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METABOLISM AND ACTION OF AMINO ACID ANALOG ANTI-CANCER AGENTS

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Abstract—The preclinical pharmacology, antitumor activity and toxicity of seven of the more important amino acid analogs, with antineoplastic activity, is discussed in this review. Three of these compounds are antagonists of L-glutamine: acivicin, DON and azaserine; and two are analogs of L-aspartic acid: PALA and L-alanosine. All five of these antimetabolites interrupt cellular nucleotide synthesis and thereby halt the formation of DNA and/or RNA in the tumor cell. The remaining two compounds, buthionine sulfoximine and difluoromethylornithine, are inhibitors of glutathione and polyamine synthesis, respectively, with limited intrinsic antitumor activity; however, because of their powerful biochemical actions and their low systemic toxicities, they are being evaluated as chemotherapeutic adjuncts to or modulators of other more toxic antineoplastic agents.

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1. INTRODUCTION

In quantitative terms, the most important function of amino acids is their role as the building blocks of proteins and peptides. It is, therefore, paradoxical that the majority of oncolytic amino acid antimetabolites do not interrupt protein synthesis or function in an important way; with few exceptions, too, they are not utilized in the assembly of proteins. Rather, these compounds achieve their antitumor effects by interfering with the transfer of nitrogen or sulfur in the course of intermediary metabolism. In the present chapter, seven such agents will be examined; namely azaserine, 6-diazo-5-oxonorleucine (DON), acivicin, L-alanosine, *N*-phosphonacetyl-L-aspartic acid (PALA), buthionine-*dl*-sulfoximine, and α-difluoromethylornithine. Obviously this list represents only a fraction of the amino acid antimetabolites reported in the scientific literature, but the agents selected are rather unique in that they have been studied—or are about to be studied—both in the laboratory and in the clinic. This feature permits a presentation of the preclinical pharmacologic properties of each antimetabolite—including what is known of its metabolism—followed by, and correlated with, its antitumor activity and toxicity in human subjects.

As a general rule the most potent amino acid antimetabolites are those which interrupt nucleic acid biosynthesis. In the assembly of the purine and pyrimidine rings, only three amino acids are required, namely, glycine, L-aspartic acid and L-glutamine. Antagonists of glycine are rare and not particularly noted for their antitumor activity. Potent oncolytic analogs of L-aspartic acid include L-alanosine and PALA; these will be discussed in a later section. Among the numerous L-glutamine antagonists reported in the literature, acivicin, DON and azaserine have been the most thoroughly investigated and will be treated first.

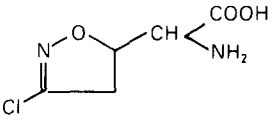
2. ANALOGS OF L-GLUTAMINE

L-Glutamine occupies an important role in intermediary nitrogen metabolism. By virtue of its amide-donating property, this amino acid regulates the production of a large number of indispensible metabolites, including several amino acids, nucleotides and complex polysaccharides. Similarly, analogs of L-glutamine with the ability to inhibit these biosynthetic reactions have assumed great importance as biochemical tools, and in cancer chemotherapy. Used *in vivo*, these agents dramatically reveal the pharmacological, toxicologic, and oncologic consequences of the interruption of L-glutamine utilization. As a group, the L-glutamine antagonists discussed in this section are believed to exert their antineoplastic effects principally by inhibiting one or more of the key enzymes in the pathways of purine and pyrimidine biosynthesis; each, however, has individuating characteristics.

2.1. ACIVICIN

2.1.1. *Mechanism of Action*

Acivicin (L-[αS,5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; NSC 163,501; Fig. 1) is a cyclic analog of L-glutamine identified in the fermentation broth of *Streptomyces sviceus* on the basis of its potent antitumor activity against L1210 leukemia *in vivo* (Hanka and Dietz, 1973; Hanka *et al.*, 1973). After isolation, purification and characterization, the structure of this unusual antibiotic agent was established as being: L-[αS,5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (Martin *et al.*, 1973; Jayaram *et al.*, 1975). Because of steric similarity to L-glutamine, but especially because L-glutamine antagonizes the cellular toxicity induced by acivicin (Neil *et al.*, 1979), the drug was labeled as an analog of that amide (Jayaram *et al.*, 1975). Indeed, both *in vivo* and *in vitro*, acivicin strongly inhibits a number of L-glutamine requiring enzymes, especially the rate-limiting enzymes of *de novo* purine (PRPP amidotransferase, FGAR amidotransferase and GMP synthetase) and pyrimidine (carbamoylphosphate synthetase II and CTP synthetase) synthesis



Acivicin

FIG. 1.

(Aoki *et al.*, 1982; Lui *et al.*, 1982; Weber *et al.*, 1982). In treated cells, such inhibitions translate into marked decreases in the CTP, GTP, dCTP, dGTP, and dTTP pools, with concomitant elevations of UTP; significant changes in the adenine ribonucleotides are not ordinarily seen.

Acivicin treatment brings about pronounced increases in the PRPP pools of the P388 leukemia, colon adenocarcinoma (Ardalan *et al.*, 1982), and Lewis lung carcinoma (Kensler *et al.*, 1982); additionally the drug is a potent inhibitor of L-asparagine synthetase both *in vitro* and *in vivo* (Cooney *et al.*, 1974a), of gamma-glutamyl transpeptidase (Allen *et al.*, 1980, 1981; Reed *et al.*, 1980), and of several other amidotransferases (Jayaram *et al.*, 1975). Although it inhibits the majority of the known L-glutamine-requiring reactions, the target enzymes responsible for its growth inhibition are thought to be CTP and GMP synthetase (Kensler *et al.*, 1982; Lui *et al.*, 1982). This contention is supported by the fact that the nucleosides, cytidine and guanosine, are each capable of reversing the antiproliferative effects of acivicin (Neil *et al.*, 1979; Lui *et al.*, 1982); moreover, the drug-induced inhibition of CPS II does not correlate especially well with its antitumor activity (Kensler *et al.*, 1982).

Not only is acivicin a competitive inhibitor of many L-glutamine-requiring enzymes (Jayaram *et al.*, 1975), it also directly inactivates these target proteins by reversible binding to the L-glutamine site, followed by alkylation of this site, probably at an L-cysteinyl residue (Tso *et al.*, 1980; Allen *et al.*, 1981). *In vivo*, acivicin causes a rapid inactivation of the PRPP amidotransferase activity of hepatoma 3924A, whereas host liver enzyme activity is only minimally affected (Prajda, 1985). This greater sensitivity of the neoplastic versus normal liver has been attributed to a ten-fold higher content of L-glutamine in the latter (0.5 mM in the hepatoma versus 5.0 mM in liver).

2.1.2. *Preclinical Pharmacology and Antitumor Activity*

Acivicin exhibits a broad spectrum of antitumor activity. The drug is effective against the L1210 and P388 leukemias (Houchens *et al.*, 1979), as well as the mammary (MX1) and lung (LX1) human tumor xenografts implanted in nude mice, but inactive against the colon xenograft (CX1) and the B16 melanoma (Houchens *et al.*, 1979; Poster *et al.*, 1981). Effective oncolytic activity was observed by the intraperitoneal, subcutaneous and even oral routes of administration, but was highly schedule-dependent. In the L1210 leukemia, intraperitoneal treatment with acivicin proved to be effective on every schedule tested. Inasmuch as intraperitoneal treatment of rats with acivicin depresses the activity of CTP synthetase and GMP synthetase in the brain, thereby decreasing the concentrations of cytidine and guanosine triphosphates (Achleitner *et al.*, 1985), and significantly increases the life-span of animals bearing responsive intracranially implanted tumors (Houchens *et al.*, 1979), it can be concluded that the drug can effectively cross the blood brain barrier.

2.1.3. *Preclinical Toxicology*

It is of interest that acivicin is more toxic to female than male mice, and also is more toxic to younger than older mice (Neil *et al.*, 1979). This differential toxicity was found to be related to a lower renal clearance of the drug, as was also evident from a prolonged plasma half-life in the more susceptible groups (Neil *et al.*, 1979). However, such sex- and age-related toxicity was not observed in monkeys and dogs, the predominant toxicity in these species being gastrointestinal (Poster *et al.*, 1981).

2.1.4. *Resistance to Acivicin*

As was mentioned, the B16 murine melanoma and the CX1 human colon carcinoma xenograft are naturally resistant to acivicin, whereas the P388 leukemia is sensitive to the drug. However, by exposing tumor-bearing mice to sub-optimal doses of acivicin (5.4 mg/kg, days 1–9) over 30 transplant generations, H. N. Jayaram of this laboratory developed a variant, P388/AC, which had entirely lost responsiveness to the drug. Although DNA synthesis was inhibited equally by acivicin in the native and resistant tumors, the V_{max} for transport of the drug was ten times higher in the parental strain (Jayaram *et al.*, 1985). Since the affinity of the activicin transporter was comparably low in both P388 and P388/AC (K_ms of 700 μM), and since therapeutic doses of the drug generally produce peak extracellular concentrations well below this value, it seems likely that the defect in acivicin transport would be amplified under pharmacologic conditions *in vivo* in the resistant line, providing a plausible basis for its resistance. Since we have found that acivicin and L-glutamine compete with one another for transport into murine lymphoblasts, it is also plausible to suggest that an elevated extracellular concentration of L-glutamine would foster resistance to the drug. Although expansion of the intracellular pool of that amide could also, in theory, lead to a refractory state by preventing or reversing binding of acivicin to the catalytic centers of the enzymes which it is known to inactivate, Jayaram and colleagues demonstrated that this mechanism was not operative in P388/AC, the concentration of L-glutamine being 425 μM in the parent P388 and 319 μM in the resistant variant (Jayaram *et al.*, 1985).

Amplification of the target amidotransferases was also examined in extracts of P388/AC. Only one such enzyme exhibited a significant increase in its specific activity: FGAR amidotransferase. Thus, in the parental line, 0.67 nmol of formylglycinamide ribonucleotide were amidated per mg protein per hour as compared to 1.8 nmol per mg protein per hour in the resistant leukemia. This result reinforces a contention to be made later in connection with the two other prototypical L-glutamine antagonists, DON and azaserine, that FGAR amidotransferase is the principal biochemical target for the class of antimetabolite. It should be cautioned, however, that subsidiary targets nearly always also exist.

TABLE 1. *Phase II Clinical Trials of Acivicin*

Schedule	I.V. Dose ($mg/M^2/d$)	Disease	Ev. pts*	Responses CR	Responses PR	References
d × 5 Bolus	12–15	Breast	22	0	0	Fleishman *et al.*, 1983
	12–15	Breast	15	0	1	Booth *et al.*, 1986
	12–21	Colorectal	33	0	1	Adolphson *et al.*, 1986
	15–26	Colorectal	17	0	0	Maroun *et al.*, 1984
	12–15	Glioma	13	0	3	Taylor and Eyre *et al.*, 1987
	12–21	Lung	33	0	2	Maroun *et al.*, 1986
	12	Lung	73	0	3	Kramer *et al.*, 1986
	12	Lung (SCLC)	7	0	0	Kramer *et al.*, 1986
	12–15	Ovarian	23	0	1	McGuire *et al.*, 1986
72 hr CI†	20–25	Breast	25	0	1	Willson *et al.*, 1986b
	20–25	Colorectal	44	1	3	Earhart *et al.*, 1987
	20–25	Colorectal	23	0	0	Eisenhauer *et al.*, 1987
	20	Mesothelioma	21	0	0	Falkson *et al.*, 1987
		Total:	349	1	15	(4.5%)

*Evaluable patients.

†CI: Continuous infusion.

Abbreviations used: bid, twice daily; qod, every other day; CR, complete response; MR, minor response; PR, partial response; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia.

2.1.5. *Metabolism of Acivicin*

It is generally agreed that the metabolism of acivicin is minor in extent—apart from its alkylation of target amidotransferase. When murine lymphoblasts are incubated with 10 μM acivicin tritiated on the α-carbon, only 10.2 ± 0.05 pmol of $[^3H]_2O$ are generated per million cells per hour (Jayaram *et al.*, 1985). This result can be interpreted to indicate that acivicin, in its capacity as a cyclic amino acid, is relatively refractory to transamination. Studies of the fate of the chlorine atom of acivicin and the metabolic stability of its ring system have not, so far, been reported.

