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DON, CONV AND DONV—II. INHIBITION OF L-ASPARAGINE SYNTHETASE *IN VIVO*

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Abstract—The inhibition *in vivo* of L-asparagine synthetase (L-glutamine hydrolyzing, EC 6.3.5.4) from L-asparaginase-resistant leukemia 5178Y (L5178Y/AR) and from mouse pancreas by the ketoamino acids DON (L-DON; 6-diazo-5-oxo-L-norleucine), CONV (L-CONV; 2-amino-5-chlorolevulinic acid; 5-chloro-4-oxo-L-norvaline) and DONV (L-DONV; 5-diazo-4-oxo-L-norvaline) was investigated using both L-glutamine and ammonium chloride as substrates. In the L5178Y/AR system, DON was shown to inhibit the utilization of L-glutamine by L-asparagine synthetase *in vivo* to a degree comparable to that previously observed *in vitro*. CONV, however, was less effective *in vivo* than *in vitro* and DONV exerted no demonstrable inhibitory effect in the intact organism. In the case of the analogous enzyme from mouse pancreas, both DON and CONV effectively inhibited the utilization of L-glutamine *in vivo*. DONV was relatively inert in this experimental setting. The pattern of inhibition of L-asparagine synthetase was generally mirrored by the effects of these ketoamino acids on the organ pool-sizes of L-asparagine. However, CONV, alone of the three, demonstrated inhibition of the incorporation of L-asparagine into tumoral and pancreatic protein *in vivo*. Both DON and CONV inhibited the incorporation of [^{14}C]formate into nucleic acid purines; this effect, in turn, most likely reflected the strong inhibition by these agents of several of the amidotransferases concerned with purine biosynthesis.

The ketoamino acids, DON (L-DON; 6-diazo-5-oxo-L-norleucine), CONV (L-CONV; 2-amino-5-chlorolevulinic acid; 5-chloro-4-oxo-L-norvaline), and DONV (L-DONV; 5-diazo-4-oxo-L-norvaline), have been shown to be effective inhibitors *in vitro* of the utilization of L-glutamine by the enzyme L-asparagine synthetase (L-glutamine hydrolyzing, EC 6.3.5.4) of several different experimental animal tumors [1–4]. In a companion paper [1] we compared the relative inhibitory potencies of these three agents *in vitro* against partially purified L-asparagine synthetase from leukemia 5178Y rendered resistant to L-asparaginase (L5178Y/AR), and against the analogous enzyme from mouse pancreas. Although it was found that DON, CONV and DONV were all capable of inhibiting L-asparagine synthetase *in vitro* [1], little has been reported of their effects on the synthesis of L-asparagine in the intact organism. Such studies are of relevance to the chemotherapy of cancer, inasmuch as agents capable of eradicating the synthesis of L-asparagine *in vivo* ought to be able to convert L-asparaginase-resistant tumors to the sensitive state [5]. For this reason, we have administered DON, CONV or DONV parenterally to mice bearing L5178Y/AR and examined the synthesis of L-asparagine by the neoplasm. In addition, the interaction of these agents with L-asparagine synthetase of pancreas has been studied pursuant to the recent observation that this gland synthesizes L-asparagine at a more vigorous rate than all other mouse organs examined [6]. We also have considered whether the inhibition of L-asparagine synthetase by these ketoamine acids is of sufficient magnitude to alter the amino acid pools and protein synthesis in tumor and pancreas. Finally, in view of their ability to inhibit L-glutamine amidotransferases other than L-asparagine synthetase *in vitro* [1], and inasmuch as purine biosynthesis is dependent on several such enzymes, experiments were carried out to determine the influence of DON, CONV and DONV on the incorporation of [^{14}C]formic acid into DNA, *in vivo*.

MATERIALS AND METHODS

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

Studies on the inhibition of L-asparagine synthetase. Male BDF$_1$ mice, weighing between 18 and 20 g, were injected by the subcutaneous route with a suspension of 10^6 cells of L5178Y/AR. Seven days later, mice bearing subcutaneous tumors, or untreated mice, were injected with the inhibitors by the intraperitoneal or intravenous route. At 0.1, 1, 2, 4, 8 or 24 hr after injection, all animals were killed by cervical dislocation, and the tumor and pancreas were collected and frozen at −70°. Homogenizations (1:10, w/v) were carried out in 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM EDTA and 1 mM dithiothreitol. The homogenates were centrifuged at 12,000 *g* for 5 min before measurement of L-asparagine synthetase activity.

