Enhancement of the Antitumor Activity of N^6-(Δ^2-Isopentenyl)adenosine Against Cultured L-1210 Leukemia Cells by Pentostatin Using a Polymeric Delivery System

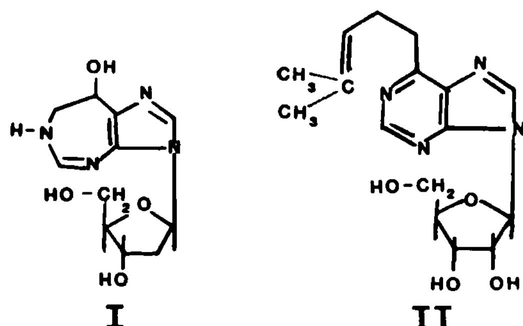
BRUCE HACKER and YUNIK CHANG*x

Received April 20, 1982, from the *Departments of Pharmacology-Toxicology and Pharmaceutics, Northeast Louisiana University, Monroe, LA 71209.* Accepted for publication July 14, 1982. *Present address: The College of Pharmacy, University of Oklahoma, Oklahoma City, OK 73190.

Abstract ☐ The adenosine deaminase inhibitor pentostatin (I), recently shown to be effective in the treatment of several types of acute and chronic human leukemias, was impregnated in a silicone polymer monolithic disk device for release *in vitro* in the presence of the antitumor nucleoside N^6-(Δ^2-isopentenyl)adenosine (II) against mouse L-1210 lymphocytic leukemia cells. Although I is ineffective alone against L-1210 cells, controlled release from the polymeric delivery matrix potentiates the antiproliferative effects of II during the midlog phase of growth (48 hr). Cytotoxicity is prolonged, leading to total cell death during the stationary phase of growth (96 hr). The present study suggests that polymeric delivery systems be used for controlled release of oncologic agents, alone or in combination with inhibitors, especially where lability is a concern.

Keyphrases ☐ Pentostatin—use to potentiate antineoplastic activity of N^6-(Δ^2-isopentenyl)adenosine against L-1210 leukemia cells, polymeric delivery system ☐ N^6-(Δ^2-Isopentenyl)adenosine—antineoplastic agent, potentiation by pentostatin against L-1210 leukemia cells ☐ Delivery systems—impregnated silicone polymer, pentostatin

The clinical use of several adenosine analogues for the treatment of various types of cancer as well as some immunodeficiency diseases has been hindered by inactivation of the oncologic agent. Elevated adenosine deaminase (adenosine aminohydrolase, E.C. 3.5.4.4), responsible for enzymatically deaminating adenosine nucleosides, has been reported in patients with acute or chronic (T-cell) lymphocytic leukemia (1–3). Certain of these patients have also been unresponsive to other conventional courses of chemotherapy (3). Treatment with pentostatin (I) (4), a tight-binding inhibitor of adenosine deaminase, has been shown to result in a successful level of clinical remission when used in single-agent therapy (1, 3, 5). It has been suggested that the antineoplastic activity of I alone when used in Phase I clinical trials is the result of accumulation of deoxyadenosine-5′-triphosphate (2, 6). The latter then interferes with DNA synthesis and/or the accumulation of 2′-deoxyadenosine which, in turn, is capable of blocking critical methylation reactions (6). Whatever the exact biochemical locus, it is clear that I potentiates certain



adenosine analogues such as the antiviral agent vidarabine (9-β-D-arabinofuranosyladenine) (3, 7) and the antitumor nucleoside N^6-(Δ^2-isopentenyl)adenosine (II) (8–10) used in the present investigation. Nucleoside II has been shown to be immunosuppressive (11) as well as capable of interfering with the transmembrane translocation of unmodified nucleosides in several mammalian systems (12).

Recently, this laboratory demonstrated that controlled release of II from a monolithic silicone polymeric delivery system results in more effective cytotoxic action against cultured L-1210 leukemia cells (8, 9). Current studies have revealed that the adenosine deaminase inhibitor pentostatin (I), itself marginally active against L-1210 leukemia, is capable of potentiating and prolonging the cytotoxic activity of II. The present investigation has sought to take advantage of both the ability of I to act as an inhibitor of adenosine deaminase and the properties of the silicone monolithic polymeric delivery system for the controllable release of I to provide maximal potentiation and regulation of the cytotoxic effects of II against cultured L-1210 leukemia cells.

