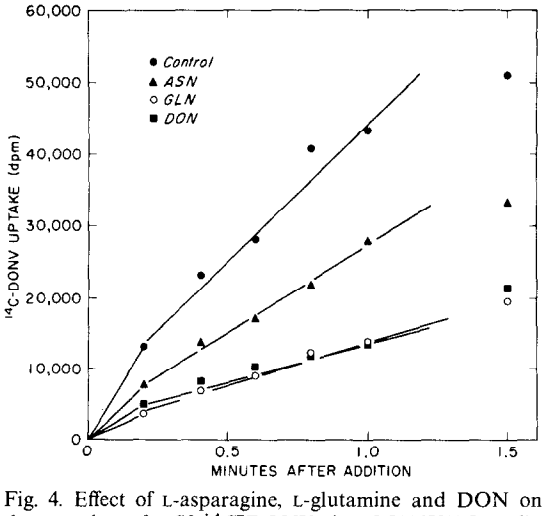

Fig. 4. Effect of L-asparagine, L-glutamine and DON on the uptake of L[5-^{14}C]DONV by L5178Y/AR cells. L5178Y/AR cells were prepared and treated as described in Materials and Methods. The rate of transport of L[5-^{14}C]DONV in this study was 0.07 nmole/min/10^6 cells.

Table 7. Relative velocity of γ-glutamyltransferase from mouse kidney with L-asparagine, L-glutamine, DON, CONV or DONV as substrates*

Substrate	Velocity (nmoles/mg protein/min)
L-Asparagine	13
L-Glutamine	500
DON	300
CONV	35
DONV	125

* Measurement of γ-glutamyltransferase was made according to the technique explained in Materials and Methods. For calculation of the rates, the velocity of hydrolysis of γ-glutamyl nitroanilide in the absence of an amino acid was subtracted from the rate seen in the presence of that amino acid. Substrate concentration was maintained at 1 mM.

Thus, homogenates of lung, spleen, kidney and testis proved to be active in the decomposition of DON as adjudged by microbiological assay while

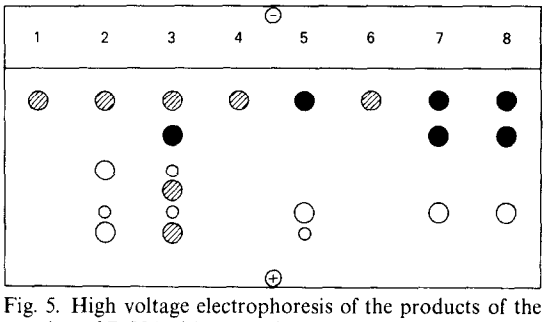

Fig. 5. High voltage electrophoresis of the products of the reaction of DON with glutathione and γ-glutamyl nitroanilide catalyzed by crude γ-glutamyltransferase from mouse kidney. The 12,000 g pellet from a 1:3 (w/v) homogenate of mouse kidney in 0.1 M Tris–HCl buffer, pH 8.4, was washed two times with the same homogenization buffer and then solubilized in 1 ml of 0.5% (w/v) sodium deoxycholate in 0.1 M Tris–HCl buffer, pH 8.4. After 12 hr at 4° the resultant suspension was centrifuged at 20,000 g for 3 min. The supernatant after centrifugation was used as the source of enzyme. A 5-μl aliquot of the following incubation mixtures (30 min at 37°) was spotted on Whatman 3MM paper and subjected to high voltage electrophoresis as described in Materials and Methods: No. 1, 20 μl of kidney enzyme; No. 2, 20 μl of kidney enzyme with 0.03 M reduced glutathione; No. 3, 20 μl of kidney enzyme with 0.03 M reduced glutathione and 0.03 M DON; No. 4, 20 μl of kidney enzyme with 0.03 DON; No. 5, 0.03 M reduced glutathione; No. 6, 0.03 M DON; No. 7, 0.03 M each of reduced glutathione and DON and No. 8, 20 μl of heated (95°, 10 min) enzyme with 0.03 M reduced glutathione and 0.03 M DON. The 0.03 M solutions of reduced glutathione and DON were prepared in 0.1 M Tris–HCl buffer, pH 8.4. Key: (○) ninhydrin positive spot, (●) ninhydrin and ultraviolet absorbing spot, and (●) ultraviolet absorbing spot.