Measurement of amino acid pools. Pancreases and tumors of animals treated as described above were homogenized in 9 vol. (w/v) of 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM EDTA and 1 mM dithiothreitol. A 200-μl aliquot of the 12,000 *g* supernatant was immediately heated at 95° for 5 min, then centrifuged at 12,000 *g* for 3 min. L-Asparagine was measured in the supernatant fluid by a radiometric

technique capable of detecting as little as 20 pmoles of the amide [7]; L-glutamine was measured by an enzymatic spectrophotometric technique, utilizing L-glutamate dehydrogenase (EC 1.4.1.2), and capable of detecting as little as 5 nmoles of this amide [8]. It is recognized that approximately 5 per cent of the L-glutamine present in these extracts will be deamidated under these circumstances. However, no correction for this loss was made. Other acidic amino acids were analyzed on the Jeolco amino acid analyzer using the lithium citrate system of Benson *et al.* [9].

Incorporation of L-*asparagine and* L-*glutamine into proteins of L5178Y/AR cells* in vitro. Cells were collected from the ascites of BDF_1 mice bearing intraperitoneal transplants of L5178Y/AR, and the cell pellet was washed twice with Hank's balanced salt solution containing 10% (v/v) fetal calf serum. One ml of washed cells (4×10^8 cells) in Hank's balanced salt solution–fetal calf serum was dispensed into Eppendorf 1500-μl conical vessels and to it, DON, CONV or DONV was added to a final concentration of 1.0 or 10.0 mM. Control vessels received Hank's balanced salt solution instead of drug. One min later, 1.0 μCi L-[U-^{14}C]asparagine (sp. act. 151 μCi/μmole) or L[U-^{14}C]glutamine (sp. act. 220 μCi/μmole) was added and the suspension incubated at 25° for 15 min. Aliquots (200 μl) were removed and the proteins precipitated with 1 ml of a 5% solution of trichloroacetic acid 5% TCA). The precipitate was washed twice with 1 ml of 5% TCA and then digested with 50 μl of 40% (w/v) KOH at 95° for 10 min. Radioactivity of the digest was counted as described previously [1].

Incorporation of L-*aspartic and* L-*asparagine into proteins* in vivo. Male BDF_1 mice bearing subcutaneous nodules of L5178Y/AR were treated intraperitoneally with 100 mg/kg of DON, CONV or DONV or with normal saline. One hr later, 10 μCi L[U-^{14}C]asparagine or L-[U-^{14}C]aspartic acid was injected intravenously. The brain, liver, pancreas and tumor were removed 30 min later and each was homogenized in 5% perchloric acid (1:10, w/v). One ml of homogenate was centrifuged at 12,000 *g* for 10 min, and the pellet was washed three times with 1 ml of 80% ethanol, resuspended and recentrifuged at 12,000 *g* for 10 min. Excess ethanol was decanted and the pellets were air dried for 16 hr at 4°. One ml of 0.1% (w/v) pronase in 0.01 M Tris–HCl buffer, pH 7.6, containing 0.001 M $CaCl_2$, was added and the suspensions were incubated for 24 hr at 25°. After heating at 95° for 12 min, the hydrolysates were treated with subtilisin (Novo, Copenhagan, Denmark) at a final concentration of 0.1% (w/v), for 16 hr at 25°, and then heated at 95° for 12 min. After a second treatment for 24 hr at 25° with pronase at a final concentration of 0.1% (w/v), the hydrolysate was heated at 95° for 20 min to inactivate the pronase. Labeled L-asparaginyl and aspartyl residues were measured by a gasometric technique [10] and unlabeled residues were measured by a spectrophotometric technique [5]; specific activities were determined accordingly.

Measurement of the incorporation of [^{14}C]*formate into nucleic acids.* Mice were treated intraperitoneally or, where indicated, intravenously with DON, CONV or DONV at a dose of 100 mg/kg. One hr later 10 μCi [^{14}C]formate (sp. act. 20 μCi/μmole; Mallinkrodt, St. Louis, Mo.) was injected intravenously. One hr later the spleen, pancreas and small intestines were removed, washed well with saline, blotted and homogenized in 2 ml of 4% (w/v) aminosalicylate. An equal volume of phenol, saturated with 4% (w/v) aminosalicylate and containing 2% (v/v) cresol, was added and the mixtures were shaken well. After 12 hr at 4° with gentle agitation, the mixtures were centrifuged at 1000 *g* for 3 hr in the cold. Five hundred μl of the milky supernatants were added to 1 ml ethanol, and after 12 hr at 4°, the nucleic acids were collected by centrifugation at 12,000 *g* for 6 min. The supernatant was discarded and the pellet washed twice with 66% ethanol. Fifty μl of 0.1 M HCl was added to the resulting pellet, and the closed vessels were heated at 95° for 20 min. The vessels were again centrifuged at 12,000 *g* for 6 min, and the radioactivity of the purines liberated by the hot acid was measured in a 10-μl aliquot by scintillation spectrometry. An additional 10 μl was subjected to high-voltage paper electrophoresis for 1 hr at 3000 V using Whatman 3MM paper, moistened with 0.1 M formic acid. The spots corresponding to authentic guanine and adenine were excised and eluted with 2 ml water. The absorbance of the eluent was measured at 260 nm, after which the entire 2 ml was counted in scintillation fluid containing toluene–ethanol–Triton-X-100-Liquifluor (16:8:12:1, by vol).