2.1.6. *Clinical Studies*

Phase I evaluation of acivicin was initiated in 1979 (O'Dwyer *et al.*, 1984). Clinical trials using schedules of single and multiple bolus intravenous injections, and one- to five-day continuous infusions (CI) produced neurotoxicity, myelosuppression, nausea and vomiting, diarrhea, stomatitis and mild hepatotoxicity (Taylor *et al.*, 1984; McGovren *et al.*, 1985; Murphy *et al.*, 1982; Sridhar *et al.*, 1983; Earhart *et al.*, 1983; Weiss *et al.*, 1982c). Dose-limiting toxicity varied according to schedule: the single bolus and continuous infusion schedules were limited by neurotoxicity, and the multiple bolus (daily × 5) schedule was limited by myelosuppression. Pharmacokinetic studies, performed in conjunction with the Phase I trials, revealed a terminal $t_{1/2}$ of 7–11 hr (Weiss *et al.*, 1982c; Earhart *et al.*, 1983; McGovren *et al.*, 1985). Clearance was predominantly non-renal. In two studies, either peak plasma levels (Weiss *et al.*, 1982c) or plasma levels above 0.9 μg/ml for over 16 hr (Earhart *et al.*, 1983) were found to correlate with the development of neurotoxicity. The daily × 5 bolus and the 72 hr continuous infusion schedules were considered the most tolerable, and for this reason were brought into Phase II screening.

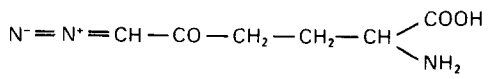
Results of single agent Phase II trials are presented in Table 1. Minimal or no activity was observed on either schedule in a variety of diseases. However, in high grade gliomas (Taylor *et al.*, 1987) acivicin has shown some promise of activity, so that confirmatory trials are planned.

In addition to the single agent trials, three combination Phase I studies have been, or are being conducted. The agents which are being tested in combination with acivicin include: (1) the nucleoside transport inhibitor, dipyridamole (Fischer *et al.*, 1986; Willson *et al.*, 1986a); (2) the amino acid solution aminosyn; and (3) cisplatin (Maroun *et al.*, 1987).

2.2. L-DON

2.2.1. *Mechanism of Action*

The keto amino acid, L-DON (6-diazo-5-oxo-L-norleucine; NSC 7365; Fig. 2), is an antitumor antibiotic also isolated from *Streptomyces* (Dion *et al.*, 1956). Because of its similarities to L-glutamine (Dion *et al.*, 1956) and because DON inhibits a number of biochemical reactions requiring L-glutamine, it is considered to be one of the prototypical antagonists of that amino acid. In mammals, the enzymatic reaction most sensitive to DON is usually considered to be the conversion of formylglycinamide ribonucleotide to formylglycinamidine ribonucleotide. Thus DON inhibits nucleic acid synthesis by limiting the biosynthesis of purines at an intermediate step (Levenberg *et al.*, 1957; Rosenbluth *et al.*, 1976), as adjudged by its repression of the flux of $[^{14}C]$



DON

FIG. 2.

formate into nucleic acid purines, an effect enhanced by its inhibition of the other two amidotransferases involved in purine biosynthesis. The specific mechanism by which DON inhibits L-glutamine utilizing enzymes is via irreversible alkylation of susceptible L-cysteinyl residues, a mechanism it shares with acivicin (Pinkus and Meister, 1972). Protein synthesis, in general, is also inhibited by the drug (Rosenbluth *et al.*, 1976); one explanation for this effect is provided by the finding that DON inhibits the utilization of L-glutamine by L-asparagine synthetase both *in vivo* (Rosenbluth *et al.*, 1976) and *in vitro* (Jayaram *et al.*, 1976; Livingston *et al.*, 1970; Chou and Handschumacher, 1972; Prusiner and Stadtman, 1973) in several experimental animal tumors. At a dose of 100 mg/kg, DON significantly increases the concentration of L-glutamine and decreases that of L-asparagine in the tumor (L5178Y/AR) and pancreas of treated mice (Rosenbluth *et al.*, 1976). Moreover, the drug inhibits the biosynthesis of D-glucosamine-6-phosphate (Ellis and Sommar, 1972), glycosaminoglycans and nicotinamide adenine dinucleotide (Barclay and Phillips, 1966). In a Chinese hamster cell line (V79.5), DON specifically inhibits GMP synthetase, resulting in a reduction of intracellular GTP pools in the wild-type, but not in drug-resistant variant cell line (*vide infra*). Drug-induced cytotoxicity can be reversed by L-glutamine and guanine, whereas adenine and hypoxanthine are relatively ineffective as antidotes.

2.2.2. *Preclinical Pharmacology and Antitumor Activity*

DON has antimicrobial (Ehrlich *et al.*, 1956), amoebicidal (Nakamura, 1956), and antitumor (Sugiura *et al.*, 1958) activity. The antitumor effects alluded to earlier were investigated in detail by the Drug Screening Program of the National Cancer Institute. DON is active against the intraperitoneally implanted L1210 leukemia in mice, producing greater than 50% increase in life span (ILS) over a dose range of 2.5 to 40 mg/kg when given by an intermittent treatment schedule (Q4D × 3). Doses of 80 mg/kg result in lethality. In addition to its activity against the leukemias (L1210 and P388), DON given weekly (Q7D × 3 or 4) at 25–50 mg/kg showed remarkable activity in reducing the tumor burden of mice bearing the subcutaneously implanted CD8F1 mammary tumor and the colon 38 carcinoma; at a dose of 12.5 mg/kg (Q4D × 3) DON was also effective against the colon 26 carcinoma implanted intraperitoneally, producing a 70% increase in life span; no activity was demonstrable against the intraperitoneally implanted B16 melanoma nor the intravenously implanted Lewis lung carcinoma. In human tumor xenografts implanted in nude mice, there were 4/4 partial regressions for Lung (LX1), and 3/4 minimal regressions for mammary (MX1) tumors. However, colon (CX1) was refractory with doses of either 25 mg/kg (for CX1 and LX1) or 50 mg/kg (for MX1) on a schedule of Q4D × 3. Since DON inhibits the synthesis of L-asparagine, attempts were made to determine whether the drug would exert therapeutic synergism with L-asparaginase: in fact, such synergism was observed with the combination, in the Ehrlich ascites test system; the B16 melanoma and Walker adenocarcinoma were also responsive to this two-drug combination (Tarnowski *et al.*, 1970).

2.2.3. *Preclinical Toxicology*

Azotomycin, a tripeptide containing two moles of DON, behaves as an effective prodrug which undergoes cleavage *in vitro* and *in vivo* to yield the proximate antimetabolite, DON (Cooney *et al.*, 1974b). Since its toxicity has been reported more extensively than DON's, data on its deleterious effects will be substituted for—but should provisionally be viewed as equivalent to—those produced by DON (Cooney *et al.*, 1974b).

Toxicologic studies of azotomycin in mice showed that, although animals can tolerate relatively large single doses of the drug, (LD_{50} = 300–400 mg/kg), very small, repeated doses are lethal (LD_{50} < 1.0 mg/kg, daily × 5). Furthermore, L-asparaginase augments the toxicity of the latter regimen but not the former (Cooney *et al.*, 1974b). Mice receiving single lethal doses of azotomycin (>400 mg/kg) exhibited ataxia and tonic–clonic convulsions from 4–24 hr after drug administration. Similar CNS toxicity is observed when cerebral synthesis of L-glutamine is inhibited by L-methionine-*dl*-sulfoximine (Lamar and Sellinger, 1965). Subcutaneous doses of L-glutamine, 1 g/kg, appear to antagonize the convulsive action of DON; however, neurotoxicity in the form of ataxia was still present. Convulsant doses of azotomycin significantly inhibited the synthesis of L-asparagine in the brain. Although L-asparagine has no known neurotransmitter function, it is possible that a significant diminution in its concentration would prevent normal neuronal protein synthesis. Brain, liver, intestine and spleen are the organs principally damaged by azotomycin (Cooney *et al.*, 1974b). One salient manifestation of the drug's toxicity is a profuse diarrhea which begins about 18 hr after large single intravenous doses and persists until death.

2.2.4. *Resistance to DON*

Transplantable tumors natively resistant to DON are, in general, also resistant to acivicin; the B16 melanoma, Lewis lung carcinoma and CX1 xenograft are cases in point; but it has not yet been determined whether, for example, the P388/AC line described earlier is, in fact, cross-resistant to DON.

The mechanisms producing resistance to DON have not been investigated extensively, but the same general attributes which render tumors refractory to acivicin might also be expected to apply in the case of DON, viz. enlargement of intracellular or extracellular pools of L-glutamine, and overproduction or altered responsiveness of the target amidotransferases. Kaufman (1985) has, in fact, documented the last-mentioned mechanism in V79.5 Chinese hamster cells selected for resistance to DON. A mutant cell line, called don801, was isolated from a wild-type population of V79.5 Chinese hamster cells by its ability to grow in the presence of 10 μM DON, a concentration which is lethal for V79.5 cells. The don801 cells were found not to be cross-resistant to

another L-glutamine analog, azaserine. It was shown that guanine but neither hypoxanthine nor adenine protected V79.5 cells from the toxic effects of DON, while hypoxanthine and adenine, but not guanine protected them against azaserine toxicity. Exposure of wild-type cells to DON was shown to result in a specific reduction of intracellular GTP pools, while in the mutant cells there was no effect on GTP levels. These results strongly suggested that DON was specifically inhibiting guanylate synthetase (GMP synthetase; xanthosine-5′-phosphate: L-glutamine amidoligase, EC 6.3.5.2) in V79.5 cells and that the enzyme in don801 cells was resistant to inhibition. *In vitro* assays of GMP synthetase activities from V79.5 and don801 cells confirmed this hypothesis. The mutant phenotype was also found to be dominant in intraspecific cell hybrids.

Unlike the case with acivicin, impaired transport of DON does not appear to be a basis for the resistance of mutant P388 cells to the drug. That a separate carrier mediates entry of DON and acivicin was confirmed by the finding that a P388 variant resistant to acivicin, and defective in acivicin uptake (cf. supra) interiorized DON normally (Huber *et al.*, 1988).

2.2.5. *Metabolism of DON*

It is well known that the diazoketone functionality of DON is chemically labile, especially under acidic conditions. By contrast, DON exhibits rather notable metabolic stability. For example, a 4 hr incubation of DON in an extract of liver produced virtually no (3%) decomposition as adjudged by microbiological assay, and an extract of kidney destroyed only 50% of the drug (Cooney *et al.*, 1976). Moreover, Huber *et al.* (1988) could demonstrate no metabolism whatsoever of DON in P388 lymphoblast/macrophages. Despite such apparent stability, it is known that the α-amino group of DON is, in fact, susceptible to transamination both by L-glutamate oxaloacetate transaminase and L-glutamate pyruvate transaminase at the rates of 225 nmol/min/mg and 202 nmol/min/mg respectively, with α-ketoglutaric acid as partner. It is not known whether the resultant α-keto acid is capable of being reduced in a reaction analogous to that catalyzed by malate dehydrogenase on oxaloacetate, but it might be expected to experience an enhanced chemical or photochemical lability by virtue of its double oxo functionalities (however, see footnote on p. 250). The most obvious chemical fate of such a metabolite would be α-decarboxylation and loss of the diazo functionality.

Although it is known that DON can inhibit protein synthesis to an important degree (Rosenbluth *et al.*, 1976), it is still uncertain if this reactive amino acid can be incorporated into protein as a fraudulent building block, despite the fact that the compound has been under study for over 30 years. With the availability of a good quality [^{14}C]-labeled drug, this point may become susceptible to direct examination.

One other metabolic reaction experienced by DON merits mention. The drug is a substrate for γ-glutamyl transpeptidase, an enzymic component of the γ-glutamyl cycle which is believed to be responsible for the active transport of select amino acids. The fact that L-glutamine is also a substrate for this enzyme might explain the finding that this amide competitively inhibits the uptake of DON by murine lymphoblasts (Cooney *et al.*, 1976).

2.2.6. *Clinical Studies*

DON originally entered the clinic three decades ago based on its novel structure and its spectrum of activity against an impressive number of murine tumors. It was also later shown to repress the growth of human lung and mammary xenografts (Catane *et al.*, 1979). In man, DON usually has been administered on a daily schedule, with both oral and parenteral routes being employed (Table 2). Mucositis and gastrointestinal toxicity characterized by nausea, vomiting and diarrhea were prominent side effects, and myelosuppression was also observed. DON showed hints of activity in breast, lung and gastrointestinal carcinomas. Li reported that three or four patients with metastatic testicular carcinoma responded to DON (Li, 1961). When the activity of 6-mercaptopurine (6-MP) plus DON was compared with 6-MP alone in patients with leukemia (Sullivan *et al.*, 1962), the combination was associated with increased toxicity without a major improvement in response rate. Clinical interest in this agent waned with the advent of more effective and better tolerated drugs such as methotrexate.

Interest was rekindled two decades later when responses in some colorectal cancer patients were seen with azotomycin (which, as was said, acts as a prodrug of DON); this tripeptide was given alone and in combination with 5-fluorouracil (5-FU) (Catane *et al.*, 1979; Kisner *et al.*, 1980).