brain, liver and spleen were similarly active with DONV (Table 8). Incubation of CONV with lung, spleen, liver and especially brain caused the generation of a product with enhanced microbiological activity. Whether this phenomenon represents metabolism of the chloroketone to a more active antimetabolite must await further experimentation. It also was observed that L5178Y/AR cells converted L[5-^{14}C]-DONV to [^{14}C]O_2 at a rate of 0.5 pmole/1 × 10^6 cells/hr. Moreover, crystalline L-glutamate oxaloacetic acid transaminase was capable of transaminating two of the three ketoamino acids in the presence of [1-^{14}C]α-ketoglutaric acid. Thus, DON and DONV were transaminated by L-glutamate oxaloacetic acid transaminase at the rate of 0.225 and 0.051 μmole/min/mg of protein respectively. The transamination of CONV was on the borderline of detection (approximately 0.010 μmole/min/mg of protein). L-Glutamate pyruvate transaminase was able to transaminate only DON (0.202 μmole/min/mg of protein) with [1-^{14}C]α-ketoglutaric acid. The transamination of CONV and DONV by this enzyme could not be detected. Under the conditions of the assay [9] L-glutamic acid was exchange-transaminated by both enzymes and the reaction went to equilibrium.

It also was possible to show that DON, in the absence of an added keto acid, permitted the transamination of L-glutamine by a crude mouse liver extract at 4 per cent of the rate seen with α-ketovaleric acid (data not shown). This finding is taken as evidence that the diazoketone is itself first undergoing transamination to the corresponding diketo acid or that the 5-keto functionality is capable of entering into a transamination.

Handschumacher *et al.* [14] documented that L[5-^{14}C]DONV was incorporated into an acid-insoluble form by cells of L5178Y; we, therefore, attempted to determine whether the compound was being incorporated into protein in lieu of L-asparagine. However, automatic amino acid analysis of pronase digests of cells of L5178Y/AR which had been incubated with L[5-^{14}C]DONV (cf. Materials and Methods) provided no evidence for the utilization of this compound in the synthesis of protein.

Table 8. Stability of DON, CONV and DONV in mouse organ homogenates as assessed by microbiological assay*

Organ homogenate	% Recovery		
	DON	CONV	DONV
Brain	73	177	40
Lung	43	117	70
Liver	97	127	53
Pancreas	83	90	103
Spleen	53	127	50
Kidney	50	97	93
Small intestine	83	77	103
Testis	60	100	80

* DON, CONV or DONV at a concentration of 3 mg/ml was incubated for 4 hr at 37° with the organ homogenates [1:10, (w/v) in physiologic saline] listed in the table, then assayed for drug concentrations using the microbiological technique outlined in Materials and Methods.

DISCUSSION

DON, CONV and DONV appear to share a common mechanism of transport into the cell, a mechanism which probably also accommodates L-asparagine and L-glutamine. In this regard it is pertinent to point out that, for most amino acids (whether fraudulent or natural) net ionic charge is the main determinant of transport [15]. Thus, these compounds are best divided into acidic, basic and neutral transport groups. L-asparagine and L-glutamine belong to the neutral transport group. Although the data on the uptake of these amides are often fragmentary, what is known can be summarized as follows.

In *Lactobacillus plantarum* the most effective competitor of the uptake of L-asparagine is L-glutamine [16]. By contrast, the most effective competitors in *Streptococcus faecalis* are small neutral amino acids such as L-alanine, L-serine, L-cysteine and L-methionine, suggesting that L-asparagine enters this organism by reaction with a catalyst having nonspecific structural discrimination among the neutral amino acids [16]. In mammalian kidney there is a transport system for neutral amino acids, another for basic amino acids and a third for acidic amino acids [15]. Moreover, glycine, L-proline and L-hydroxyproline are transported by the system for neutral amino acids as well as by a specific system of their own. L-Norvaline, L-asparagine and L-glutamine are trasnsprted by the system for neutral amino acids both in kidney and in the small intestine [15]. However, in brain, these three amino acids can also be transported by other systems; for example, L-norvaline and L-norleucine are transported along with L-leucine, L-isoleucine, L-tyrosine and L-tryptophan, while L-asparagine and L-glutamine are transported with L-serine and L-threonine [15]. In Ehrlich ascites carcinoma cells, also, there are three main transport systems, but many subgroups exist. Furthermore, there are transport systems dependent on chain length, one for short chains and another for long-chain amino acids [15]. There is also a separate system for acidic amino acids by which L-glutamic acid, but not L-aspartic acid, is taken up. Paradoxically, L-glutamine competes with L-glutamic acid in this system [17].