RESULTS

Inhibition of L-*asparagine synthetase.* DON given intraperitoneally 4 hr before sacrifice at doses of 10 and 100 mg/kg completely inhibited the utilization of L-glutamine by L-asparagine synthetase in extracts of tumor and pancreas of BDF_1 mice bearing L5178Y/AR as well as by L-asparagine synthetase from pancreas of non-tumor-bearing mice (Table 1). CONV was a less effective inhibitor of the enzymes from both sources, and DONV was inert. It is noteworthy that ammonia utilization by L-asparagine synthetase from pancreas of tumor-bearing mice was significantly inhibited by doses of 100 mg/kg of DON and CONV.

To ascertain whether the markedly reduced inhibitory potency of CONV was related to the arbitrary practice of removal of the tissue 4 hr after administration of drug, the inhibition of L-asparagine synthetase by DON, CONV and DONV at time periods ranging from 0.1 to 24 hr after an intraperitoneal dose of 100 mg/kg was examined, with the results presented in Table 2.

DON was a potent inhibitor of L-glutamine utilization by both the tumoral and pancreatic enzymes at all time periods, although maximal inhibition of the tumoral enzyme was delayed until 1 hr after dosing. The utilization of ammonia was less impressively inhibited by DON. CONV inhibited the utilization of L-glutamine by the pancreatic enzyme strongly, but its inhibition of the tumoral enzyme was modest and transitory. The utilization of ammonia by both enzymes was also modestly inhibited. By contrast, DONV was consistently without any effect.

During the course of these studies *in vivo*, it was noted that CONV, given intraperitoneally, produced marked sclerotic effects in the peritoneum. To ascer-

Table 1. Inhibition *in vivo* by DON, CONV and DONV of L-asparagine synthetase of L5178Y/AR and mouse pancreas*

		Per cent inhibition of L-asparagine synthetase				
		Tumor-bearing mice				Normal mice
		Tumor		Pancreas		Pancreas
Inhibitor	Dose (mg/kg)	Substrate L-Glutamine	Ammonia	Substrate L-Glutamine	Ammonia	Substrate L-Glutamine
DON	100	99†	28	99†	79†	97†
	10	90†	19	99†	40	
CONV	100	34†	0	80†	65†	63†
	10	23	0	0	0	
	2	0	0	0	0	0
DONV	100	0	0	0	0	0
	10	0	0	0	0	

† $P \leq 0.05$.

* Groups of five normal BDF_1 mice or L5178Y/AR tumor-bearing BDF_1 mice were injected with DON, CONV or DONV at the indicated doses or with normal saline. After 4 hr, the tumor and pancreas were collected, homogenized and centrifuged at 12,000 *g* as described. Then, 5 μl of the resulting 12,000 *g* supernatant was added to a final reaction volume of 10 μl containing 5.6 nmoles L[U-^{14}C]aspartic acid, 0.2 μmoles L-glutamine or 0.5 μmoles NH_4Cl, 0.1 μmoles ATP, 0.25 μmoles $MgCl_2$, and 2.5 μmoles Tris–HCl buffer, pH 8.4 and assayed for L-asparagine synthetase activity as described in Materials and Methods. Uninhibited pancreatic and tumoral L-asparagine synthetase activities were 40 nmoles/mg of protein/hr.

tain whether the relatively feeble inhibition *in vivo* of tumoral L-asparagine synthetase observed with CONV resulted from the use of this route of administration, CONV, as well as DON and DONV, was administered intravenously to tumor-bearing mice at a dose of 100 mg/kg. Animals were sacrificed at 0.1, 1, 4, 8 and 24 hr after dosing, and L-asparagine synthetase was assayed in both tumor and pancreas. Essentially identical results were produced by all three drugs after their intravenous injection with the notable exception that DONV, given by this route, did inhibit the synthesis of L-asparagine in pancreas with L-glutamine or ammonia as substrates, by 60 and 49 per cent respectively, at the 6-min samplings.