Based on the interesting preclinical activity of DON, new Phase I trials were instituted with this amino acid based on the experience gained with

TABLE 2. *Early Clinical Experience with DON*

Dose (mg/kg)	Schedule	Disease	Ev. pts	Clinical response	References
0.1–1.1	p.o. qd	Pooled results:	47	7	Magill *et al.*, 1957
0.2–0.6	i.m. or i.v. qd	Solid tumor	42	6	
1.6	i.v. q 4 d	Leukemia/lymphoma	5	1	
0.2	p.o. qd × 30	Pooled results:	41	2	Veterans Admin. Cancer
		Solid tumor	23	0	Chemotherapy Study Group,
		Lymphoma	18	2	1959
0.2–0.3	p.o. qd	Testicular	4	3	Li, 1961
0.25 + 6-MP, 2.5	p.o. qd	Leukemia	94	63 CR	Sullivan *et al.*, 1962

azotomycin, but using shorter courses with higher individual doses (Table 2). Initial pharmacokinetic methodology involved a non-specific microbiological assay, but Powis and Ames (1980) reported a specific and sensitive reverse-phase HPLC method for the analysis of DON. Plasma elimination showed dose-dependency, with the $t_{1/2}$s ranging from 76–230 min (Kovach *et al.*, 1981). At doses of 300–450 mg/m^2, the plasma clearance (Cl_p) was 3.4–3.6 ml/min/kg, and the volume of distribution (V_d) was 0.45–0.77 L/kg (Kovach *et al.*, 1981). When [^{14}C]-DON was given to patients, 90% of the radiolabel was recovered in the urine by 72 hr (Rahman *et al.*, 1981); parent drug, however, was not detectable in the urine, a result which indicates extensive metabolism or chemical decomposition (Kovach *et al.*, 1981). In pediatric patients, there was no evidence of dose-dependent pharmacokinetics; the $t_{1/2}$ was 161 ± 47 min, the Cl_p was 6.8 ± 2.4 ml/min/kg, and the V_d was 1.2 ± 0.45 L/kg (Sullivan *et al.*, 1988).

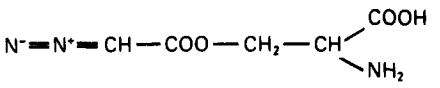
Prolonged, severe nausea and vomiting proved to be dose-limiting in adult patients on all schedules. Other toxicities included mucositis, leukopenia and thrombocytopenia. Sullivan *et al.* (1988) recently reported the results of a Phase I trial in children. Premedication with chlorpromazine markedly reduced nausea and vomiting, permitting dose escalation to achieve myelosuppression as the limiting toxicity. No complete or partial responses were seen, although minor responses were observed in half the patients.

Several Phase II trials in solid tumors have also been performed (Table 3). No meaningful activity was observed in adult patients using these more toxic regimens, and further clinical investigation in this area has been halted.

2.3. Azaserine

2.3.1. *Mechanism of Action*

The antibiotic azaserine (*O*-diazoacetyl-L-serine; NSC 742; Fig. 3) was isolated from *Streptomyces fragilis* and characterized in 1954 (Fusari *et al.*, 1954a,b). In common with the two L-glutamine antagonists discussed earlier, the cytotoxic effects of azaserine are believed to be due, in large measure,



Azaserine

FIG. 3.

to inhibition of *de novo* purine synthesis (Bennett *et al.*, 1956; Fernandes *et al.*, 1956; Levenberg *et al.*, 1957; Baker, 1959), specifically, at the level of the conversion of formylglycinamide ribotide (FGAR) to formylglycinamidine ribotide (FGAM). A small number of other L-glutamine requiring reactions are also inhibited (Bentley and Abrams, 1956; Goldthwait, 1956; Kammen and Hurlbert, 1959; Narrod *et al.*, 1959). In experiments using [^{14}C]-labeled formate as a tracer, the drug was found to inhibit purine and nucleic acid biosynthesis in the Ehrlich ascites carcinoma (Henderson *et al.*, 1957; Moore and LePage, 1957), sarcoma 180 (Bennett *et al.*, 1956; Henderson *et al.*, 1957), as well as in a number of normal mouse tissues (Moore and LePage, 1957); L-glutamine partially prevents, but cannot reverse this effect (Greenlees and LePage, 1956). In addition, treatment with azaserine results in a marked reduction of NAD and NADP levels in mouse (Narrod *et al.*, 1959, 1960) and rat liver (Slater and Sawyer, 1966), possibly as a consequence of depleted adenine pools. Although the principal action of azaserine is inhibition of the L-glutamine-dependent conversion of FGAR to FGAM, which leads, in turn, to a dose-dependent reduction of purine nucleotides, addition of preformed purines to the culture medium does not reverse the drug's cytotoxicity (Vandevoorda *et al.*, 1964).

2.3.2. *Preclinical Pharmacology and Antitumor Activity*

In animal tumor models, azaserine is most effective against the Ehrlich ascites carcinoma (Sugiura, 1955; Fernandes *et al.*, 1956; Sassenrath, 1958; Sassenrath *et al.*, 1958), and a number of lymphosarcomas implanted in mice (Sugiura and Stock, 1955; Sassenrath, 1958; Sassenrath *et al.*, 1958); it is also

TABLE 3. *Second Generation Phase I and II Clinical Trials with DON*

Dose (mg/m^2)	IV schedule	Disease	Ev. pts	Activity CR	Activity PR	References
Phase I trials						
100–500	biw × 3 wk	Solid tumor	26	0	0	Sklaroff *et al.*, 1980
300–550	d × 1 q 21 d	Solid tumor	26	0	0	Kovach *et al.*, 1981
50–200	d × 3 q 21 d	Solid tumor	26	0	0	Kovach *et al.*, 1981
7.5–97.5	d × 5 q 21 d	Solid tumor	25	0	0	Earhart *et al.*, 1982
50–600	CI × 24 h q 21 d	Solid tumor	25	0	0	Rahman *et al.*, 1981
150–520	biw × 4 wk	Leukemia	9	0	0	Sullivan *et al.*, 1988
150–520	biw × 4 wk	Solid tumor	8	0	0	Sullivan *et al.*, 1988
Phase II trials						
160	d × 3 q 21 q	Colon	30	0	0	Lynch *et al.*, 1982
300	biw × 2 wk	Colon	14	0	1	Rubin *et al.*, 1983b
160	d × 3 q 21 d	Lung	22	0	0	Eagan *et al.*, 1982
50	d × 5 q 21 d	Sarcoma, mesothelioma	36	0	0	Borden *et al.*, 1986

moderately active against several murine sarcomas (Stock *et al.*, 1954; Sugiura, 1955; Clarke *et al.*, 1957), and carcinomas; for example, the Novikoff hepatoma treated over a dose range of 1.0–6.0 mg/kg/day (QID × 7), underwent 89–100% growth-inhibition by day 8 of therapy. Significant inhibition was also observed in a spontaneous mammary carcinoma model, but only at toxic doses (Scholler *et al.*, 1955; Tarnowski, 1955; Tarnowski and Stock, 1955). Combination therapy of mouse tumors revealed that azaserine and 6-thioguanine (6-TG) induced additive therapeutic effects against the Ehrlich ascites carcinoma, Sarcoma 180 ascites and the TA3 ascites carcinoma, with 10/20, 12/20, and 9/39 cures respectively, despite the fact that neither agent was effective alone (Sartorelli and LePage, 1958). Other studies have also supported this synergism. Combination with 6-mercaptopurine produced 45/60 complete regressions in an adenocarcinoma model versus 2/60 and 6/60 for azaserine and 6-mercaptopurine alone, respectively (Tarnowski and Stock, 1957).

2.3.3. *Preclinical Toxicology*

The single dose LD_{50} of azaserine in mice is 100–150 mg/kg after intraperitoneal or peroral administration respectively (Sternberg and Phillips, 1957); death usually occurs 2–5 days after the treatment. When the drug is given intraperitoneally on a 5-day schedule, the LD_{50} drops to 10–40 mg/kg. Similar LD_{50} values are observed in rats, but dogs seem to be more sensitive to the drug than rodents; thus, at a dose of 10 mg/kg, given for 6–8 doses, an LD_{100} was reached in 6–13 days (Sternberg and Phillips, 1957).

Drug-induced toxicity in rats and mice includes damage to the pancreas, liver, kidney, bone marrow and intestinal epithelium; in dogs, toxicity is confined mainly to the gastric and intestinal mucosa (Sternberg and Phillips, 1957). In addition to the above dose-limiting toxicities, azaserine is known to be carcinogenic in the rat, with the pancreas being especially susceptible. Reports (Lhoste *et al.*, 1987a,b) have shown that steroid sex hormones are involved in the induction of this effect. In hamster and rat acinar cell cocultures, azaserine is also capable of producing transformation (Schaeffer *et al.*, 1987) and is a direct-acting mutagen in microbial test system (Longnecker *et al.*, 1974).

2.3.4. *Metabolism of Azaserine*

It is likely that three separate types of enzyme can initiate the metabolic decomposition of L-azaserine: L-amino acid oxidase, one or more transaminases, and one or more enzymes catalyzing α,β eliminations.* That the last mentioned reaction is the preponderant activity is shown by studies in which no oxygen or α-keto acid is added: under these conditions, vigorous decomposition of azaserine is still demonstrable. Mouse liver is the most active source of this activity (>8 mol of azaserine degraded per hour) but canine salivary gland (an organ heavily damaged by parenteral azaserine) is also energetic with 3.9 mol of drug decomposed/hr (Rosenkrantz *et al.*, 1972). The products of this decomposition, identified by enzymatic and colorimetric methods, proved to appear in close to stoichiometric amounts. They were ammonia, pyruvate (or hydroxypyruvate), and glycolic acid; the nitrogen gas which was presumed to arise from the diazo end of the molecule was not identified. It is of interest that 0.025 M ammonium sulfide totally blocks the decomposition of azaserine catalyzed by mouse liver whereas equimolar cyanide is without effect.

The decomposition of azaserine via an α,β elimination presumably is a consequence of its structural homology to several β esters of L-serine; these are attacked simultaneously on carbons 2 and 3 by a pyridoxal enzyme catalyzing a concerted α,β elimination. The resulting amino acrylate intermediate would then spontaneously decompose to ammonia, pyruvate, and diazoacetate, which in turn would be hydrolyzed to yield nitrogen gas and glycolic acid.

Lastly, it should be pointed out that azaserine, like DON, is a good substrate for γ-glutamyl transpeptidase (Perantoni *et al.*, 1979). Indeed, this transferase has been suggested to mediate transport of the drug. A positive correlation was observed between cellular γ-glutamyl transpeptidase levels and sensitivity to azaserine. Moreover, azaserine-resistant strains exhibited generally reduced activities of enzyme (Perantoni *et al.*, 1979).

2.3.5. *Clinical Studies*

In 1954, Ellison *et al.* tested a crude filtrate of the culture broth of *Streptomyces fragilis* (which, as was said, had antitumor activity in preclinical models) in 51 patients prior to isolation of the active principle, azaserine. Eight minor responses of brief duration were noted in 37 patients with leukemia, while no objective responses were seen in 14 patients with solid tumors. Clinical trials with purified azaserine were conducted under the sponsorship of the Division of Cancer Treatment (DCT), National Cancer Institute (NCI) from 1964 to 1975. The drug was administered orally as a single agent at doses of 3–12 mg/kg daily for 5 to 20 days. Holland *et al.* (1967) reported

*While this chapter was being prepared, we resolved to reinvestigate the metabolism of azaserine using the techniques originally deployed for this purpose (mainly paper chromatography), as well as a more up to date system. These studies, while by no means exhaustive, established: that fresh, centrifugally clarified extracts of liver quantitatively decomposed azaserine to a pyruvate-like material readily reducible by LDH in the presence of NADH, with concomitant loss of all UV (diazo) absorbance; that GOT did not appear capable of transaminating the drug; that L-amino acid oxidase attacked L-azaserine readily, generating a ninhydrin-negative product with full retention of UV absorption; this product was readily separated from azaserine itself on C-18 columns using ion-pairing chromatography with tetrabutylammonium phosphate and an acetonitrile gradient, or on paper (Whatman 3MM, ascending, using alcohol/1 M ammonium acetate, 700:300, as solvent); that the aforementioned diketo product lost all UV absorption on illumination for 20 sec with 254 nm UV light—as did azaserine—and, that commerical (Sigma) γ-glutamyl transpeptidase did accept azaserine as substrate but at very sluggish velocity.