EXPERIMENTAL

Drug Agents—Pentostatin (I), (*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol, (2′-deoxycoformycin; NSC-218321) was supplied[1]. The preparation of N^6-(Δ^2-isopentenyl)adenosine (II) was as previously described (6, 9, 12). Both agents are routinely stored at −20° over silica gel.

Culturing of L-1210 Cells and Determination of the Antileukemic Activity of I and II—Mouse lymphocytic L-1210 leukemia cells were routinely cultured in RPMI-1640 medium with L-glutamine plus 10% fetal bovine serum[2] in tissue culture flasks[3] at 37° under controlled conditions using a digital incubator[4] containing a 5% carbon dioxide–air atmosphere, and grown to various densities as previously described (8, 9). Total cell number and cell viability values were determined using Turk's solution and trypan blue exclusion, respectively (8, 9). After the addition of I, I–silicone polymer, or II (singly or in combination), aliquots of each type of cell suspension were aseptically removed at various intervals for the determination of cell number. In addition, a portion of each cell suspension was centrifuge-filtered[5] to produce cell-free filtrates for high-performance liquid chromatographic (HPLC) analyses as previously described (8, 9), with certain modifications.

HPLC Analyses for I and II in L-1210 Cell Culture Medium—HPLC using a reverse-phase column[6] eluted at designated flow rates with various concentrations of aqueous or phosphate-buffer methanolic solutions was used to quantitatively analyze I and II in each cell-free culture

[1] Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.
[2] Grand Island Biological Co., Grand Island, N.Y.
[3] Corning No. 25100; 25 ml.
[4] Forma Scientific, Marietta, Ohio (Model 3029).
[5] Bioanalytical System, Inc., West Lafayette, Ind.
[6] Micropak MCH-10 (300 mm × 0.4 mm i.d.), Varian Instrument Co., Palo Alto, Calif.