The possibility was considered that the comparatively feeble potency of parenteral DONV as an inhibitor of L-asparagine synthetase *in vivo* might be a consequence of the fact that the doses of this keto-amino acid used in the studies presented in Table 2 were considerably less toxic than the doses used of DON and CONV. Therefore, all three drugs were compared at their LD_{50}. One hr after the intraperitoneal injection of DONV at a dose of 1000 mg/kg, only marginal and insignificant inhibition (7 per cent) of pancreatic L-asparagine synthetase and no inhibition of the tumoral enzyme were seen, whereas an equitoxic dose of DON, 220 mg/kg, powerfully inhibited L-asparagine synthetase from both sources (92 per cent in tumor, 93 per cent in pancreas) and an

Table 2. Inhibition *in vivo* of L-asparagine synthetase of L5178Y/AR and of mouse pancreas by intraperitoneal administration of DON, CONV or DONV as a function of time, using L-glutamine and ammonia as substrates*

	Per cent inhibition of L-asparagine synthetase by:					
Time after injection (hr)	DON		CONV		DONV	
	L-Glutamine	Ammonia	L-Glutamine	Ammonia	L-Glutamine	Ammonia
			L5178Y/AR			
0.1	50	12	32	24	20	0
1	83†	0	30	20	0	0
2	85†	35†	50†	30†	0	0
4	99†	28	34	9	17	0
8	88†	0	0	0	0	
			Pancreas of normal mice			
0.1	100†	50†	90†	17	9	0
4	99†	79†	65†	65†	0	0
24	90†		‡	‡	0	0

* Groups of five BDF_1 mice bearing L5178Y/AR or normal mice were injected intraperitoneally with 100 mg/kg of DON, CONV or DONV or with normal saline. At the times indicated, tumors or pancreas were collected, and assayed for L-asparagine synthetase activity as described in the legend to Table 1. Uninhibited pancreatic and tumoral L-asparagine synthetase activities were approximately 40 nmoles/mg of protein/hr.

† $P \leq 0.05$.

‡ Animals died of drug toxicity.

Table 3. Influence of a single intraperitoneal dose of DON, CONV and DONV on the pool size of selected amino acids in the pancreas and tumor of L5178Y/AR bearing mice*

Amino acid	Concentration (nmoles/g wet weight) of amino acids after treatment with: DON	CONV	DONV	Saline
		Pancreas		
L-Aspartic acid	1312 ± 145	681 ± 141	638 ± 106	986 ± 144
L-Threonine	1624 ± 187†	266 ± 59	295 ± 47	278 ± 22
L-Serine	1095 ± 121†	397 ± 50†	627 ± 76	792 ± 85
L-Asparagine	152 ± 22	94 ± 12†	317 ± 29	251 ± 14
L-Glutamic acid	4373 ± 732	1531 ± 221†	3518 ± 499†	4824 ± 637
L-Glutamine	4947 ± 373†	1143 ± 100	930 ± 145	1464 ± 215
Glycine	7515 ± 364	2998 ± 390†	3242 ± 580†	6318 ± 789
L-Alanine	2137 ± 344	1203 ± 78†	1270 ± 60	2018 ± 232
		Tumor		
L-Asparagine	158 ± 46	159 ± 33	296 ± 6	216 ± 33
L-Glutamine	1081 ± 191†	368 ± 25†	424 ± 57	562 ± 76

* BDF_1 mice, in groups of three, were given single intraperitoneal injections of DON, CONV or DONV at a dose of 100 mg/kg. After 4 hr, the mice were killed by cervical dislocation, their organs removed, and amino acid pools measured using the techniques outlined in Materials and Methods. All values shown represent the mean ± S.E.M. of six animals and twelve determinations. Statistical analysis of group differences was made using Tukey's Standardization Range Test as described by Snedecor [11].

† $P \leq 0.05$.

equitoxic dose of CONV, 65 mg/kg, powerfully inhibited the utilization of L-glutamine by the pancreatic enzyme (71 per cent).

Measurement of amino acid pools in animals treated with DON, CONV or DONV. The influence of treatment with DON or DONV on the L-glutamine and L-asparagine pools of tumor and pancreas of tumor-bearing mice was studied in order to determine the ultimate effect of inhibition of L-asparagine synthetase on the homeostasis of L-glutamine and L-asparagine in the intact organism. DON, at the dose of 100 mg/kg, significantly increased the concentration of L-glutamine in both pancreas and tumor (Table 3). CONV depressed the pool size of L-asparagine in pancreas and of L-glutamine in tumor, whereas DONV was consistently without significant effect on the concentration of both amino acids, in both organs. Pursuant to these observations, the pool size

Table 4. Influence of DON, CONV and DONV on the synthesis and incorporation of L[U-^{14}C]asparagine and L[U-^{14}C]aspartic acid into protein*