TABLE 4. *Clinical Trials of Azaserine in Combination Regimens*

Dose/schedule azaserine*	Dose/schedule second agent	Disease	No. responders/ total no. pts	References
0.2 mg/kg p.o. bid	6-TG 1 mg/kg/d p.o.	Myeloma	2 of 11 MR	Hayes *et al.*, 1967
16 mg/m^2/d × 42 p.o.	6-TG 40 mg/m^2/d × 42 p.o.	CML-blast crisis	2 of 18	Hayes *et al.*, 1974
1.7 mg/kg/d × 5–7 i.v. bid	6-TG 1.7 mg/kg/d × 5–7 i.v. bid	Solid tumor	6 of 16 MR	Schroeder *et al.*, 1964
2.5 mg/kg/d p.o.	2.5 mg/kg/d p.o.	Acute leukemia (no prior Rx)	20 of 29 CR	Burchenal *et al.*, 1955
2.5 mg/kg/d p.o.	6-MP 2.5 mg/kg/d p.o.	Acute leukemia (no prior Rx) (prior 6-MP)	24 of 39 CR (18/25 CR) (6/14 CR)	Burchenal *et al.*, 1956
2.5 mg/kg/d p.o.	6-MP 2.5 mg/kg p.o.	Acute leukemia	28 of 58 CR	Heyn *et al.*, 1960
None	6-MP 2.5 mg/kg p.o.	Acute leukemia	30/67 CR	Heyn *et al.*, 1960
60–90 mg/m^2/4d p.o. × 28 d	L-Asp 6000 U/m^2 i.v. 3 of 4 d × 28 d	ALL	2 of 7 CR	Leventhal *et al.*, 1970
90–150 mg/m^2/4d p.o. × 28 d	L-Asp 6000 U/m^2 i.v. 3 of 4 d × 28 d	ALL	7 of 9 CR	Leventhal *et al.*, 1970
60–150 mg/m^2/4 d i.v.	L-Asp 15,000 U/m^2 i.v.	ALL	1 of 5 Cr	Leventhal and Henderson, 1971
300 mg/m^2/4 d i.v. × 28 d	L-Asp 15,000 U/m^2 i.v. 3 or 4 d × 28 d	ALL	6 of 9 CR	Leventhal and Henderson, 1971

*Azaserine generally given in divided doses, often with oral bicarbonate.

absence of activity in nine patients with myeloma. Catane *et al.* (1979) reported that four responses were observed in 61 patients with solid tumors (7%) treated in a Phase I trial conducted by the Central Oncology Group.

Clinical trials using azaserine in combination with other agents are presented in Table 4. Toxicity included anorexia, epigastric distress, nausea and vomiting, stomatitis, myelosuppression, malaise, weakness, jaundice and electrolyte disturbances. Although modest activity was seen with azaserine plus 6-thioguanosine against solid tumors, toxicity was severe (Schroeder *et al.*, 1964). A randomized trial in acute leukemia comparing azaserine plus 6-MP versus 6-MP alone failed to demonstrate an advantage of the combination (Heyn *et al.*, 1960). Another study, comparing the activity of L-asparaginase alone or in combination with azaserine was performed in children with acute lymphoblastic leukemia (Leventhal *et al.*, 1970); although the response rate for the combination was high, the rate was comparable to that observed with L-asparaginase alone and responses were of brief duration. Eight of 14 children with acute lymphocytic leukemia achieved complete remissions (CR) in a subsequent study employing intravenous administration of higher doses of azaserine given in conjunction with L-asparaginase, but, again, the combination appeared no better than L-asparaginase alone, and was associated with a greater incidence of hepatotoxicity (Leventhal and Henderson, 1971). In summary, although azaserine showed some activity in leukemia, it appeared to be less effective than other available agents; furthermore, azaserine given in combination with other agents failed to produce noteworthy therapeutic enhancement. Hence, clinical investigation of the drug has been largely abandoned.

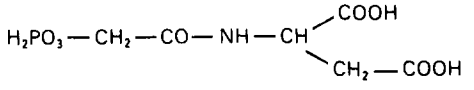
3. ANALOGS OF L-ASPARTIC ACID

L-Aspartic acid, although a non-essential amino acid, plays an indispensable role in the construction of both purine and pyrimidine ring systems. The whole molecule is incorporated during the formation of *N*-carbamyl-L-aspartate, the second committed step in the biosynthesis of pyrimidines, whereas in purine biosynthesis it gives up its amino nitrogen at two critical points: first in the formation of 5-AICAR (5-aminoimidazole-4-*N*-succinocarboxamide ribonucleotide) and then in the formation of adenylosuccinic acid. Analogs of L-aspartic acid function as antitumor agents principally because they inhibit these steps.

3.1. PALA

3.1.1. *Mechanism of Action*

N-(Phosphonacetyl)-L-aspartate (PALA) (NSC 224,131) (Fig. 4) is a rationally-designed transition-state analog of the two components of the reaction catalyzed by L-aspartate transcarbamylase (ATCase), the second enzyme in *de novo* pyrimidine synthesis. The drug inhibits ATCase from both microbial (Collins and Stark; 1971) and mammalian cells (Hoogenraad, 1974) and at submicromolar concentrations has cytotoxic and cytostatic effects on the growth of many cell types in culture. These effects can be reversed by uridine, *in vitro*. Moreover *in vivo*, in mice the toxicity and antitumor activity of the drug can be reversed both by uridine and by *N*-carbamyl-DL-aspartate showing, in both cases, that PALA specifically blocks *de novo* pyrimidine nucleotide biosynthesis (Swyryd *et al.*, 1974; Johnson, 1977; Tsuboi *et al.*, 1977). According to Jayaram (Jayaram *et al.*, 1979a), ACTase activity is significantly lower in PALA-sensitive tumors (241 ± 126 nmol of product



PALA

FIG. 4.

formed/mg protein/hr) than in their PALA-refractory counterparts (526 ± 99 nmol/mg/hr); this correlation has been confirmed by Johnson (Johnson *et al.*, 1978). Acquired resistance to PALA *in vitro* is also characterized by elevated ATCase levels (Kempe *et al.*, 1976; Johnson *et al.*, 1978; Kensler *et al.*, 1981a). However, among tumors sensitive to PALA there is no strict correlation between ATCase activity and degree of sensitivity to the drug (Jayaram *et al.*, 1979a; Leyva *et al.*, 1981). For this reason a number of other biochemical parameters have been explored: kinetics of inhibition of ATCase, capacity for restitution of ATCase activity after drug treatment, detoxification of PALA by tumor cells, uptake and accumulation of PALA in tumors, and pyrimidine salvage enzyme activities (Jayaram *et al.*, 1979a). Among these parameters, the specific activity of pyrimidine salvage enzymes correlates best with the sensitive or resistant nature of any given tumor, higher enzyme activity consistently being seen in the latter.

Another intriguing aspect of PALA's mechanism of action is the pertinacity with which it inhibits ATCase. Several laboratories have shown sustained inhibition both of the enzyme's activity and of pyrimidine synthesis for several days after a single dose of the drug (Yoshida *et al.*, 1974; Tsuboi *et al.*, 1977; Jayaram *et al.*, 1979a; Moyer and Handschumacher, 1979; Moyer *et al.*, 1982). Biotransformation of PALA apparently does not occur *in vivo*.

3.1.2. *Preclinical Pharmacology and Antitumor Activity*

In experimental systems, PALA has proven to be a potent antitumor agent. It exhibits outstanding activity against a variety of rodent solid carcinomas, including several which had been rather consistently refractory to conventional chemotherapy. The Lewis lung carcinoma is a case in point. By contrast, PALA is ineffective against most transplantable murine leukemias (Johnson *et al.*, 1976, 1978). Inasmuch as ATCase activity correlates directly with the cell proliferation rate in a series of transplantable hepatomas (Sweeney *et al.*, 1970) and normal tissues (Young *et al.*, 1967), it is understandable why slow-growing tumors (ovarian teratocarcinoma, Lewis lung carcinoma, colon adenocarcinoma, B16 melanoma), with their lower levels of ATCase (Jayaram *et al.*, 1979a), are more sensitive to PALA than rapidly proliferating leukemias. In the aggregate, these results indicate that PALA has an unusual, but limited, spectrum of antitumor activity against transplantable murine tumors.

Expansion of the therapeutic uses for PALA was attempted by giving it in combination with other inhibitors of pyrimidine biosynthesis. PALA and 5-fluorouracil were markedly synergistic in inhibiting the growth of human mammary carcinoma cells, whereas in a number of test systems (Johnson *et al.*, 1978) PALA and pyrazofurin were ineffective. The most favorable results have been obtained by the combination of PALA and acivicin used against a PALA-resistant variant of the Lewis lung carcinoma. Compared to its sensitive counterpart, this tumor had a two-fold elevated level of carbamyl phosphate synthetase II (Kensler *et al.*, 1981a). Although acivicin and PALA were completely ineffective as single agents in this system, their use in combination provided a significant therapeutic advantage; indeed, under optimal dosing conditions, inhibition of tumor growth approached 100% (Kensler *et al.*, 1981b). This is a case, then, where a rationally designed drug combination enhanced therapeutic results (Kensler *et al.*, 1981b).

In mice, PALA causes striking gestational-stage-specific embryolethal and teratogenic effects at comparatively low doses (Sieber *et al.*, 1980b). Embryos were most sensitive to the lethal effects of PALA on days 7 and 8 of gestation, with embryonic LD_{50}s of 9 and 8 mg/kg, respectively (Sieber *et al.*, 1980a). In contrast, the embryonic LD_{50} on day 10 of gestation was 144 mg/kg (Sieber *et al.*, 1980a). Uridine was ineffective in reducing the day 8 embryolethality of PALA but its precursor, *N*-carbamyl-L-aspartic acid did function as a partial antidote. By contrast, both of these agents reversed PALA-induced embryolethality in day 10 embryos (Sieber *et al.*, 1980a).

Even though PALA is not a myelosuppressive antimetabolite, it does accumulate in substantial quantities (up to 400 μM after an intraperitoneal dose of 400 mg/kg) in the bony matrix (not the marrow) of treated mice (Ardalan *et al.*, 1981). Ardalan *et al.* (1981) suggest that bone matrix serves as a reservoir from which PALA is released slowly into the circulation. The prolonged plasma half-life (8 days) observed in mice after a single dose of 400 mg/kg is consistent with this suggestion.

3.1.3. *Metabolism of PALA*

PALA is fully refractory to metabolism in the systems examined to date. Such metabolic stability is very likely a consequence of the occlusion of its α-amino nitrogen with the *N*-phosphonacetyl functionality, a feature which precludes oxidation, transamination, etc.

3.1.4. *Clinical Studies*

Phase I clinical trials with PALA have been conducted on several schedules. Bolus administration was evaluated using a daily × 1, daily × 5 and weekly × 3 schedule (Gralla *et al.*, 1980; Valdivieso *et al.*, 1980; Erlichman *et al.*, 1979; Kovach *et al.*, 1979). Continuous infusion for 24 hr and 120 hr was also explored (Hart *et al.*, 1980; Ervin *et al.*, 1980). Dose-limiting toxicities included skin rash, diarrhea, and mucositis. Nausea and vomiting was mild, and myelosuppression was infrequent. Neurotoxicity, in the form of paresthesias and seizures, also occurred. Three partial responses were observed in patients with melanoma, carcinoid and chondrosarcoma (Valdivieso *et al.*, 1980; Erlichman *et al.*, 1979; Hart *et al.*, 1980).

The pharmacokinetic behavior of PALA has been studied by enzymatic assays and chromatographic techniques. Using an enzymatic assay, the plasma elimination of PALA was described by a biexponential model, with a short initial $t_{1/2}$ (56–100 min) and a $t_{1/2}$ beta ranging from 4.8–8 hr (Ohnuma *et al.*, 1979; Loo *et al.*, 1980; Erlichman *et al.*, 1979). Elimination

of PALA was found to be triexponential when an HPLC assay was used, with $t_{1/2}$s alpha, beta and gamma of 0.7, 2.7 and 12.7 hr, respectively (Lankelma *et al.*, 1981). The V_d ranged from 290–333 ml/kg (Ohnuma *et al.*, 1979; Loo *et al.*, 1980; Erlichman *et al.*, 1979). PALA was excreted primarily in the urine as unchanged drug, with total body clearance (C_{TB}) ranging from 1.4–1.6 ml/kg/min (Loo *et al.*, 1980; Lankelma *et al.*, 1981). High plasma levels are obtained following a 1 hr infusion of PALA; e.g. a dose of 4 g/m^2 resulted in a peak level of 900 μM (Lankelma *et al.*, 1981).

Karle *et al.* (1980) reported that PALA depressed the concentration of uridine in plasma by 7–65% within 24 hr of a single bolus dose; however, the circulating levels of this nucleoside rarely fell below the lower limits of normal. Moore *et al.* (1982) reported that ACTase activity of biopsied tumors was inhibited in a dose-responsive way following PALA administration; but the maximal reduction in enzyme activity was 87%. UTP pools were also decreased by 16–72% (relative to purine nucleotide pools) in nine of ten tumor specimens, but were decreased by more than 50% in only 3 specimens (Moore *et al.*, 1982). Casper *et al.* (1983) measured the effect of PALA on pyrazofurin-induced orotic aciduria and orotidinuria as an index of inhibition of 'total body pyrimidine synthesis', and found that a low dose, 0.25 g/m^2, was as effective as a higher dose, 2 g/m^2, in decreasing the amount of urinary orotate/orotidine. These observations provide evidence that, as used, PALA is not apparently capable of producing total inhibition of ACTase activity in human tumor and normal tissues. Moreover, it is not clear, at present, whether the drug can produce selective depletion of UTP and CTP pools in neoplastic as opposed to normal tissue.