0022-3549/83/0800-0902$01.00/0
© 1983, American Pharmaceutical Association

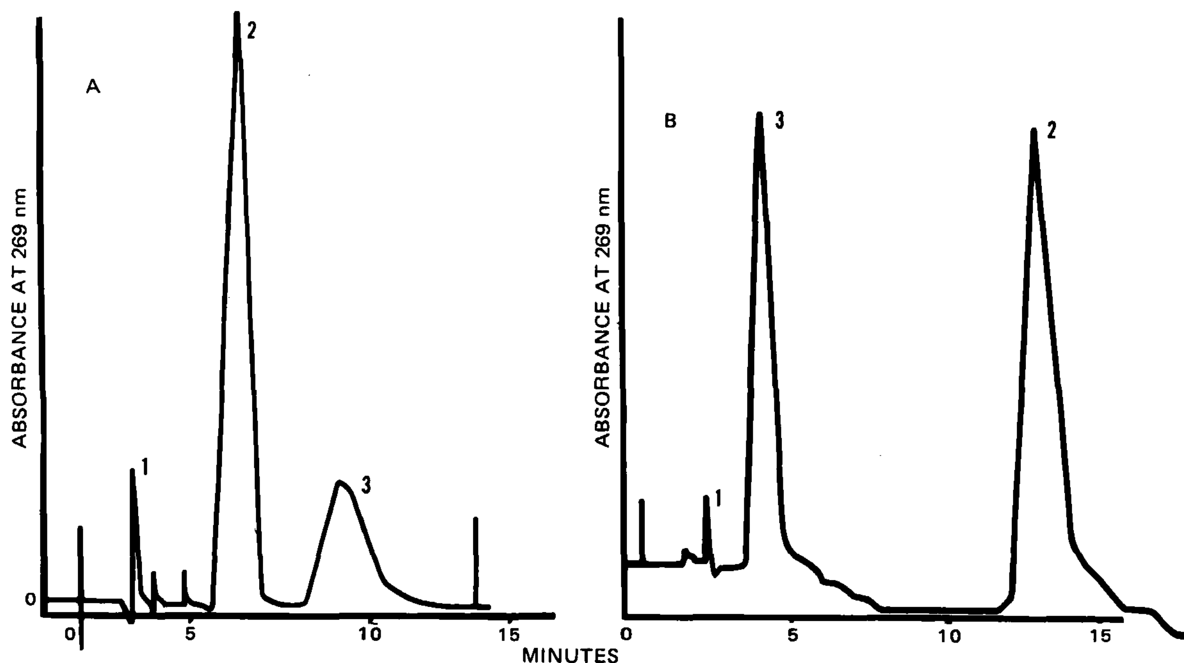


Figure 1—*Representative reverse-phase chromatograms of cell-free growth medium fractions from cultured L-1210 leukemia cells: (A) mobile phase, 60% methanol-water; column temperature, 25°; injection volume, 30 µl; (B) programmed mobile phase, 25% methanol–3 mM phosphate buffer, pH 7.0, increased to 70% methanol–3 mM phosphate buffer, pH 7.0, at a rate of 4.5%/min. Key: (1) inosine or adenosine; (2) II; (3) I.*

extract. Representative chromatograms are given in Fig. 1A and B. Three different calibration curves (stored in computer memory) were established to quantitate the results using a spectrophotometric–computer interface system[7] as previously described (8, 9). Standard curves for both I and II constructed using a detector sensitivity of 0.05–0.5 AUFS were found to be linear over the concentration ranges detected in the samples. Correlation coefficients for the standard curves were usually 0.99 (n = 7). The preparation of samples for HPLC employed cellulose prefilters, as detailed previously (9).

RESULTS AND DISCUSSION

The nucleoside N^6-(Δ^2-isopentenyl)adenosine has been shown to interfere with the transport of unmodified nucleosides in several types of mammalian cells at the level of transmembrane translocation (11). Following its metabolism in mouse spleen lymphocytes, II, or its phosphorylated form, interferes with the transport of unmodified nucleosides thereby resulting in inhibition of cellular RNA and DNA synthesis (12). It has been possible to prepare a serologically specific antibody to II (13) as well as to demonstrate an immunosuppressive response to the parent nucleoside (12). Although at first II appeared to be potentially useful for treating leukemia in humans (10), its antitumor activity was found subsequently to be limited by its low solubility and short half-life (14) due to rapid deamination to inosine by adenosine deaminase (15, 16).

This laboratory recently demonstrated (8, 9) that it is possible to use a silicone polymeric delivery system to control the release rate of II against cultured L-1210 leukemia cells, avoiding the usual peak and trough concentrations encountered with direct single additions of antitumor agents. Moreover, information was acquired in that study concerning the interaction of II and silicone polymers. In other recent studies using pentostatin, information has been acquired showing that I potentiates the antileukemic effects of II when added in combination directly to L-1210 cells grown in culture[8]. The enhanced cytotoxicity of this synergistic combination has not only been demonstrated in these studies *in vitro*, but has also been shown to substantially increase the mean survival time (MST) using tumorgenicity assays in CD2F1 mice *in vivo*[9].

In the present investigation, analyses for I and II in cell-free extracts from serum-containing growth medium have been accomplished using two separate reverse-phase HPLC systems (Fig. 1). When 60% aqueous methanol was used as described previously (8, 9), it was possible to resolve I and II with a retention time difference of ~3 min (Fig. 1A). On occasion, the peak for II had a tendency to broaden in that system, thereby reducing the sensitivity for detecting I and obtaining less than satisfactory results. Use of a computer-programmed gradient, initially at a concentration of 25% methanol in 3 mM phosphate buffer, pH 7.0, and increased to 70% methanol at a methanol rate change of 4.5%/min, gave much greater sensitivity for detecting I (Fig. 1B). In addition to increasing the retention time difference to 13 min, II became more lipophilic, thereby reversing the elution order for I and II when using the programmed gradient system. Use of the programmed mobile phase system, however, gave some tailing of the peak of II. In practice both systems were used throughout this investigation for routinely separating I and II to confirm and generate the data given in Table I and elsewhere.

