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SHORT COMMUNICATIONS

Inhibition of dihydrofolate reductases by derivatives of 2,4-diaminopyrroloquinazoline

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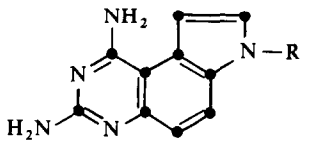
Elslager and Davoll [1] have synthesized a large number of derivatives of 2,4-diaminoquinazoline, some of which exhibit a high degree of antiparasitic activity. Several such diaminoquinazolines are very effective inhibitors of dihydrofolate reductases from mammalian and bacterial sources [2]. 2,4-Diamino-6-(3,4,5-trimethoxyanilinomethyl)-5-methylquinazoline was reported, in 1975, to be comparable to methotrexate in terms of potency as an inhibitor of murine leukemia dihydrofolate reductase [3], and more recent studies [4] have reported that this compound is a strong inhibitor of dihydrofolate reductase from human leukemia cells, is able to suppress the growth of leukemic cells *in vitro*, and exerts potentially useful antileukemic effects *in vivo*. We have evaluated the activity of a series of new diaminoquinazolines, synthesized at Wyeth Laboratories [5], as inhibitors of dihydrofolate reductases from several sources, and wish to report the results of such studies that suggest possible use of compounds of this type as antineoplastic agents. Compounds to be tested were dissolved in dimethylsulfoxide (DMSO; Aldrich Chemical Co., Milwaukee, WI) to prepare stock solutions (1×10^{-3} M); at the dilutions used, DMSO had no effect on the enzymatic assays. Dihydrofolate reductase was assayed at 37° and pH 7.0 (0.1 M phosphate buffer) with dihydrofolate (5×10^{-5} M), NADPH (1×10^{-4} M) and mercaptoethanol (1×10^{-2} M) in the reaction mixture. All inhibitors were preincubated for 5 min with the enzyme before initiation of the reaction. Enzymatic activity was estimated by measuring the decrease in absorbance at 340 nm using a Beckman model 25 kinetic spectrophotometric system. Extracts of acetone powders prepared from rat liver [2] and *Crithidia oncopelti* [6] were used as sources of normal mammalian and protozoal reductases, respectively. Dihydrofolate reductase, partially purified from *Lactobacillus casei*, was obtained from the New England Enzyme Center (Tufts University School of Medicine, Boston, MA). Dihydrofolate reductase from L1210 mouse leukemia was extracted from acetone powders provided by Dr. J. A. R. Mead (National Cancer Institute, Bethesda, MD). In each case, enzymatic activity was adjusted to give an absorbance change of approximately 0.200 over 10 min. Under the reaction conditions used, methotrexate, a standard inhibitor of dihydrofolate reductase, produces 50 per cent inhibition of the enzymes employed, at a concentration of $2–8 \times 10^{-9}$ M.

Table 1 summarizes the results of our studies. It is clear that an aromatic function in the substituent attached to the pyrrole nitrogen is associated with a large increase in inhibitory potency evaluated against all four of the enzyme systems tested; for example, compound 3 with a benzyl substituent is approximately three orders of magnitude more potent than the corresponding unsubstituted or methyl-substituted analog as an inhibitor of dihydrofolate reductases from the mammalian and protozoal sources. It is interesting to note that a heteroaromatic substituent in the side chain attached to the pyrrole nitrogen (compounds 8 and 9) confers an increase in inhibitory potency (relative to 1) quite comparable to that observed with a phenyl substituent. The relatively high inhibitory potency of compound 7, which differs from compound 2 by having a cyclopropyl substituent, was surprising, in view of the small size of the substituent; it is possible that the enhanced activity of 7 relative to 2 is associated with the comparatively high chemical reactivity of the strained cyclopropyl substituent. There are no examples of marked species selectivity of enzyme inhibition among the compounds we have studied; thus, compounds which are strong inhibitors of

Table 1. Inhibition of dihydrofolate reductases by 2,4-diaminopyrroloquinazolines