Broad Phase II screening of PALA indicated minimal single agent activity (Table 5). Cutaneous and gastrointestinal toxicity predominated, but neurotoxicity in the form of seizures, headaches, lethargy, and confusion also occurred sporadically. Myelosuppression was infrequent except in one trial involving patients with lymphoma (Muggia *et al.*, 1984).

Clinical interest shifted to combination regimens with PALA employed as a 'biochemical modulator' of the activity of other antimetabolites. PALA in combination with 5-fluorouracil has been the most extensively studied combination (Table 6). The majority of the trials used a high dose of PALA, compromising the dose of 5-fluorouracil. Biochemical endpoints were not incorporated into most trials, but pharmacokinetic analysis indicated that PALA did not alter the plasma clearance of the fluorinated pyrimidine (Erlichman *et al.*, 1982). Diarrhea and mucositis were dose-limiting in most studies; however, in a recent Phase I trial of low-dose PALA followed 24 hr later by high-dose 5-fluorouracil given as a 24 hr infusion, ataxia and myelosuppression proved to be dose-limiting (Ardalan and Singh, 1987). Single arm studies have yielded response rates comparable to those anticipated from 5-fluorouracil alone. Two prospective randomized trials compared PALA plus 5-fluorouracil (given on a daily × 5 schedule) to 5-fluorouracil alone; neither revealed an advantage for the combination (Mann *et al.*, 1982; Buroker *et al.*, 1985). Ardalan and Singh (1987) reported a 48% response rate in 21 patients receiving sequential PALA (250 mg/m^2) and 5-fluorouracil (750–3400 mg/m^2/24 hr beginning 24 hr post PALA) in a Phase I trial. Confirmatory Phase II trials in colorectal, pancreatic and gastric carcinoma have been initiated.

Other clinical trials have used PALA in combination with methotrexate and 5-fluorouracil, thymidine and 5-fluorouracil, and 6-methylmercaptopurine ribonucleoside. A pilot trial of PALA, 4 g/m^2 given 24 hr prior to thymidine, 15 g, and 5-fluorouracil,

TABLE 5. *Phase II Clinical Trials with PALA Alone*

Schedule	Dose (g/m^2)	Disease	Ev. pts	CR	PR	References
d × 1	5–6	Ovary	32	0	0	Muss *et al.*, 1984b
		Cervix	33	0	0	Muss *et al.*, 1984a
		Head and neck	19	0	0	Creagan *et al.*, 1981a
		Lung	29	0	1	Ettinger *et al.*, 1984
		Lung	57	0	2	Creagan *et al.*, 1981b
		Lung (SCLC*)	17	0	0	Creech *et al.*, 1984
		Melanoma	16	0	2	Creagan *et al.*, 1981c
		Sarcoma	20	0	0	Kurzrock *et al.*, 1984
d × 2	2.5	Breast	29	0	2	Paridaens *et al.*, 1982
		Lymphoma	31	0	0	Muggia *et al.*, 1984
		Melanoma	36	0	4	Kleeberg *et al.*, 1982
		Sarcoma	27	0	1	Bramwell *et al.*, 1982
d × 5	1.2–1.5	Breast	30	0	3	Taylor *et al.*, 1982
		Colon	19	0	0	Van Echo *et al.*, 1980
		Colon	32	0	1	Rubin *et al.*, 1981
		Renal	43	0	2	Earhart *et al.*, 1983
wk × 3	3.7–4.5	Colon	19	0	0	Carroll *et al.*, 1980
		Head and neck	52	0	3	Kaplan *et al.*, 1983
		Lung	18	0	0	Casper *et al.*, 1980
		Bladder	17	0	0	Natale *et al.*, 1982
		Renal	15	0	0	Natale *et al.*, 1982
		Total:	591	0	21	(3.5%)

*SCLC—Small-cell lung carcinoma.

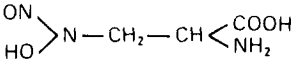
200 mg/m^2, repeated at four-week intervals, was conducted by O'Connell *et al.* (1984). One complete remission and nine partial responses were observed in 37 evaluable patients with colorectal carcinoma (27%); several of these responses were seen in patients with anaplastic histology. These investigators are currently evaluating the three-drug regimen in colorectal cancer patients with unfavorable histology as well as in those with gastric carcinoma, but results are not yet available. A detailed review of such multidrug regimens is beyond the scope of this review; further information can be found in the following references: Leyland-Jones and O'Dwyer (1986); O'Dwyer *et al.* (1987); Grem *et al.* (1988).

A Phase I trial of PALA (0.5–4.5 g/m^2) in combination with dipyridamole (50 mg/m^2 p.o. q 6 hr), an inhibitor of nucleoside transport, has been conducted (Markman *et al.*, 1987). Phase II trials of the combination are ongoing in soft-tissue sarcoma and lung carcinoma. It remains speculative, however, whether salvage of uridine and cytidine is an important mechanism of resistance to PALA in the clinic.

In summary, the current clinical plans with PALA focus on its potential role as a biochemical modulator. Lower doses of PALA are being used in an effort to permit administration of the highest dose possible of the effector agent (5-fluorouracil in most cases), with the hope that PALA may selectively enhance the effect of the fluorinated pyrimidine against malignant cells without enhancing toxicity to normal tissues; whether this will prove to be a fruitful strategy remains to be determined.

3.2. L-ALANOSINE

During the course of a systematic search for antibiotics with antiviral activity, Murthy and colleagues (Murthy *et al.*, 1966) isolated L-alanosine from cultures of *Streptomyces alanosinicus*. Subsequently, the structure of the antibiotic was established as being L-2-amino-3-(*N*-hydroxy-*N*-nitrosamino) propionic acid (NSC 153,353) (Fig. 5). Notable in this natural product is the presence of an *N*-nitrosamino functionality.



L-Alanosine

FIG. 5.

3.2.1. *Mechanism of Action*

Gale and Schmidt (1968) were the first to observe that L-alanosine inhibits the incorporation of [^{14}C] formate into adenine but not into guanine nucleotides. Since that observation was made, many other workers have established that parenteral doses of this antitumor antibiotic interrupt the biosynthesis of AMP, ATP, dATP and DNA (Tyagi *et al.*, 1979, 1981b; Anandaraj *et al.*, 1980). A substantial body of evidence indicates that this interruption takes place at the level of adenylosuccinate synthetase, the penultimate enzyme involved in the biosynthesis of purine nucleotides (Gale and Schmidt, 1968). It also seems clear that L-alanosine by itself is not the proximate inhibitor of this enzyme, but rather an anabolite of the drug, resulting from its conjugation with AICOR (5-amino-4-imidazole carboxylic acid ribonucleotide), functions as the final antimetabolite (Hurlbert *et al.*, 1977).

Because of the structural similarity of L-alanosine to L-aspartic and L-glutamic acids, Tyagi and Cooney (1984) also evaluated the interaction of the drug with both of these dicarboxylic amino acids and their amides, emphasizing transport, enzymology and metabolic effects. At concentrations attainable after therapeutic doses of the antibiotic, no major impact on these several systems was observed. It can be concluded, therefore, that L-alanosine functions as an antimetabolite principally at those steps of *de novo* purine biosynthesis in which L-aspartic acid donates its α-amino nitrogen.

It may also be relevant to point out that L-alanosine can also function as a copper-chelator. The contribution of chelation to its antineoplastic activity is not, at present, clear.

TABLE 6. *Clinical Trials of PALA in Combination with FUra*

Dose/schedule PALA (g/m^2)	Dose/schedule FUra (mg/m^2)	Disease	Ev. pts	CR	PR	References
0.625/d × 5	100–350/d × 5	Solid tumor	14	0	2	O'Connell *et al.*, 1982
0.625/d × 5	300/d × 5	Colorectal	34	0	4	Buroker *et al.*, 1985
0.4/d × 5	300/d × 5	Breast	35	1	10	Mann *et al.*, 1982
0.7–1.5/d × 5	150–250/d 2–5	Solid tumor	17	0	1	Erlichmann *et al.*, 1982
0.4–0.8/d × 5	200–400/d × 5	Colorectal	26	0	3	Bedikian *et al.*, 1980
0.94/d × 5 CI	180–325/d × 5 CI	Solid tumor	18	0	2	Ardalan *et al.*, 1984
0.5–1.175/d × 5 CI	185–430 d 2–6	Solid tumor	16	0	3	Meshad *et al.*, 1981
0.85/d × 5 CI	300–630/d × 5 CI	Solid tumor	21	0	1	Weiss *et al.*, 1982b
0.85/d × 5 CI	300 d 2–6	Solid tumor	43	0	6	Weiss *et al.*, 1982a
0.94/d × 5 CI	250–400/d × 5 CI	Colorectal	11	0	2	Presant *et al.*, 1983
0.25/wk @ hr 0	750–3400/d × 1 CI @ hr 24	GI tumors	21	2	8	Ardalan and Singh, 1987
1–2/wk × 4 @ hr 0	240–480/wk × 4 @ hr 3	Colorectal	24	0	4	Bedikian *et al.*, 1981
1.5 @ hr 0 d 1	400–800 d 1, 8 @ hr 0.5	Colorectal	23	0	6	Muggia *et al.*, 1987
1.0 d 1, 8	300–600 d 2, 9	Colorectal	14	0	3	Muggia *et al.*, 1987
1–2/wk × 3 @ hr 0	200–500/wk × 3 @ hr 3	Solid tumor	68	0	1	Casper *et al.*, 1983
and 0.25–2/qow	400–1200 @ hr 24	Solid tumor	68	0	1	Casper *et al.*, 1983

3.2.2. *Preclinical Pharmacology and Antitumor Activity*

The antitumor activity of L-alanosine was first demonstrated in a transplantable fibrosarcoma induced by the SV-40 virus in hamsters (Murthy *et al.*, 1966). More detailed therapeutic studies with the drug were then carried out in the Drug Screening Program of the National Cancer Institute. Several leukemias (P388, L1210, L5178Y) and the CD8F1 mouse mammary tumor all proved to be sensitive to L-alanosine, whereas the B16 melanoma, the MX1 mammary carcinoma xenograft, and the CX2 colon carcinoma xenograft were refractory (Tyagi and Cooney, 1984). Interestingly, both the P388 and L1210 leukemias could be rendered rapidly resistant by exposure to subcurative doses of the drug (Tyagi *et al.*, 1981a). The concentrations of L-alanosyl-AICOR achieved in these refractory tumors—and in their sensitive counterparts—correlated, in a general way, with their response to the parent drug (Tyagi *et al.*, 1981a): 84 and 94 μM in the case of the sensitive P388 and L1210 tumors, respectively, but only 25 and 27 μM in resistant P388 and L1210 tumors, respectively. It was also observed that the activity of purine nucleoside salvage enzymes was approximately 200% higher in the resistant cell lines compared to their parental strains; moreover, the levels of SAICAR synthetase (the enzyme responsible for the generation of L-alanosyl-AICOR) was depressed in the P388 resistant variant resistant to L-alanosine but not in the L1210/resistant line. These observations suggest that two mechanisms can determine resistance to L-alanosine: a significantly diminished ability to accumulate L-alanosyl-AICOR, and/or a significantly enhanced ability to reutilize preformed purines.

3.2.3. *Preclinical Toxicology*

Large single doses of L-alanosine are required to produce lethality in mice (LD_{50} = approximately 2 g/kg), with males being somewhat more susceptible than females. Although no lesion was universally present at a given dose, the small intestine was the principal site of toxicity, followed by lung, liver and spleen (Tyagi and Cooney, 1984). L-Alanosine, administered to dogs at single intravenous doses up to 800 mg/kg, produced neurological toxicity immediately after drug adminstration. Other dose-dependent toxicities were seen on or after day 2, with gastrointestinal lesions being most prominent (Tyagi and Cooney, 1984). L-Alanosine terminates pregnancies in rats and hamsters after single or multiple treatments given subcutaneously or orally. Maximal contragestational effects occur 3 to 4 days after implantation (Galliani *et al.*, 1985), with both placenta and fetus appearing to be target sites (Galliani *et al.*, 1985). Parenterally administered drug is excreted mainly via the renal route in mice, rats, dogs, and monkeys, whereas in rodents a significant fraction of the dose is eliminated as CO_2 (Kelley *et al.*, 1977).