Experiments conducted to assess the antileukemic effects of I alone and in combination with II revealed some profound information. Al-

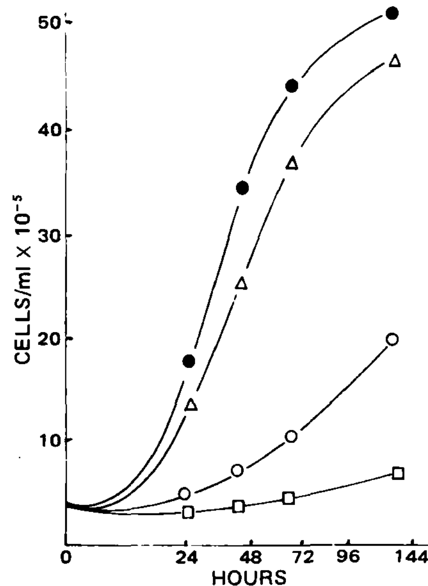


Figure 2—*Effects of I and II on the proliferation of L-1210 mouse leukemia cells. Aliquots of each cell suspension were removed at designated intervals for HPLC and determination of cell number. Key: (●) no additions; (○) plus 25 µg/ml of II; (△) plus 2 µg/ml of I; (□) plus 2 µg/ml of I and 25 µg/ml of II.*

[7] UV-50, Varian Instrument Co., Palo Alto, Calif.
[8] B. Hacker and Y. Chang (presented at the 73rd American Association of Cancer Research, St. Louis, Mo., April 1982); *Cancer Treat. Reports* (submitted for publication).
[9] B. Hacker and Y. Chang, unpublished results.

Table I—HPLC Determination of N^6-(Δ^2-Isopentenyl)adenosine and Pentostatin in Medium from Cultured L-1210 Leukemia Cells

	Sample Time, hr							
	0		72		96		144	
	Concentration in Growth Medium[b], µg/ml							
Addition at Time Zero[a]	I	II	I	II	I	II	I	II
None	0	0	0	0	0	0	0	0
I (10 µg/ml)	8.6	0	5.1	0	3.5	0	2.8	0
I (3 µg/ml) plus II (25 µg/ml)	2.3	25.3	3.0	24.2	1.3	19.2	0.9	17.1
I (10 µg/ml) plus II (25 µg/ml)	9.4	20.6	5.4	21.9	4.3	14.0	3.0	13.3
II (25 µg/ml)	0	21.2	0	25.3	0	25.1	0	19.3
Silicone–I (1.96 mg of I per 88.8 mg silicone device; 1 × 7 cm)	11.7	0	90.3	0	80.6	0	76.8	0
Silicone–I (2.18 mg of I per 98.6 mg silicone device; 1 × 7 cm) plus II (25 µg/ml)	23.0	27.2	98.7	20.0	79.2	21.8	75.4	16.0

[a] Each T-flask contained 5 ml of growth medium (RPMI-1640 plus 10% fetal bovine serum) plus 1×10^5 L-1210 leukemia cells. The experiments were initiated (time zero) by the addition of each agent in RPMI-1640 without serum in a volume of 0.05–0.10 ml with prior sterile-filtration before their introduction into the cell cultures. [b] Aliquots (0.20 ml) of each cell suspension were removed at each designated sample time for the determination of cell number, viability, and HPLC analyses. Values reflect the results of duplicate determinations.

though I is a tight-binding inhibitor of adenosine deaminase and has demonstrated antitumor effects against certain human lymphocytic leukemias (3, 6), it is by itself marginally inhibitory (≤4%) toward cultured L-1210 cells (Figs. 2 and 3). On the other hand, when I (2 µg/ml) is added directly to cultured L-1210 lymphocytic leukemia cells, it dramatically potentiates the effects of II (25 µg/ml) within 24 hr. At 144 hr after each addition of I and II in combination, 85% inhibition is achieved. This represents an additional 30% compared with II used alone (Fig. 2). The most dramatic result here is that without concurrent use of I and II, the ability of II to be cytostatic gradually declines from a 73% value at 24 hr to 56% at 144 hr. The combined use of I and II not only potentiates the latter, but also prolongs its cytostatic effects against L-1210 cells in culture. Recently, Cass *et al.* (17) have shown that several sugar-substituted homologues of tubercidin and adenosine, which are deaminase-sensitive, were also active in combination with I.