Compound No.	R	ID_{50} (nM)			
		Rat liver	L1210	*C. oncopelti*	*L. casei*
1	H	1000	4000	1600	420
2	Methyl	1500	3400	2300	1900
3	Benzyl	5.1	2.5	3.4	3.4
4	3,4-Dichlorobenzyl	3.4	4.2	2.1	15
5	4-Methylbenzyl	0.9	2.5	0.7	1.7
6	3,4,5-Trimethoxybenzyl	4.5	3.3	3.9	4.6
7	Cyclopropylmethyl	62	9	22	100
8	2-Picolyl	8	3.2	10	16
9	4-Picolyl	11	14	2.4	10
10	4-Cyanobenzyl	2.1	4	0.7	6.4
11	Methotrexate*	8	2	6	4

* A 2,4-diaminopteridine.

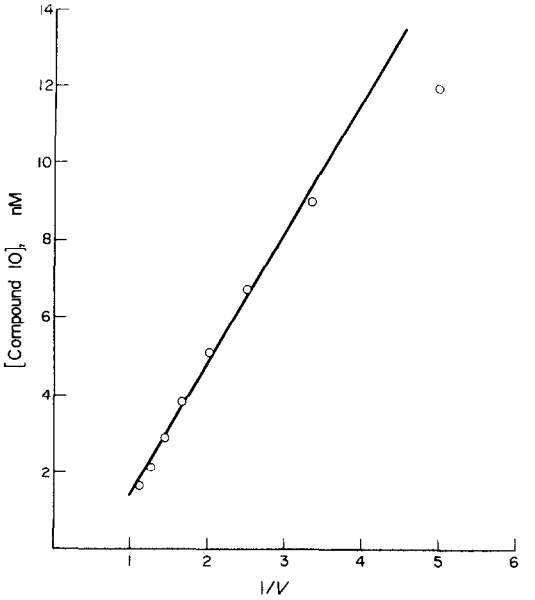


Fig. 1. Relationship between degree of inhibition of rabbit liver dihydrofolate reductase by compound 10 and the concentration of compound 10. $V = V_I/V_C$ where V_C = velocity in the absence of inhibitor and V_I = velocity in the presence of inhibitor.

the mammalian reductases also produce comparable inhibition of the protozoal and bacterial enzyme. The fact that several compounds in this series have inhibitory activity against mammalian reductases, similar to that observed for methotrexate, suggests that these compounds may exhibit useful antineoplastic activity.

In anticipation of possible pharmacokinetic studies of compounds of this type, we have developed relatively simple assay procedures based on two important properties of these molecules, namely, inhibitory activity against dihydrofolate reductase and intense native fluorescence. A number of investigators have exploited the ability of methotrexate and other compounds to inhibit dihydrofolate reductase, as a basis for sensive assays for such drugs [7–9]; using crude dihydrofolate reductase obtained by extracting acetone powders of rabbit liver (obtained from Pel-Freez Inc., Rogers, AR), we have shown that compound 10 produces a degree of inhibition which is closely correlated with concentration (Fig. 1). The curve that we obtained compares favorably with that recently reported for enzymatic analysis of a 2,4-diaminopyrimidine using rat liver dihydrofolate reductase [10]. In principle, the relationship between concentration and enzyme inhibition described for compound 10 will hold for any of the compounds in Table 1 and, of course, the assay procedure can be modified by use of another enzyme, e.g. *L. casei* or beef liver reductase [11]. The strong fluorescence, characteristic of compounds of this type, should also facilitate pharmacokinetic studies. As shown in Fig. 2 (using compound 5 as prototype), a linear relationship is observed between fluorescence readings (Aminco spectrophotofluorimeter; ethanol as solvent; activation 340 nm; emission 450 nm) and concentration in the range of 10^{-9} to 3×10^{-8} M. The intense fluorescence of this type of compound may prove useful in studies of quantitation of levels of dihydrofolate reducatase similar to those recently described by Kaufman *et al.* [12].

In order to establish that members of this series of quinazoline derivatives are not only good inhibitors of dihydrofolate reductase but also are capable of penetrating cellular barriers, we examined the ability of compound 10 to suppress cellular growth (L1210 cells in culture; 1×10^5 cells/ml at zero time; RMPI 1630 medium; assay at 48 hr). We find the compound inhibits cell growth approximately as well ($ED_{50} = 4 \times 10^{-8}$ M) as the widely used antineoplastic agent methotrexate.