3.2.4. *Metabolism of L-Alanosine*

Among all the amino acid antimetabolites, L-alanosine undergoes the most extensive metabolism—anabolism, as well as catabolism (Fig. 6). On a structural basis, it was conceivable that L-alanosine could replace L-aspartic acid in enzymatic situations involving transfer of its α-amino nitrogen. Hurlbert *et al.* (1977) were first to observe that one metabolite of L-alanosine is, in fact, an analog of SAICAR, presumably formed by the condensation of L-alanosine with AICOR (5-amino-*N*-imidazole carboxylic acid ribonucleotide). The enzyme responsible for this unique reaction is SAICAR synthetase (5-amino-4-imidazole-*N*-succinocarboximide ribonucleotide synthetase), and the product, L-alanosyl-AICOR (L-alanosyl-5-amino-4-imidazole carboxylic acid ribonucleotide, compound 8 in Fig. 6), is a powerful inhibitor of adenylosuccinate synthetase (Fig. 7). For example, studies on the inhibition of a partially purified preparation of adenylosuccinate synthetase from leukemia L5178Y by L-alanosyl AICOR showed that this nucleotide exerts noncompetitive inhibition with L-aspartic acid and GTP as variable substrates, the apparent K_is being in the high micromolar range; by contrast, formally competitive inhibition is observed versus IMP, with an apparent K_i of 0.228 μM (Tyagi and Cooney, 1983, 1984). This antimetabolite, L-alanosyl-AICOR, has also been isolated from L5178Y nodules (implanted in mice) after parenteral treatment with radiolabeled L-alanosine (Tyagi and Cooney, 1983, 1984). Measurements of the concentration of the putative active metabolite of L-alanosine and related enzymologic studies in susceptible mouse organs suggest that L-alanosyl-AICOR is the molecule likely to be responsible for the therapeutic and toxicologic effects of the drug (Tyagi *et al.*, 1981b).

In addition to the anabolic fate discussed earlier, L-alanosine is also subject to extensive catabolism. A scheme illustrating the relevant reactions is presented in Fig. 6. Transamination or α-oxidation is believed to inaugurate this sequence of catabolic events. The resulting α-keto analog of L-alanosine can then either undergo reduction or decarboxylation with further oxidation of the α-carbon to the level of a carboxylate. Evidence for the operation of these reactions was provided mostly by the work of Jayaram *et al.* (1979b) and Kelley *et al.* (1977).

3.2.5. *Transamination of L-Alanosine*

In mice, dogs, and monkeys, a substantial fraction of the administered dose of DL-[1-^{14}C] alanosine is excreted in urine. Chromatographic analysis showed that the majority of this radioactivity eluted away from the L-alanosine peak. Pursuant to this observation, and in view of the high concentration of radioactivity associated with this metabolite (Kelley *et al.*, 1977), attempts were made to characterize this compound. DEAE-Sephadex chromatography of homogenates of the principal organs of BDF_1 mice given a single parenteral injection of DL-[1-^{14}C] alanosine (Jayaram *et al.*, 1979b) showed the ubiquitous presence of a compound whose chromatographic behavior corresponded to that of the principal urinary metabolite. In quantitative terms, this metabolite was most abundant in extracts of liver, lung, and kidney, and least abundant in heart. Electrophoresis, paper and column chromatography

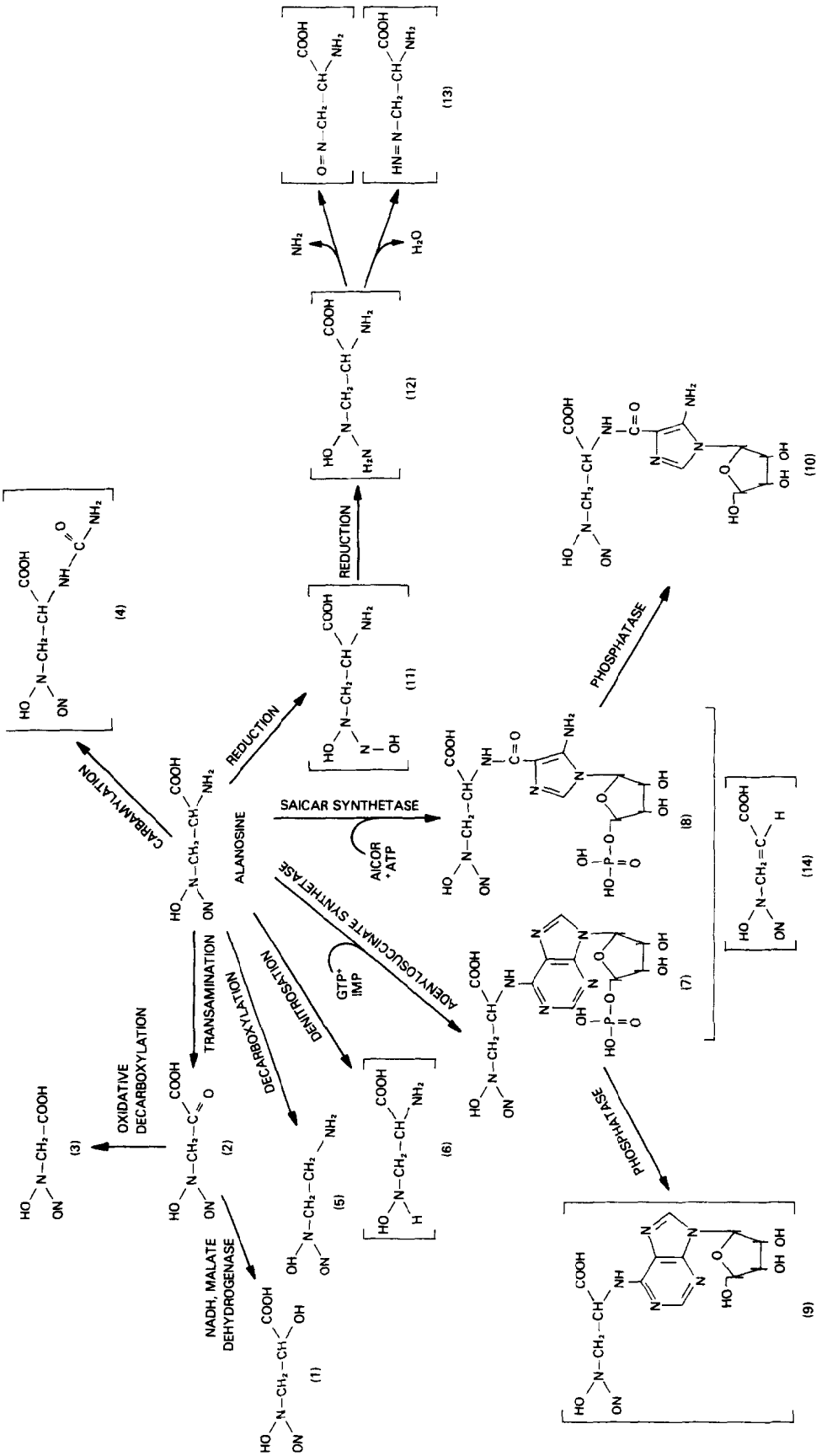


FIG. 6. Metabolites and degradation products of L-alanosine: (1) α-hydroxylanosine; (2) α-ketoanalosine; (3) α-*N*-hydroxy,*N*-nitrosoglycine; (4) α-*N*-carbamoyl-L-alanosine; (5) α-amino,β-*N*-nitroso,*N*-hydroxy ethane; (6) dinitrosated alanosine; (7) L-alanosyl analog of adenylosuccinic acid; (8) L-alanosyl AICOR; (9) product of 5′-dephosphorylation of (7); (10) product of 5′-dephosphorylation of (8); (11), (12) postulated reduction products of L-alanosine; (13) (upper and lower) postulated chemical degradation products of (12); (14) postulated fumaric acid analog of L-alanosine; bracketed compounds have not been identified definitively.

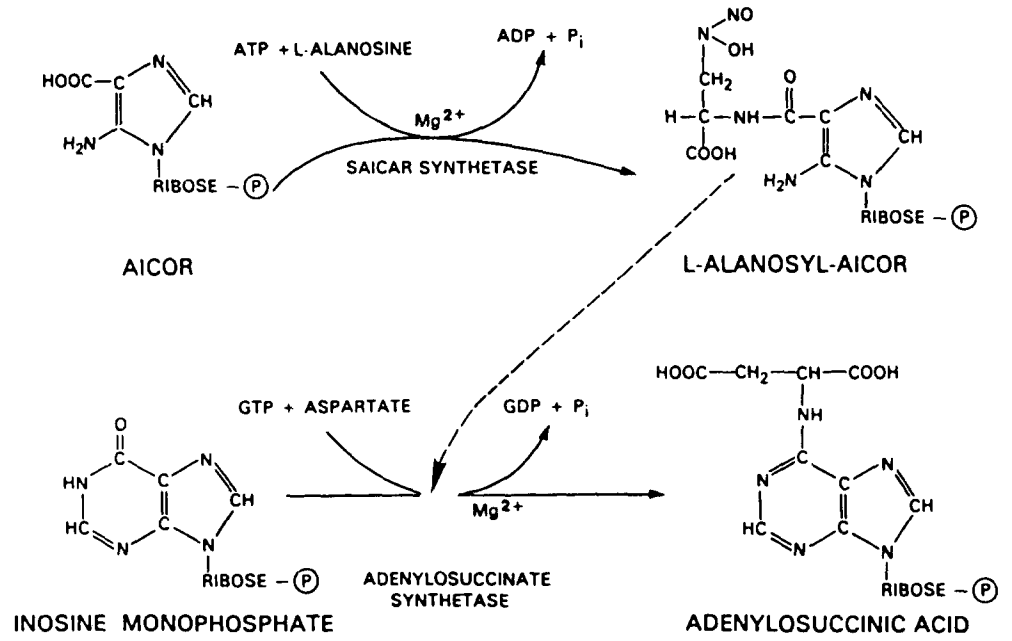


FIG. 7.

of this compound revealed it to be more negatively charged than L-alanosine (Jayaram *et al.*, 1979b). Further characterization revealed that this molecule retained the carbon skeleton of the antibiotic, and was devoid of a free α-amino functionality; the chromophoric *N*-hydroxy-*N*-nitroso group was also probably intact. Utilizing chromatographic and enzymatic means, Jayaram *et al.* (1979b) established this metabolite to be the α-hydroxy analog of L-alanosine, 2-hydroxy-3[(*N*-hydroxy-*N*-nitroso)-amino] propionic acid (compound 1 in Fig. 6).

3.2.6. α-*Decarboxylation of* L-*Alanosine*

Kelley *et al.* (1977) examined the expiration of $[^{14}C]O_2$ by normal and gut-sterilized mice following administration of DL-[1-^{14}C] alanosine. In both cases as much as 18% of the dose was expired as $[^{14}C]O_2$ in 24 hr. This result established that the α-decarboxylation occurs *in vivo*, and excluded the intestinal flora as a source of such catabolism. On the other hand, liver was found to play an important role in decarboxylation, as about 1% of the drug was degraded to CO_2 per hr per gram of this tissue. Indeed, Tyagi *et al.* (1981a,b) have demonstrated the presence of α-decarboxylated L-alanosine (compound 5 in Fig. 6) in tumor and in various other tissues after the administration of the antibiotic to mice.

3.2.7. *Oxidation of* L-*Alanosine*

The foregoing discussion suggests that compound 2 (Fig. 6) could arise as a consequence of transamination. This product might also be generated *in vivo* by the action of D- or L-amino acid oxidases on DL-alanosine. Jayaram *et al.* (1979b) have reported that exposure of DL-[1-^{14}C] alanosine to L-amino oxidase from *Crotalus adamanteus* resulted in the formation of a product which could be readily α-decarboxylated by mildly acidic hydrogen peroxide. This oxidation proceeded at about 5% the rate observed with L-methionine, giving rise to compound 2 in Fig. 6.

3.2.8. *Denitrosation of* L-*Alanosine*

Jayaram *et al.* (1979b) documented the presence of a material chromatographically identical to HAPA (hydroxylamino propionic acid, compound 6 in Fig. 6), the product of denitrosation of L-alanosine, in the urine of rodents given the antibiotic parenterally. However, efforts to generate this compound *in vitro* have not been successful.

3.2.9. *Reduction of* L-*Alanosine*

In vivo and *in vitro* work of Jayaram *et al.* (1979b) suggest that the *N*-nitroso moiety of L-alanosine is not reduced to an appreciable degree in the course of its metabolism.