Drug	Treatment	Specific activity of L-asparaginyl residue (pCi/μmole ± S.E.M.) Tumor	Pancreas	Liver	Brain
Saline	L[U-^{14}C]aspartic acid	31.6 ± 2.9	320.3 ± 52.5	19.0 ± 2.3	6.7 ± 1.7
DON	L[U-^{14}C]aspartic acid	6.8 ± 2.4†	3.9 ± 0.9†	2.0 ± 1.0†	1.5 ± 0.3†
CONV	L[U-^{14}C]aspartic acid	3.3 ± 1.0†	24.5 ± 12.1†	18.1 ± 7.2	1.2 ± 0.5†
DONV	L[U-^{14}C]aspartic acid	32.6 ± 4.8	460.6 ± 127.6	17.0 ± 1.9	4.7 ± 1.3
Saline	L[U-^{14}C]asparagine	2192.0 ± 201.0	16959.0 ± 5323.5	4757.0 ± 1155.5	262.4 ± 27.4
DON	L[U-^{14}C]asparagine	2136.0 ± 474.7	35182.0 ± 7813.8	5468.0 ± 789.3	210.2 ± 40.3
CONV	L[U-^{14}C]asparagine	400.0 ± 75.5†	6022.0 ± 394.2	4044.0 ± 680.7	271.2 ± 71.9
DONV	L[U-^{14}C]asparagine	2214.0 ± 303.3	13470.0 ± 1149.3	3208.0 ± 655.2	293.2 ± 38.2
		Specific activity of L-aspartyl residue (pCi/μmole ± S.E.M.)			
Saline	L[U-^{14}C]aspartic acid	75.0 ± 32.2	384.6 ± 185.6	122.3 ± 32.6	23.3 ± 6.8
DON	L[U-^{14}C]aspartic acid	44.0 ± 12.1	35.8 ± 9.1†	20.8 ± 6.8†	5.7 ± 1.3†
CONV	L[U-^{14}C]aspartic acid	10.7 ± 3.7†	15.5 ± 5.9†	50.6 ± 5.9†	6.5 ± 0.7†
DONV	L[U-^{14}C]aspartic acid	39.8 ± 4.1	142.2 ± 11.0	97.5 ± 33.9	8.6 ± 1.6†
Saline	L[U-^{14}C]asparagine	33.0 ± 8.4	99.2 ± 21.3	76.8 ± 19.7	19.8 ± 4.3
DON	L[U-^{14}C]asparagine	39.8 ± 9.9	95.4 ± 13.0	89.6 ± 22.3	19.0 ± 3.0
CONV	L[U-^{14}C]asparagine	12.2 ± 3.3†	34.4 ± 4.1†	41.0 ± 1.4	18.8 ± 2.7
DONV	L[U-^{14}C]asparagine	20.6 ± 1.6	53.0 ± 5.1†	68.4 ± 8.1	15.3 ± 4.3

* BDF_1 mice bearing L5178Y/AR received intravenous injections of 10 μCi L[U-^{14}C]aspartic acid or L[U-^{14}C]asparagine. Measurement of the incorporation of these amino acids into proteins was conducted as described in Materials and Methods. Values represent means ± S.E.M. of five animals and ten determinations. The efficiency of the pronase-subtilisin digestion, calculated on the basis of percentage of recovery of L-aspartyl and L-asparaginyl residues released from 3 mg L-asparaginase carried through the entire process, was 68 per cent of the theoretical value.

† $P \leq 0.05$.

of related amino acids was measured in the pancreas of animals treated with saline or with one of the three drugs under study (Table 3). In addition to confirming the influence of CONV and DON on the amides of the dicarboxylic acids, these analyses revealed that DON greatly increased the concentrations of L-threonine and L-serine in this organ. Two other L-glutamine antagonists, azotomycin [12] and L-(αS,5S)-α-amino-3-chloro-4,5,-dihydro-5-isoxazoleacetic acid [13], have been observed to induce a similar change in the pool size of L-threonine, but the metabolic reasons for this action are obscure.

Incorporation of amino acids into proteins. Inasmuch as DON and CONV exerted notable effects on the pool size of L-asparagine or L-glutamine in both tumor and pancreas, it seemed relevant to consider the effects of the ketoamino acids on incorporation of these amino acids into proteins. The specific activities of L-asparaginyl residues in the organs of mice pretreated with DON, CONV or DONV and then given radiolabeled L-aspartic acid or L-asparagine are presented in Table 4. When L-aspartic acid was the injectate, DON reduced the specific activity of L-asparaginyl residues of every organ; CONV exerted comparable inhibition except in the case of liver, whereas DONV exerted only marginal effects. When L-asparagine was the injectate, CONV inhibited the uptake and/or incorporation of the amide into the protein of tumor and pancreas, whereas CONV and DON were either without effect or exerted stimulatory activities. For example, DON doubled the extent of incorporation of exogenous L-asparagine into pancreatic proteins, most likely because of the fact that endogenous synthesis of the amides had been inhibited. There is precedent for such stimulatory activity in the case of L-glutamine analogs [2].