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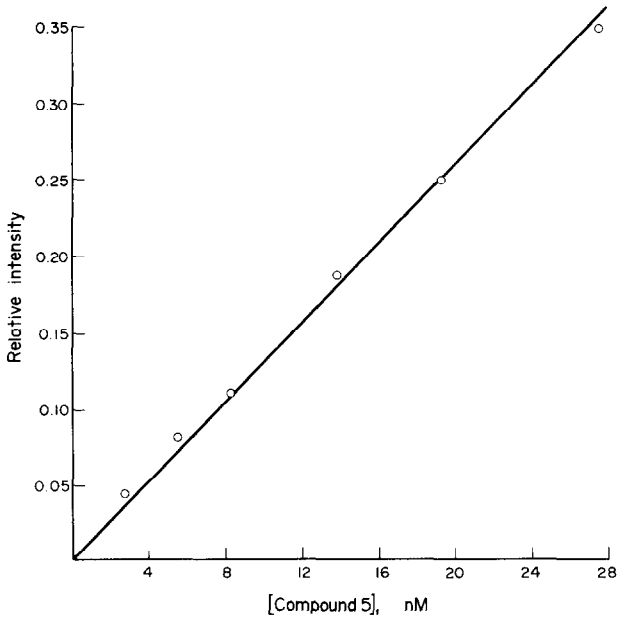

Fig. 2. Relationship between relative fluorescent intensity and concentration for compound 5. Fluorescence measurements were made in ethanol with activation at 340 nm and emission at 450 nm.

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Decreased protein degradation in the skin of glucocorticoid-treated newborn rats*

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The administration of pharmacological doses of glucocorticoids results in decreased growth of experimental animals [1,2] and humans [3,4]. Protein synthesis [2,5–8] and DNA synthesis [2] are decreased in skin following glucocorticoid treatment. Reports in the literature of anti-anabolic effects of glucocorticoids on protein synthesis in skin are numerous [2,5–8]. Although glucocorticoids markedly decrease protein synthesis in skin, we previously observed that the administration of three daily pharmacological doses of the synthetic glucocorticoid, triamcinolone, did not result in the loss of either total body weight or skin weight as compared to zero day controls [2].

Glucocorticoids have been shown to increase catabolic processes in skin. Reports in the literature demonstrate that glucocorticoids increase both collagenolytic and proteolytic activities [9–11] in skin as well as collagenolytic activity in corneal tissue [12] and corneal fibroblasts [13]. Protein degradation in skeletal muscle is also increased by cortisone treatment [14]. The marked decrease of protein synthesis and the increase of catabolic processes by glucocorticoids in the skin should have resulted in an appreciable decrease of protein content. However, the content of total protein did not change as compared to zero day controls [8]. The present study was undertaken to determine the half-lives of skin protein of control and glucocorticoid-treated neonatal rats *in vitro*. Skin proteins labeled with either radioactive proline or tryptophan from triamcinolone-treated newborn rats had longer half-lives *in vitro* than labeled proteins from control animals. During these protein degradation studies, cycloheximide was present at a level which indicated protein synthesis by 91 per cent, thus negating the problem of radioactive precursor reutilization. The present report indicates that, besides having an anti-anabolic effect, glucocorticoids also have an anti-catabolic effect on protein metabolism in skin.

Sprague–Dawley rats (1 to 2-days-old) were used throughout these studies. Powdered triamcinolone diacetate was kindly supplied by Dr. E. W. Cantrell of Lederle Laboratories (Pearl River, NY). Steroid was suspended in 0.9% NaCl. [^{3}H]Tryptophan (3 Ci/mmole) and [2-3-^{3}H]proline (20 Ci/mmole) were purchased from New England Nuclear, (Boston, MA). Eagle's minimal essential medium (F-12) with Hank's balanced salt solution (pH 7.4), 2 × glucose and 2 × $NaHCO_3$, and penicillin–streptomycin solution were obtained from Grand Island Biologicals (Grand Island, NY).

In order to determine the pulse time which gave maximum labeling of skin protein, newborn rats were given radioactive proline for various times before death (Table 1). Skin protein

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