3.2.10. *Clinical Studies*

Phase I studies of L-alanosine administered as a bolus intravenous injection were conducted in patients with solid tumors using a daily × 1, 3 or 5 schedule (Dosik *et al.*, 1982; Goldsmith *et al.*, 1983; Creagan *et al.*, 1983a). Pharmacokinetic analysis, using HPLC methodology, revealed a rapid plasma disappearance of the drug, with $t_{1/2}\alpha$ and $t_{1/2}\beta$ equal to 14 and 99 min, respectively (Powis and Ames, 1979; Creagan *et al.*, 1983a; Powis, 1983). Transamination was the primary route of metabolism, and excretion of unchanged L-alanosine (< 5%) and metabolites (95%) was principally renal. L-Alanosine is a strong chelator of copper, and transient decreases in plasma copper (up to 30% depression) accompanied by an increase (up to 20-fold) in urinary zinc,

occur in cancer patients receiving the drug (Powis and Kovach, 1981).

Two patients given a 5000 mg/m^2 intravenous bolus of L-alanosine developed acute renal failure, so this schedule was abandoned. Stomatitis was dose-limiting on the daily × 3–5 schedules. Other toxicities included nausea and vomiting, diarrhea, skin rash, leukopenia, thrombocytopenia, hepatic dysfunction, and CNS disturbances including headache, confusion and disorientation. Based upon *in vitro* preclinical evidence of synergy, Phase I studies of L-alanosine in combination with PALA were conducted; stomatitis was the dose-limiting toxicity with both the single dose (maximum tolerated dose (MTD) 3000 mg/m^2 for L-alanosine and 5000 mg/m^2 for PALA, respectively) and daily × 5 (MTD 60 mg/m^2/d for L-alanosine and 500 mg/m^2/d for PALA) schedules, respectively (Creagan *et al.*, 1983b).

L-Alanosine as a single agent displayed minimal activity in Phase II testing in solid tumors and leukemia (Table 7). A single Phase II trial of the drug (60 mg/m^2/d × 5) given in conjunction with PALA (500 mg/m^2/d × 5) has been conducted in patients with melanoma: one partial response was observed among 21 patients with no prior chemotherapy (Morton *et al.*, 1987). However, due to the lack of activity, further clinical investigation is not being pursued.

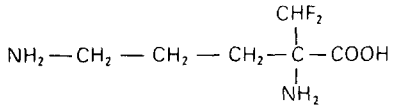
4. OTHER AGENTS

The therapeutic potential of the antimetabolites discussed above is compromised by their pronounced toxicities, for which reason these drugs have enjoyed only limited use in man, at least as single agents. More recently the amino acid analogs α-difluoromethylornithine and buthionine-*dl*-sulfoximine, have progressed towards clinical use as rationally designed enzyme-inhibitory biochemical modulators. Although these newer agents have limited antitumor activity in their own right, because of their low toxicities they are being used with increasing frequency in combination chemotherapeutic strategies.

4.1. DFMO

4.1.1. *Mechanism of Action*

The observation that the increased levels of polyamines in tumors are reduced by antitumor agents (Heby and Russell, 1973), and that polyamine metabolism might play a role in tumor development (Boutwell *et al.*, 1979; Scalabrino and Ferioli, 1981, 1982; Verma and Boutwell, 1987), led to a search for agents which could effectively deplete intracellular polyamines.



α-Difluoromethylornithine

FIG. 8.

The biosynthesis of the polyamines involves conversion of L-arginine to L-ornithine, followed by decarboxylation of the latter molecule to putrescine; putrescine in turn is converted to spermidine and spermine. Decarboxylation of L-ornithine is carried out by a highly regulated (Pegg *et al.*, 1981; Jänne *et al.*, 1978; McCann, 1980) and rate-limiting enzyme, ornithine decarboxylase (ODC) (Pegg and Williams-Ashman, 1968). A number of reports have indicated an association between increased ODC activity or polyamine levels, and human carcinogenesis: a 6.5-fold increase in ODC activity has been reported in colon adenocarcinoma (Rozhin *et al.*, 1984), and a 2- to 4-fold increase in polyamine level is found in certain colorectal neoplasms (Kingsnorth *et al.*, 1984) including adenocarcinomas as well as benign polyps (Takenoshito *et al.*, 1984). Since cells treated with inhibitors of polyamine synthesis respond by increasing their *de novo* synthesis of ODC, irreversible enzyme inhibitors (the so-called suicide substrates) had to be designed in order to block polyamine synthesis effectively. Toward this end, α-difluoromethyl-L-ornithine (DFMO) (Fig. 8) was designed as a specific, potent, enzyme-activated irreversible inhibitor of ODC (Metcalf *et al.*, 1978; Bey *et al.*, 1987; Pegg, 1988). The specificity of the drug is due to its mechanism of enzyme inactivation; DFMO readily binds to the active site of ODC and, during enzymatic decarboxylation, a carbanionic species is generated which alkylates a nucleophilic residue at or near the active site of the enzyme. *In vitro* and *in vivo* treatments with DFMO result in a marked decrease in the intracellular levels of putrescine and spermidine, but not of spermine. This reduction in polyamine levels, albeit partial, translates into a strong antiproliferative effect in a great variety of cells, normal as well as cancerous (Pegg and McCann, 1982; Pegg, 1986; Porter and Sufrin, 1986; Danzin and Mamont, 1987; Sunkara *et al.*, 1987; Pera *et al.*, 1986; Mamont *et al.*, 1984). Reversal of the antiproliferative effect of the

TABLE 7. *Phase II Clinical Trials with L-Alanosine*

Dose (mg/m^2)	Schedule I.V. (q 21 d)	Disease	Ev. pts	Activity CR	Activity PR	References
250	d × 3	Breast	26	0	0	Data on file, DCT, NCI
250	d × 3	Head and neck	26	0	0	Creagan *et al.*, 1983b
250	d × 3	Melanoma	39	0	2	Creagan *et al.*, 1984
160	d × 5	Colon	30	0	0	Rubin *et al.*, 1983a
160	d × 5	Renal	36	1	0	Data on file, DCT, NCI
125–150	d × 5 CI	Leukemia	22	0	0	Weick *et al.*, 1983
		Total	179	1	2	

drug can be achieved by addition of putrescine (the intracellular precursor of spermidine), a result which confirms that the cytostatic effects of DFMO are, in fact, due to depletion of polyamines.

4.1.2. *Preclinical Pharmacology and Antitumor Activity*

Initial studies in HTC cells, L1210 leukemia cells, human prostatic adenoma cells (Mamont *et al.*, 1978), human embryo fibroblasts (Höltta *et al.*, 1979), HeLa cells (Oredsson *et al.*, 1980; Sunkara *et al.*, 1983) and rat 9L gliosarcoma cells in culture (Seidenfeld *et al.*, 1980) indicated that DFMO had antiproliferative properties in a broad range of systems. Cytotoxicity has also been shown in small-cell lung carcinoma cells (Luk *et al.*, 1981, 1982b) and in HL-60 human promylocytic leukemia cells (Luk *et al.*, 1982a). These reports indicate that DFMO is cytostatic or cytotoxic depending on the cell line or conditions of the study.

DFMO produces antineoplastic effects *in vivo*, versus the EMT6 solid tumor of mice, where tumor growth was found to be inhibited by 80% on day-35 (Prakash *et al.*, 1978, 1980). The drug inhibits the growth of the Morris 5123 hepatoma in rats by 70% (Kellen *et al.*, 1980), and was even moderately active against L1210 leukemia (Prakash *et al.*, 1978; Prakash and Sunkara, 1983). Human renal (Kingsnorth *et al.*, 1983a) and small cell lung xenografts implanted in nude mice (Luk *et al.*, 1983a, 1986) are also responsive. Perhaps the most impressive antitumor activity of DFMO was observed in mice bearing the Lewis lung tumor, a carcinoma which—as was said earlier—is resistant to most chemotherapeutic agents; administration of DFMO, given as a 2% solution in the drinking water, not only retarded growth of the primary subcutaneous tumor by 43%, but also decreased pulmonary metastases by as much as 80%, with 25% of the animals showing no visible metastases (Sunkara and Prakash, 1984). Because of the short plasma half-life of DFMO ($t_{1/2}$: 1–2 hours), and because mammalian ODC turns over rapidly, with an apparent half-life of 15–20 min (McCann, 1980), the most effective way to keep the drug continually present at the target site is by administering it *per os*; most *in vivo* preclinical studies have, therefore, used this route of adminstration.

DFMO treatment can also reduce the incidence of a number of chemically induced tumors (Fozard and Prakash, 1982; Thompson *et al.*, 1984, 1985; Rozhin *et al.*, 1984; Kingsnorth *et al.*, 1983b; Takigawa *et al.*, 1982, 1983; Weeks *et al.*, 1982). Furthermore, the very low *in vivo* toxicity of the drug makes it a good candidate for combination chemotherapy. Indeed, an enhanced cytotoxicity of S-phase specific drugs such as arabinosyl cytosine can be shown when they were used in combination with DFMO, both *in vitro* and *in vivo* (Prakash *et al.*, 1981; Sunkara *et al.*, 1980, 1981). A pronounced synergistic effect using combinations of DFMO and Interferon has also been observed against the B16 melanoma (Sunkara *et al.*, 1983). Lastly, DFMO has been reported to induce terminal differentiation of tumor cell lines, including both teratocarcinomas (Heby *et al.*, 1984; Schindler and McCann, 1983; Schindler *et al.*, 1985), and neuroblastomas in culture (Chen *et al.*, 1983), ostensibly by promoting reversion to a non-malignant phenotype.

Although beyond the scope of this chapter, inhibition of *S*-adenosylmethionine decarboxylase—the second key enzyme in the polyamine synthetic pathway—deserves mentioning. Various bis(guanylhydrazones) are potent inhibitors of this enzyme and are also, therefore, potential chemotherapeutic agents. The antitumor activity of these agents has been under investigation since 1958 (Freedlander and French, 1958). Of a number of bis(guanylhydrazones) tested, only glyoxal bis(guanylhydrazone) (GBG) and methylglyoxal bis(guanylhydrazone) (MGBG) appear to have *in vivo* antitumor activity (Jänne *et al.*, 1986; Mihich, 1975). Recently, Elo *et al.* (1988) have reported synthesis and antileukemic activity of a novel analog of MGBG, namely diethylglyoxal bis(guanylhydrazone) (DEGBG). The latter molecule is the most potent inhibitor of *S*-adenosylmethionine decarboxylase reported so far (K_i approx. 9 nM). DEGBG was also found to enhance the antiproliferative effects of DFMO in L1210 leukemia cells in culture.

4.1.3. *Preclinical Toxicology*

An important feature of DFMO is its low *in vivo* toxicity. No change in the pattern of weight gain, compared to controls, and no organ-toxicity was observed when DFMO was administered to rats and mice at a daily dose of 4 g/kg for up to 4 weeks. Rats, however, did develop significant thrombocytopenia after 3 weeks of treatment (Luk *et al.*, 1983b; Ota *et al.*, 1986a,b). The most common side effect of DFMO administration in all animal species so far tested has been diarrhea, but this was reversible on cessation of treatment. Dogs given DFMO showed atrophy of the intestinal mucosa (Yarrington *et al.*, 1983).

4.1.4. *Metabolism of DFMO*

Although metabolism of DFMO has not been studied in detail, it does seem clear that the majority of the administered drug is eliminated *via* the renal route as unchanged parent molecule. It is also clear that the pharmacologic and toxicologic consequences of DFMO administration are directly related to depletion of polyamines engendered by the parent molecule. The oral bioavailability of 10 and 20 mg/kg doses in humans is 58% and 54% respectively (Haegele *et al.*, 1981). Of the orally absorbed dose, 86% is recovered in the urine as unmetabolized drug; 81% of intravenously administered drug is also recovered as intact molecule in urine (Haegele *et al.*, 1981).

4.1.5. *Clinical Studies*

DFMO is currently undergoing clinical investigation under the sponsorship of Merrell Dow Pharmaceuticals, Inc. Maddox *et al.* (1984) studied the clinical pharmacology of DFMO administered either intravenously or perorally at doses ranging from 4 to 32 g/m^2 in 14 patients with leukemia or myeloma.