The effect of these ketoamino acids on the specific radioactivity of L-aspartyl residues was next examined in the organs of mice pretreated with DON, CONV or DONV and then given radiolabeled L-aspartic acid or L-asparagine (Table 4). CONV reduced the specific activity of L-aspartyl residues in every organ examined, when L-aspartate was the injectate. DON produced a reduction in pancreas, liver and brain, while DONV reduced the specific activity of L-aspartyl residues only in brain. When L-asparagine was the injectate, CONV reduced the specific activity of L-aspartyl residues of every organ examined except brain while DONV did so only in pancreas; DON was without effect.

After the injection of radioactive L-aspartic acid, pronase-hydrolysates of carcass proteins (all tissues remaining after the removal of brain, liver, pancreas and tumor) were also examined by the methodology used to generate Table 4 in order to determine if DON, CONV and DONV perturbed the conversion of this amino acid to L-asparagine throughout the body under the experimental conditions used. In fact, DON and CONV reduced the specific activity of L-asparaginyl residues by 92 and 70 per cent respectively. DONV exerted no inhibition in carcass protein. Thus, the inhibition of L-asparagine biosynthesis was general, and did not appear to be proceeding via the alternate substrate, ammonia, whose utilization is not strongly or irreversibly inhibited by these drugs.

In further studies, isolated cells of L5178Y/AR were also used to assess the degree to which each of these agents interfered with incorporation of L-asparagine and L-glutamine into proteins. From the data in Table 5, it is clear that again CONV significantly inhibited the incorporation of both amino acids into tumor cell proteins. DONV exerted prominent inhibitory effects only at a concentration of 10 mM and these effects were roughly equal with both precursors. DON, however, while less potent than CONV is this system, did appear to impair the utilization of L-glutamine preferentially.

With the foregoing in mind, it became of interest to consider whether the marked inhibitory effect of CONV on amino acid incorporation into proteins results from its behavior as a fraudulent amino acid, structurally similar to L-glutamine and L-asparagine, or whether it exerted a direct toxic effect on protein synthesis in general, independent of its structural analogy to these amino acids. To this end, the effect of CONV on the incorporation of L[U-^{14}C]leucine into tumor cell proteins was considered under conditions of L-asparagine and L-glutamine depletion and repletion. From Table 6, it is clear that CONV inhibited the incorporation of L[U-^{14}C]leucine into tumor cell proteins whether or not pharmacological concentrations of L-asparagine were present. In fact, the presence of L-glutamine, L-asparagine or a combination of the two augmented the degree to which CONV prevented L[U-^{14}C]leucine incorporation. This may indicate that CONV exerts a direct effect

Table 5. Effect of DON, CONV and DONV on the incorporation of L[U-^{14}C]asparagine and L[U-^{14}C]glutamine into proteins of L5178Y/AR cells *in vitro**

Inhibitor	Concn (mM)	Percent inhibition of incorporation of: L[U-^{14}C]asparagine	L[U-^{14}C]glutamine
DON	1	0	33†
DON	10	35†	57†
CONV	1	81†	86†
CONV	10	95†	97†
DONV	1	0	12
DONV	10	39†	42†

* L5178Y/AR cells were prepared and treated as described in Materials and Methods. Per cent inhibition was calculated by comparison of the saline control vessels with those receiving a drug.

† $P \leq 0.05$.

Table 6. Effect of CONV on the incorporation of L[U-^{14}C]leucine into proteins of L5178Y/AR cells *in vitro**

Treatment	Amino acid(s) added (2 mM)	L[U-^{14}C]leucine incorporated (pmoles/2 × 10^8 cells/30 min ± S. E.)	Per cent inhibition of incorporation
None		186 ± 18	0
	L-Asparagine	183 ± 6	0
	L-Glutamine	185 ± 10	0
	L-Asparagine + L-glutamine	159 ± 21	0
CONV		134 ± 2	28
	L-Asparagine	109 ± 11†	40†
	L-Glutamine	114 ± 5†	38†
	L-Asparagine + L-glutamine	90 ± 1†	43†

* L5178Y/AR cells were prepared as described in Materials and Methods. Aliquots of 2 × 10^8 cells were resuspended in 1 ml of Hanks' balanced salt solution (HBSS) in Eppendorf vessels. The cell suspensions then received 20 μl of 0.1 M L-asparagine, 20 μl of 0.1 M L-glutamine or 20 μl of a solution of 0.1 M L-asparagine plus 0.1 M L-glutamine all dissolved in HBSS, or 20 μl of HBSS alone. After 10 min of equilibration at 4°, the vessels received CONV at a final concentration of 1 mM. Two μCi L[U-^{14}C]leucine (sp. act. 300 μCi/μmole) was added to each vessel and the assemblies were incubated at 25° for 15 min. The protein was then precipitated with 5% TCA, washed twice with 5% TCA and twice with 80% ethanol, resuspended in 200 μl of 40% (w/v) KOH and heated for 10 min at 95°. The entire vessel was then immersed in scintillation fluid and counted as described in Materials and Methods. The incorporation of L[U-^{14}C]leucine was calculated as pmoles of L[U-^{14}C]leucine/per 2 × 10^8 cells/30 min. The per cent inhibition of L[U-^{14}C]leucine incorporation was calculated by comparison of the control vessels with those receiving CONV.