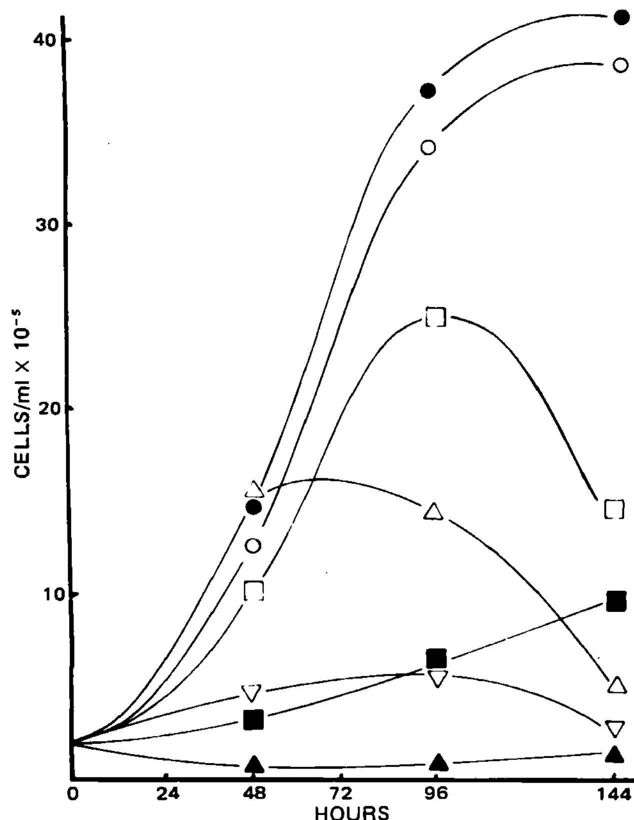


Figure 3—*Effects of pretreatment of cultured L-1210 cells. Additions of I (5 µg/ml) and II (25 µg/ml) to cultures of L-1210 cells were made at time zero and 48 hr. Aliquots of each cell suspension were removed at designated intervals for HPLC and determination of cell number. Key: (●) no additions; (○) plus I at time zero; (□) plus I at time zero and II at 48 hr; (■) plus II at time zero; (▲) plus I and II at time zero; (▽) plus II at time zero and I at 48 hr; (△) plus I and II at 48 hr.*

In subsequent experiments, when the concentration of I was increased to 5 µg/ml and given in combination with II, it was found to further potentiate the latter to an ~96% level within 144 hr (Fig. 3). The few cells which remained had viability values <50%. Use of higher concentrations of I did not result in significantly greater cytotoxicity[10]. If the addition of II (25 µg/ml) is delayed for 24 hr after the introduction of I into the cultured L-1210 cell suspension, inhibition is reduced by about one-fourth compared with the inhibition when both agents are added simultaneously (Fig. 3). This suggests the importance of administering I at the onset to maximize its inhibition of adenosine deaminase, thereby reducing the overall deamination of II. When this occurs the antileukemic effects of II are potentiated and prolonged. Under these conditions it may be possible to reduce the concentration or dose of II required for maximally effective cytotoxicity as an antitumor agent.

With these initial observations, experiments were designed to establish the feasibility of preparing a silicone polymeric delivery form for I that was perhaps similar to the system used previously for II (8, 9). The underlying rationale was that the controlled release (if possible) of I from such a device might serve to block the deamination of II, thereby making