If little is known about the transport of the amides of the normal dicarboxylic acids, less is known about the transport of their analogs. Jacquez and Hutchinson [18] observed that the uptake of azaserine, a structural analog of L-glutamine, by L1210 cells sensitive to the drug did not differ from that seen in resistant cells. Pine [19] showed that the azaserine-sensitive 70429/S plasma cell tumor accumulated azaserine to intracellular concentrations as high as thirty times the external concentration. Two azaserine-resistant sublines, 70429/AZ-R-1 and 70429/AZ-R-5, concentrated azaserine approximately equally well, but the spontaneously resistant 6C3HED lymphosarcoma cells were less efficient. This observation indicates that not all tumor cells will exhibit the same V_{max} with a given amino acid. It is noteworthy that L-glutamine was a good inhibitor of the uptake of azaserine by the azaserine-sensitive 70429/S plasma cell tumor, by the AZ-R-5 resistant subline, and by the DBA/2 thymoma [19] as it was of the uptake of the ketoamino acids treated in the present paper. Although

kinetic analyses of the transport of DON, CONV and DONV have not been undertaken and although it does appear that these agents enter cells along with the neutral amino acids, it is highly likely that the velocity of transport of each ultimately will be found to be different, and also very likely will differ from organ to organ. Evidence touching on this supposition is provided by the uniquely high and sustained concentration of DONV found in pancreas after parenteral administration of the drug.

Further evidence for the diversity of DON, CONV and DONV can be seen from the particular pattern of toxicity of each drug in the mouse. Not only were the single-dose LD_{50} values widely different on a molar basis, but the repeated-dose toxicity also was distinctive, with DON confirmed as exhibiting extraordinary cumulative toxicity (analogous to that of azaserine [12]) while DONV and CONV were devoid of this property. Moreover, DON produced its principal damage on the GI tract and liver, whereas CONV attacked the peritoneum and all abdominal viscera. In therapeutic studies, DON proved to be the most active of the three agents examined. This oncolytic activity of DON is, of course, in accord with published results [20]. However, our failure to observe synergism of DON or CONV with L-asparaginase is at variance with the reports of other workers [21, 22]. Differences in the doses and schedules used may explain this discrepancy. Moreover, it is worthwhile pointing out that the dose of CONV used by Burchenal *et al.* [23] was 2 mg/kg and that this dose is insufficient to inhibit L-asparagine synthetase significantly so that the synergism observed by these workers with L-asparaginase is unlikely to have been mediated by depression of the biosynthesis of L-asparagine.

It has been hoped that pharmacologic studies of the fate and distribution of DON and CONV would explain why these two agents, equipotent *in vitro* as inhibitors of L-asparagine synthetase, are of unequal potency *in vivo*. After intraperitoneal administration, CONV produces sclerosis and necrosis of the membranes and viscera with which it comes into contact. This interaction doubtless consumes a portion of the injectate. That some drug escapes random fixation to the peritoneal contents is, however, proven by the finding that intraperitoneal CONV is an excellent inhibitor of purine biosynthesis [1, 2] as well as of L-asparagine synthetase of mouse pancreas. In the former case, intraperitoneal administration of the drug inhibits the incorporation of formic acid into DNA to a greater degree than does intravenous administration.

Nevertheless, the unequal potency of DON and CONV by the intravenous route still requires explanation. Less than 5 per cent of these drugs is bound to the plasma protein of the mouse as adjudged by the procedures outlined in Materials and Methods. Moreover, neither was found to enter the erythrocyte to a significant degree, and both agents were removed from the blood of the mouse with a half-life of several min. However, direct measurements of the concentrative abilities of isolated cells of L5178Y/AR have shown that the intracellular molarity of DON is nearly two times that of CONV under comparable conditions of incubation. This finding makes it likely that DON is a better inhibitor than CONV of L-asparagine synthetase in tumor because higher intracellular concentrations of the diazoketone are obtained *in vivo*. The minimal inhibitory activity of DONV, on the other hand, probably is a result of the reversibility of the inhibition this drug produces.

It is pertinent to point out that man appears to dispose of DON somewhat differently than the mouse does. Thus, after an intravenous dose of DON of 0.2 to 0.3 mg/kg (7.4 to 11.11 mg/m^2), peak plasma levels of between 250 and 500 ng/ml were reached in man [24] within 15 min. After a very rapid decline, presumably representing distribution of the drug in the body fluids, DON was removed from the plasma with a half-life of approximately 1–2 hr. In addition, although urinary excretion was negligible in man [24] it was found to be significant in mice (Fig. 3). The reasons for these species differences are unclear at present.

Inasmuch as DON, CONV and DONV have been found to exhibit such distinctive attributes, it would probably be unwise to ascribe their oncolytic activity wholly to a common mechanism. Moreover, since none of the drugs eradicates the synthesis of L-asparagine *in vivo*, it is unlikely that these antagonists can be of use in preventing the emergence of resistance to L-asparaginase or in converting resistant cells to the sensitive state. Nevertheless, in view of their diverse reactivity, and their partial success as inhibitors of L-asparagine synthesis, further structural modifications seem warranted.

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