† $P \leq 0.05$.

on protein synthesis that is partly independent of its role as a fraudulent amino acid analog of L-glutamine and L-asparagine. Nevertheless, the incorporation of L[U-^{14}C]asparagine and L[U-^{14}C]glutamine was inhibited by CONV to a greater degree (85 per cent) than the incorporation of L[U-^{14}C]leucine (28 per cent).

Incorporation of [^{14}C]formate into nucleic acids. Inasmuch as these ketoamino acids inhibit one or more of the L-glutamine amidotransferases *in vitro* [1], and inasmuch as purine biosynthesis is dependent on several such enzymes, measurements of incorporation of [^{14}C]formate into the adenine and guanine residues of nucleic acids were undertaken. In this study, DONV produced only equivocal inhibition of [^{14}C]formate incorporation and hence nucleic acid synthesis—a finding in accord with its failure to inhibit the enzymes responsible for the biosynthesis of purines *in vitro* [1]. On the other hand, DON and CONV strongly inhibited the incorporation of [^{14}C]formate in spleen, pancreas and small intestine (Table 7), reflecting the powerful and equal potency of these agents on the isolated enzymes of purine biosynthesis. It is notable that CONV given intraperitoneally was a better inhibitor of this process than CONV given by the intravenous route.

DISCUSSION

DON, CONV and DONV are ketoamino acids bearing a structural resemblance both to L-glutamine and L-asparagine (Fig. 1). Several investigators have shown that these agents are capable of inhibiting the utilization of L-glutamine by L-asparagine synthetase in various animal tumors [1–4]. In a companion paper [1], we have demonstrated that at a concentration of 1 mM, DON and CONV almost completely inhibited the utilization *in vitro* of L-glutamine by partially purified L-asparagine synthetase of L5178Y/AR and of mouse pancreas, while DONV inhibited both enzymes by only 50 per cent. To gain insight into the effect of these agents *in vivo*, we undertook the study of DON, CONV and DONV as inhibitors of L-asparagine synthetase in the intact organism, in expectation that effective inhibition of this enzyme might convert L-asparaginase-resistant tumors to the sensitive state [5].

The pattern of inhibition of L-asparagine synthetase from L5178Y/AR or mouse pancreas by intraperitoneally administered DON, CONV and DONV, *in vivo*, was not altogether consonant with the pattern

Table 7. Measurement of incorporation of [^{14}C]formate into nucleic acids after a single dose of saline, DON, CONV or DONV*

Group	Organs	Per cent inhibition of incorporation of: Adenine	Guanine
DON (i.p.)	Spleen	97†	99†
	Pancreas	99†	84†
	Small intestine	95†	95†
DONV (i.p.)	Spleen	0	30
	Pancreas	29	0
	Small intestine	0	29
CONV (i.p.)	Spleen	98†	97†
	Pancreas	98†	83†
	Small intestine	94†	93†
CONV (i.v.)	Spleen	68†	71†
	Pancreas	84†	62†
	Small intestine	95†	82†

* BDF_1 mice were treated with 100 mg/kg of the drugs listed. One hr later, 10 μCi [^{14}C]formate was injected intravenously. One hr later, the organs were removed and treated as described.

† $P \leq 0.05$.