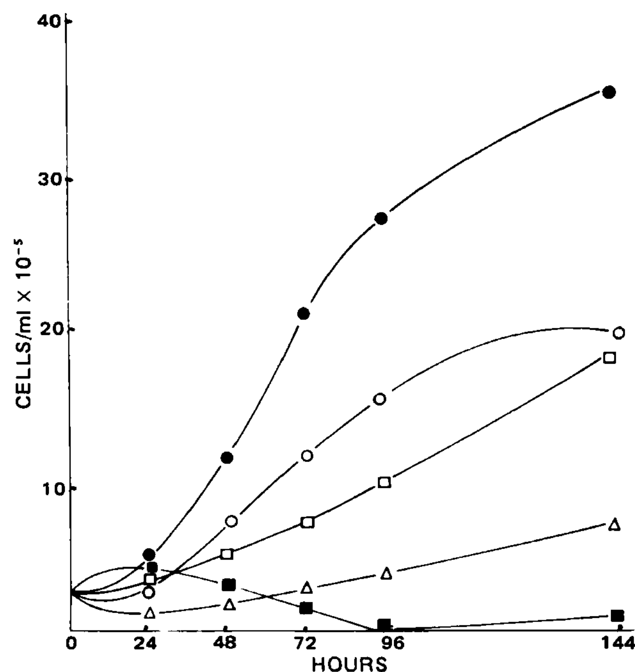


Figure 4—*Enhancement of antiproliferative activity of II against cultured L-1210 leukemia cells by I and I-silicone polymeric monolithic membrane (SPMM) in the presence of II. Aliquots of each cell suspension were removed at designated intervals for HPLC and determination of cell number. Key: (●) no additions; (○) plus 25 µg/ml of II; (□) plus 25 µg/ml of II and 2 µg/ml of I; (△) plus 25µg/ml of II and 5 µg/ml of I; (■) plus 25 µg/ml of II and I-SPMM (2.18 mg of I/98.6 mg of SPMM, 1 × 7 cm).*

[10] B. Hacker, unpublished results.

II a more potent cytotoxic agent. As the release of I begins to occur (Fig. 4 and Table I), its protective effect *via* binding and inhibition of adenosine deaminase is initiated. This occurs within 48 hr with the concurrent potentiation of the cytotoxic property of II. At 72 hr, inhibition by II is almost complete with remaining cells at 30% viability. At 96 hr, when the silicone polymer–I is present initially, inhibition by II is total. Although it was not possible to achieve total cell death when I was added directly (at concentrations as high as 100 μg/ml) with II (Figs. 2 and 3), that end was reached when lower concentrations of I were released into the growth medium from the silicone polymeric delivery device in the presence of II. The few remaining cells at 144 hr were found to be nonviable (Fig. 4) when the combination of the I–silicone polymer plus II was used.

Although both I and II are separately stable when incubated in cell-free medium, analyses of the data using HPLC (Table I) suggest that L-1210 cells have the capacity to metabolize I and/or II. Concentrations of I and II gradually decrease at longer sample times. Previously it was demonstrated that cell-free enzyme extracts of L-1210 cells converted II to its nucleoside monophosphate (18). Similarly, recent studies by Venner and Glazer (19) have shown that L-1210 cells convert I to its mononucleotide to the extent of ≤16%, while most of the parent drug is excreted unchanged in the urine of tumor-bearing mice. Studies by two groups have sought to develop models and data for I alone to describe the pharmacokinetics in normal and leukemic (L-1210) mice (20, 21).

The present study has demonstrated that the adenosine deaminase inhibitor I is capable of greatly potentiating the antileukemic effects of II, particularly when the former is released at a controllable rate from a silicone monolithic polymeric matrix. The clinical application of protection labile oncologic agents in this manner will be explored in the near future.