TABLE 8. *Selected Clinical Trials with DFMO*

Dose/schedule DFMO (g/m^2 p.o.)	Dose/schedule 2nd drug (mg/m^2)	Disease	Ev. pts	CR	PR	References
2.25 q 6 hr daily	None	Small-cell lung	22	0	1	Abeloff *et al.*, 1986
2.25 q 6 hr daily	None	Colon	12	0	0	Abeloff *et al.*, 1986
2 q 8 hr daily	None	Melanoma	21	1	0	Meyskens *et al.*, 1986
4 q day	MGBG 200–700 i.v. d 4 qow	Solid tumor	22	0	1	Warrell *et al.*, 1983
3–9 q 8 hr d × 5 repeat q 2 wk	None	Solid tumor	19	0	0	Natale *et al.*, 1985
2–6 q 8 hr d × 5 repeat q 2 wk	MGBG 400 i.v. d 5	Solid tumor	25	3	2	Natale *et al.*, 1985
1 q 6 hr d × 42	MGBG 350 i.v. d 14, 28, 42	Brain	12	0	0	Levin *et al.*, 1987
1.33 q 8 hr d 1–14 21–35, 42–56	MGBG 200 i.v. d 14, 35, 56	Brain	21	0	4	Levin *et al.*, 1987
1.5 q 6 hr daily	IFN-a 0.4–3.2 × 10^6 U/m^2 i.m. daily	Melanoma	12	0	2	Talpaz *et al.*, 1986
1.3–2 q 8 hr d × 11	1.5–9 × 10^6 U/m^2 i.m. d × 11	Solid tumor	17	0	3	Croghan *et al.*, 1987
2.25 q 6 hr d 1–7 q 28 d	3–48 × 10^6 U/m^3 i.m. d 3–7 q 28 d	Solid tumor	24	0	0	Edmonson *et al.*, 1987

Seven to ten days of daily oral dosing are usually required before detectable levels ($\geqslant 1\ \mu M$ using a bioassay) of DFMO are achieved in plasma; by contrast, DFMO became measurable within three days when the drug was given as a continuous infusion. Polyamine concentrations were depressed in mononuclear cells from peripheral blood and bone marrow (measured with HPLC methodology) when intracellular DFMO exceeded 2–5 μM (Maddox *et al.*, 1984). With prolonged daily dosing, cellular levels of the drug approached 100 μM with the oral route, while millimolar concentrations were attained after intravenous administration.

Abeloff *et al.* (1984) conducted a Phase I trial of DFMO given orally every 6 hr for 28 days. Thrombocytopenia was dose-limiting, although nausea, vomiting and audiometric abnormalities were also reported. The recommended dose was 2.25 g/m^2/q 6 hr. A total of 20 leukemic patients were treated in a Phase I trial of DFMO given at doses ranging from 5.5–6.4 g/m^2 by continuous infusion (Maddox *et al.*, 1985). Dose-related nausea and vomiting were noted, and four patients experienced reversible hearing loss. No remissions were seen. Griffin *et al.* (1987) reported that the MTD of DFMO was 3.75 g/m^2 every 6 hr × 4 days, with nausea and vomiting as the limiting toxicities; diarrhea also occurred. Lipton *et al.* (1987) reported that the MTD of DFMO administered as a continuous hepatic arterial infusion was 2 g/m^2/d, with tinnitus and reversible high frequency hearing loss being dose-limiting. One of seven patients evaluable for antitumor response achieved a partial remission.

The results of two Phase II trials in cancer patients have been reported (Table 8). One of 21 evaluable patients with melanoma achieved a complete regression of a large soft tissue mass lasting for 11 months (Meyskens *et al.*, 1986). Mabry *et al.* (1987) conducted a pilot study of DFMO given at 2.25 g/m^2/6 hr for 21 of 28 day cycles in patients with small cell lung cancer achieving remission with standard induction chemotherapy. Auditory toxicity and gastrointestinal intolerance led to patient refusal to continue DFMO therapy. DFMO has been reported to have some activity against *Trypanosoma brucei gambiense* and *Pneumocystis carinii* infections (McCann *et al.*, 1986).

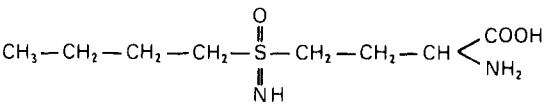
The current clinical emphasis is on the evaluation of DFMO in combination with other agents (Table 8). For example, synergistic or additive increases in survival have been observed with the combination of DFMO and alpha-interferon in animal tumor models (Sunkara *et al.*, 1983). Methylglyoxal-bis-guanylhydrazone (MGBG), also an inhibitor of polyamine biosynthesis at the level of *S*-adenosylmethionine decarboxylase, has been studied in combination with DFMO, but it is too early to determine whether DFMO is enhancing the therapeutic activity of this regimen.

4.2. L-BUTHIONINE-*S,R*-SULFOXIMINE

4.2.1. *Mechanism of Action*

One of the objectives of cancer drug development is to discover rational techniques for potentiating the antineoplastic effects of existing chemotherapeutic drugs without significantly enhancing host cell toxicity (Roizin-Towle, 1985; Siemann, 1984; Green *et al.*, 1984; Arrick and Nathan, 1984). L-Buthionine-*S,R*-sulfoximine (BSO) (*S*-*n*-butyl-homocysteine-*S,R*-sulfoximine) (Fig. 9) alone is an ineffective oncolytic agent but it is an important adjuvant drug in a wide range of chemotherapies.

BSO is a rationally synthesized, selective inhibitor of gamma-glutamylcysteine synthetase (Griffith and Meister, 1979a), the first of the enzymes involved in glutathione synthesis. Intracellular levels of gluta-



L- Buthionine Sulfoximine

FIG. 9.

thione, GSH, the major tripeptide thiol in the cells, are effectively depleted by treatment with BSO, both *in vivo* and *in vitro* (Griffith and Meister, 1979a,b). This depletion is seen after oral or subcutaneous administration of BSO to mice and rats, where reduced levels of GSH were reported in most of the tissues examined, without observable adverse effects (Griffith *et al.*, 1979; Arrick *et al.*, 1981). Injecting mice with the drug produces a rapid decline in renal and hepatic levels of GSH to about 20% of control (Griffith *et al.*, 1979). In addition, BSO reduces the glutathione concentration of murine tumors to 20–30% of control levels (Yu and Brown, 1985), eradicates GSH from macrophages after a 30 hr exposure (Rouzer *et al.*, 1981), and sharply reduces the concentration of the tripeptide in cultured human lymphoid cells (Dethmers and Meister, 1981).

When cellular levels of GSH are depressed by BSO (Griffith and Meister, 1979a), the antitumor activity of many other chemotherapeutic agents is enhanced (Russo and Mitchell, 1985; Russo *et al.*, 1984; Green *et al.*, 1984); BSO also potentiates radiotherapy (Mitchell *et al.*, 1983). By contrast, raising the cellular levels of GSH by 2-oxothiazolidine-4-carboxylate (Williamson *et al.*, 1982) reverses the cytotoxic effects of adriamycin and bleomycin (Russo and Mitchell, 1985; Russo *et al.*, 1984). In an analogous approach, protection against radiation can be achieved in human lymphoid cell line by treatment with glutathione monoethyl ester (Wellner *et al.*, 1984; Anderson *et al.*, 1985), a prodrug of GSH itself; it is thus evident that glutathione plays an important role in determining sensitivity or resistance of many tumors to chemotherapeutic agents.

4.2.2. *Preclinical Antitumor Activity*

The growth of L1210 leukemia, human RPMI 8226 myeloma, MCF-7 breast carcinoma, and WiDr colon carcinoma cells in a soft agar culture was reduced by 50% during continuous exposure to 0.4–1.4 μM BSO (Dorr *et al.*, 1986). However, the drug was inactive against the L1210 leukemia implanted in mice (Dorr *et al.*, 1986). Even so, BSO is gaining attention in combination chemotherapy. Pretreatment with BSO enhances the effectiveness of cyclophosphamide in EMT6/SF tumors without enhancing bone marrow toxicity (Ono and Shrieve, 1987), and increases the antitumor activity as well as the therapeutic index of melphalan (Kramer *et al.*, 1987; Ozols *et al.*, 1987). In nude mice bearing human ovarian cancer cells, pretreatment with BSO followed by a single melphalan treatment (5 mg/kg) increases the median survival time by 72% compared to animals receiving melphalan alone (Ozols *et al.*, 1987). This pretreatment restores the sensitivity of murine leukemia cells, which had been rendered resistant to phenylalanine mustard (Suzukake *et al.*, 1982), increases the radiosensitizing effects of misonidazole (Ono *et al.*, 1986; Guichard *et al.*, 1986), and enhances the susceptibility of human lymphoid cell lines to subsequent radiotherapy (Dethmer and Meister, 1981).

4.2.3. *Preclinical Toxicology*

Preclinical toxicology studies with BSO have been performed in mice and beagle dogs. Repetitive oral dosing of BSO in mice indicated that 100–800 mg/kg of BSO administered every 8 hr for 15 doses was well tolerated (Page *et al.*, 1987). Although oral bioavailability was estimated to be only 2% based on plasma BSO levels, comparable decreases in plasma and liver glutathione levels were seen by the oral or intravenous route. The effects on GSH depletion suggest that BSO undergoes rapid distribution into tissues. BSO was rapidly eliminated from mouse plasma in a biexponential manner with a terminal half-life of 40 min (Page *et al.*, 1987). Combination studies of oral BSO and melphalan have also been performed. Mice given 800 mg/kg of BSO orally every 8 hr × 5 doses, a regimen which produced depletion of liver glutathione to 20% of control levels, tolerated the drug well (Smith *et al.*, 1988). Melphalan given at 1 mg/kg and 5 mg/kg (3 mg/m^2 and 15 mg/m^2 respectively) i.v. 1 hr following the last dose of BSO produced leukopenia (Smith *et al.*, 1988). Ozols *et al.* (1987) have shown that oral BSO given prior to melphalan 5 mg/kg i.v. was superior to melphalan alone in terms of antitumor activity in nude mice bearing NIH: Ovcar 3 ascites, but was not more toxic in terms of lethality than melphalan alone.

Preclinical pharmacokinetic analysis in dogs confirmed that plasma elimination was rapid (plasma clearence 5.2 ± 0.5 ml/min/kg) with a terminal half-life of 36 min (Smith *et al.*, 1987). Repetitive oral dosing at 100 mg/kg/dose (2000 mg/m^2) was nontoxic. A dose of 400 mg/kg/dose (8000 mg/m^2/dose), which depleted GSH in liver to 20% of control, produced reversible gastrointestinal toxicity. Hematuria, recurrent convulsions and gastrointestinal toxicity were observed following oral administration of 800 mg/kg every 8 hr for 15 doses (Smith *et al.*, 1987).

Although BSO concentrations of up to 2 mM have no significant effect on embryo viability, exposure has caused malformations in cultured rat embryos (Slott and Hales, 1987a). *In vitro* treatment with BSO, 100 μM, significantly enhances the embryolethal, and teratogenic effects of acrolein but not of phosphoramide mustard (Slott and Hales, 1987b). [Both acrolein and phosphoramide mustard are toxic and reactive metabolites of cyclophosphamide (Mirkes, 1985).]

4.2.4. *Metabolism of Buthionine Sulfoximine*

Metabolism of buthionine sulfoximine (BSO) was studied by Griffith (1982) in rats and mice. Administration of L-[^{35}S] buthionine-*S,R*-sulfoximine (5 mmol/kg) to these animals resulted in greater than 90% of the radiolabel being excreted via the renal route in a 24 hr period. About 60% of this activity was identified as the parent molecule on an amino acid analyzer. Another major radioactive peak which accounted for most of the remainder of the activity was found to be ninhydrin negative, but after hydrolysis with 6 M HCl at 100°C it was converted to ninhydrin positivity. The hydrolyzed molecule

coeluted with BSO on the amino acid analyzer. Moreover, the urinary metabolite was also converted to BSO after treatment with hog kidney acylase I, which indicated, therefore, that primary metabolism of the drug involves acetylation of the α-amino nitrogen.

Unlike methionine sulfoximine (Rao and Meister, 1972) BSO is not significantly catabolized *in vivo* (Griffith, 1982). However, treatment of mice with BSO significantly enhances the catabolism of subsequently administered L-[1-^{14}C] cysteine. After i.p. administration of DL-[1-^{14}C] buthionine-*S,R*-sulfoximine to rats or mice, less than 3% of the radiolabel is recovered in the expired CO_2 (Griffith, 1982).

4.2.5. *Plans for Clinical Development*

Initial clinical trials will be conducted with intravenous BSO in combination with intravenous melphalan. Multiple doses of BSO will be given the first week to permit assessment of acute toxicity and effects on glutathione levels. A single dose of melphalan will be given following BSO pretreatment the second week, with the time of melphalan administration to coincide with maximal glutathione depletion. If the combination proves tolerable, a weekly × 3 schedule will be pursued.

5. CONCLUSIONS AND PROSPECTS

The present chapter, admittedly not exhaustive, treats seven of the more important amino acid antimetabolites endowed either with bona fide activity against experimental tumors or with important chemosensitizing properties. In some cases, as for example with PALA and DFMO versus the Lewis lung carcinoma, such activity is striking. Nevertheless, none of the amino acid analogues examined here is active as a single agent against human cancer, and several (e.g. DON and azaserine) are prohibitively toxic in the clinic. Although this result is disheartening, it is emblematic of the difficulties inherent in cancer chemotherapy. However, it is likely that several of the amino acids discussed here will ultimately find another niche in the therapeutic armamentarium: as biochemical modulators. Looking ahead, it is still to be hoped that rational screens for amino acid antimetabolites, like those of Hanka and his colleagues (Hanka and Dietz, 1973), will ultimately succeed in discovering an agent capable, on its own, of arresting the growth of human cancer.

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