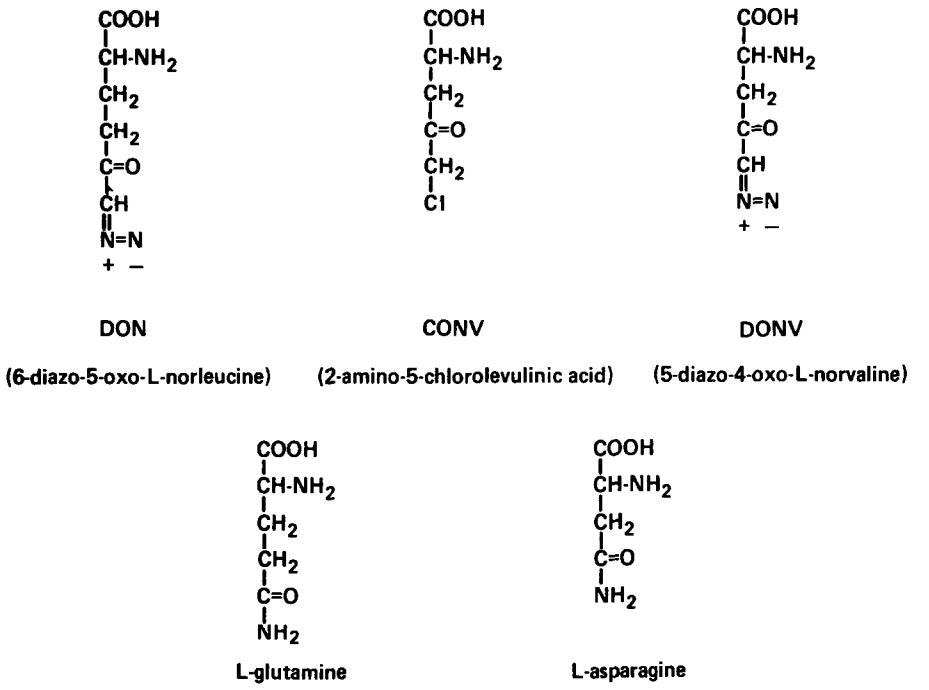

Fig. 1. Chemical structures of DON, CONV, DONV, L-glutamine and L-asparagine.

seen *in vitro*. Thus, although DON inhibited the utilization of L-glutamine by the tumoral enzyme to an equivalent degree *in vivo* and *in vitro*, CONV and DONV were generally feeble inhibitors of this enzyme in the intact organism. Also, whereas DON and CONV were potent inhibitors of the utilization of L-glutamine by L-asparagine synthetase of mouse pancreas *in vitro* and *in vivo*, DONV was active only *in vitro*.

The discrepancy between the degree of inhibition of the pancreatic enzyme and the tumoral enzyme by CONV might be related to the vascular supply of these two organs. It may be speculated that CONV gains access to the entire pancreas by virtue of that organ's plentiful and homogeneous vasculature, but is excluded from the interior of the tumor by virtue of the poor blood supply to that region. On similar grounds one might explain the early lag in inhibition of the tumoral enzyme by DON in contrast to the rapid, total inhibition observed in the case of the pancreatic enzyme. Only with DONV did the route of administration affect the pattern of inhibition observed: thus, intravenous injection of DONV was required to demonstrate its rather strong but transitory effect on the L-asparagine synthetase of pancreas.

The reduction in pancreatic and tumoral L-asparagine pools observed in animals treated with DON and CONV, but not in animals treated with DONV, supports the observation of significant inhibition *in vivo* of L-asparagine synthetase by the former two agents (Table 3). That DON alone elevated the organ pool size of L-glutamine might reflect stronger inhibition of amide transfer *in vivo* by DON as opposed to CONV or DONV [1]. The mechanism by which DON alone, of the three agents examined in the present work, produced such a striking increase in the free pool of L-threonine and L-serine in the pancreas of L5178Y tumor-bearing mice is obscure. Skipper and Thompson [14] reported that DON given to mice on a diet deficient in L-threonine produced tumor regression far greater that that seen when mice on a normal diet were given the drug. Also discernible in those studies was the tendency of DON to produce greater lethal toxicity in L-threonine-deficient mice than in their normal-diet counterparts.

The observation that these agents altered the organ pools of free amino acids prompted our consideration of their ability to affect the incorporation of these amino acids into proteins. CONV, alone of the three, demonstrated persistent inhibition of the incorporation of L-asparagine into tumoral and pancreatic proteins, *in vivo*. Similarly, in experiments with intact tumor cells, CONV was the most potent inhibitor of the incorporation of both L[U-^{14}]asparagine and L[U-^{14}C]glutamine into cell proteins. That the effect on protein synthesis by CONV was not wholly dependent on its activity as an L-glutamine or L-asparagine analog was demonstrated by the inhibition of L[U-^{14}C]leucine incorporation into tumor-cell proteins in the presence of of pharmacologic concentrations of L-asparagine, L-glutamine or both. A possible mechanism for this effect on protein synthesis was further suggested by the CONV-induced inhibition of [^{14}C]formate incorporation into purines in the several mouse organs studied. Inhibition of purine biosynthesis would be expected to lead to reduced nucleic acid synthesis and ultimately to reduced protein synthesis. That this inhibition of purine biosynthesis, in turn, resulted from inhibition of several of the amidotransferases concerned with purine biosynthesis is suggested by the fact that the pattern of inhibition of [^{14}C]formate incorporation by the three ketoamino acids mirrors the observed effects *in vitro* on these enzymes [1].

In order to determine the basis for the discrepancy between the activities of these three drugs *in vitro*

and *in vivo*, and in the hope of further characterizing their biological activity, additional therapeutic, toxicologic and pharmacologic studies were undertaken in mice. The results of these experiments will be presented in the third paper [15] of the series.

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