REFERENCES

(1) A. Yu, B. Bakay, S. Matsumoto, W. Nyhan, M. Green, and I. Rayston, *Proc. Am. Assoc. Cancer Res.*, **22,** 226 (1981).
(2) D. G. Poplack, S. E. Sallan, G. Rivera, *Cancer Res.*, **41,** 3343 (1981).
(3) D. P. Gray, M. R. Grever, M. F. E. Slaw, M. S. Coleman, and S. P. Balcerzak, *Cancer Treat. Rep.*, **66,** 253 (1982).
(4) P. W. K. Woo, H. W. Dion, S. M. Lange, and D. F. Dahl, *J. Heterocycl. Chem.*, **11,** 641 (1974).
(5) D. D. Von Hoff, J. Kuhn, and G. J. Harris, "New Anticancer Drugs in Cancer Chemotheropy Annual 2," H. M. Pinedo, Ed., Elsevier, New York, N.Y. 1981, pp. 122, 130.
(6) R. L. Wortmann, J. Holcenberg, and D. G. Poplack, *Cancer Treat. Rep.*, **66,** 387 (1982).
(7) C. E. Cass and T. H. Au-Yeung, *Cancer Res.*, **36,** 1486 (1976).
(8) B. Hacker and Y. Chang, *Proc. J. Pharm. Sci.*, **11,** 136 (1981).
(9) Y. Chang and B. Hacker, *J. Pharm. Sci.*, **71,** 328 (1982).
(10) J. T. Grace, M. T. Hakala, R. H. Hall, and J. Blakeslee, *Proc. Am. Assoc. Cancer Res.*, **8,** 23 (1967).
(11) D. Hare and B. Hacker, *Phys. Chem. Phys.*, **4,** 275 (1972).
(12) B. Hacker and T. L. Feldbush, *Cancer*, **27,** 1384 (1971).
(13) B. Hacker, H. Vunakis and L. Levine, *J. Immunol.*, **108,** 1726 (1972).
(14) G. B. Chheda and A. Mittelman, *Biochem. Pharmacol.*, **21,** 27 (1972).
(15) B. M. Chassy and R. J. Suhadolnik, *J. Biol. Chem.*, **242,** 3655 (1967).
(16) R. H. Hall and G. Mintsioulis, *J. Biochem.*, **73,** 739 (1973).
(17) C. E. Cass, M. Seiner, T. H. Tan, W. H. Muhs, and M. J. Robins, *Cancer Treat. Rep.*, **66,** 317 (1982).
(18) B. Hacker, *Biochim. Biophys. Acta*, **224,** 635 (1970).
(19) P. M. Venner and R. I. Glazer, *Biochem. Pharmacol.*, **28,** 3239 (1979).
(20) W. R. McConnell, R. L. Furner, and D. L. Hill, *Drug Metab. Dispos.*, **7,** 11 (1979).
(21) F. G. King and R. L. Dedrick, *J. Pharmacokinet. Biopharm.*, **9,** 519 (1981).

ACKNOWLEDGMENTS

Supported in part by Faculty Research Grants from the State of Louisiana to B. Hacker and Y. Chang.

The authors wish to acknowledge the Lord Jesus Christ who inspired and directed this investigation (Proverbs 3:6). We thank Mrs. Beverly Coody for typing the manuscript.

Time-Dependent Kinetics VII: Effect of Diurnal Oscillations on the Time Course of Carbamazepine Autoinduction in the Rhesus Monkey

PETER J. WEDLUND and RENÉ H. LEVY ˣ

Received January 7, 1982, from the *Departments of Pharmaceutics and Neurological Surgery, Schools of Pharmacy and Medicine, University of Washington, Seattle, WA 98195.* Accepted for publication July 8, 1982.

Abstract ☐ Extensive blood sampling and repeated long-term carbamazepine infusions were carried out in four rhesus monkeys to examine the time course of carbamazepine autoinduction in detail and assess the intraanimal variability in the rate constant of induction. Diurnal oscillations in carbamazepine blood levels were observed during all infusions and these prevented a good data fit for the biochemical model previously proposed for describing the decline in drug blood levels during induction by carbamazepine. An attempt at fitting only selected blood samples to the model resulted in variable (and perhaps questionable) induction rate constants, even in the same animal. Previous variability in calculated induction rate constants may be due to the presence of diurnal oscillations superimposed on the autoinduction phenomenon. It is proposed that the simultaneous expression of diurnal oscillations and autoinduction are the result of effects on drug metabolism at two independent levels.

Keyphrases ☐ Carbamazepine—autoinduction in the rhesus monkey, rate constant determination, effect of diurnal oscillations on drug metabolism ☐ Metabolism—of carbamazepine in the rhesus monkey, autoinduction rate constants, effect of diurnal oscillations ☐ Diurnal Oscillation—effect on metabolism of carbamazepine in the rhesus monkey

The ability of carbamazepine to induce self-elimination during chronic administration in the rat, dog, monkey, and human is well established (1–10). This increased elimination is reflected in a decline in carbamazepine steady-state blood levels. To describe this time-dependent decline, equations have been proposed based on a biochemical model of exponentially increasing levels of drug-metabolizing enzymes in the liver (11–14). These equations have

0022-3549/83/0800-0905$01.00/0

© 1983, American Pharmaceutical Association