Although the amount of heparin injected may not be large compared to therapeutically administered doses, it is much more than would reach the general circulation by using indwelling heparinized catheters. The effect of lower amounts should be carefully assessed prior to studies using such catheters not only if protein binding determinations are planned, but in kinetic studies in general. This is important because altered protein binding can have a profound effect on kinetic parameters such as clearance (19) and apparent volume of distribution (20) or the interpretation of such data.

An earlier report (12) raised the possibility that protein binding of quinidine, at least in rabbits, might be capacity limited. However, Table II shows that there is no such effect discernible over the concentration range of 250–3000 ng/ml. Similarly, a previous study (9) found no concentration-dependence in the binding of quinidine to human serum over the clinically relevant range.

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Antineoplastic Effects of N^6-(Δ^2-Isopentenyl)adenosine against L-1210 Mouse Lymphocytic Leukemic Cells Using a Polymeric Delivery System

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Abstract □ N^6-(Δ^2-Isopentenyl)adenosine (I), a nucleoside previously shown to be cytotoxic against several types of tumor cells, was impregnated in silicone polymer monolithic disc devices for release *in vitro* against lymphocytic mouse leukemia cells. Plotting the cumulative amount of N^6-(Δ^2-isopentenyl)adenosine released per unit area of the device *versus* the square root of time revealed a linear relationship. However, the higher loading dose tended to rapidly release any drug deposited on the polymer surface. The optimum loading dose of the device for the most effective antileukemic activity in 24 hr was calculated based on a plot of the release rate *versus* the square root of an initial loading dose. The silicone polymer–I delivery system enabled a sustained and controllable release of additional agent. It was thus possible to achieve virtually total inhibition of leukemic cell replication using the polymeric delivery system. Increased concentrations of I, without the use of the polymeric system, resulted in maximum 24 hr inhibition of only ~81%, followed by a decline in overall antileukemic activity. It is possible to achieve a more predictable release rate of N^6-(Δ^2-isopentenyl)adenosine and corresponding antileukemic activity using a polymeric delivery system against L-1210 mouse leukemic cells *in vitro*. The relative data indicate the ED_{50} concentrations to be considerably less using the polymeric delivery system.

Keyphrases □ Delivery systems—impregnated silicone polymer, N^6-(Δ^2-isopentenyl)adenosine □ Release rates—impregnated silicone polymer delivery system, N^6-(Δ^2-isopentenyl)adenosine □ N^6-(Δ^2-Isopentenyl)adenosine—antineoplastic, improved delivery using impregnated silicone polymer

N^6-(Δ^2-Isopentenyl)adenosine (I), a nucleoside previously shown to be both an inhibitor and cytotoxic to human leukemic myeloblast and sarcoma-180 cells (1), was prepared entrapped in the polymeric delivery form of a silicone polymer[1] monolithic disk to evaluate its relative antineoplastic properties.

Earlier studies demonstrated that I, found also in several isoaccepting species of tRNA, can interfere with the transport of unmodified nucleosides through the cytoplasmic membrane of mouse embryo cells at the level of the transmembrane translocation function (2). This membrane transport inhibition is believed to be responsible for its ability to alter RNA synthesis in phytohemagglutinin-stimulated mouse spleen lymphocytes as well as to be immunosuppressive in nature (3). In the latter context, it was possible to prepare an antibody with serologic specificity for I (4). Previous studies (5) demonstrated that L-1210 mouse leukemic cells possess the necessary

[1] Silastic, 382 Medical Grade Elastomer, Dow Corning Corp., Midland, Mich.

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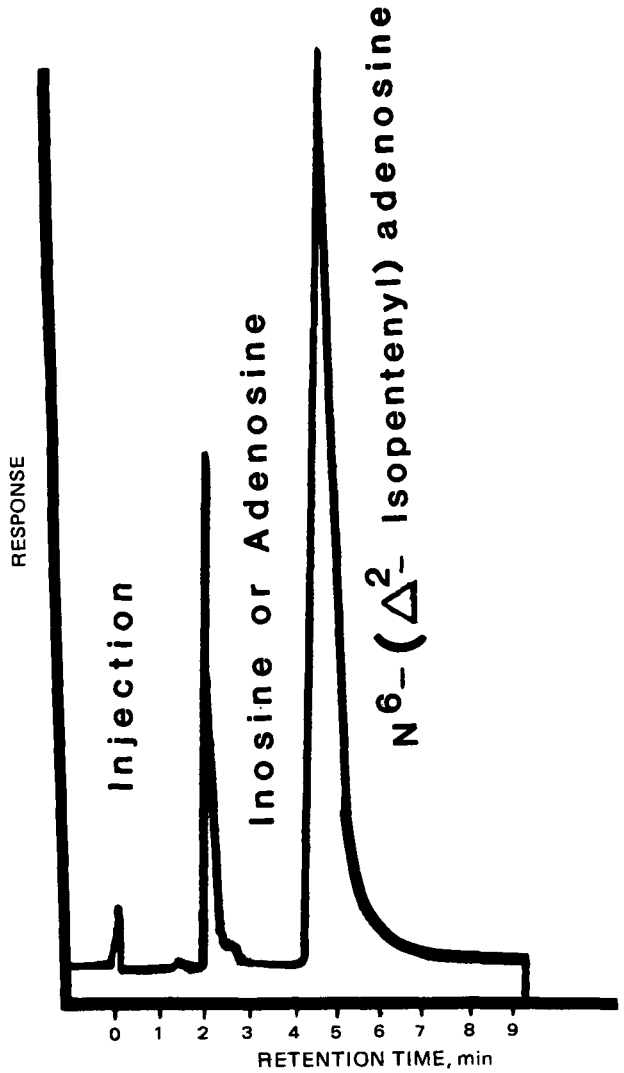

Figure 1—*High-pressure liquid chromatogram of a sample prepared by adding inosine or adenosine to the cultured sample containing 182 μg of I/ml.*

enzyme systems to phosphorylate I to the nucleotide level *in vitro*. It is not yet certain whether this biotransformation is a prerequisite for its antileukemic property.

The chemical reactions, biosynthesis, and metabolism of I, as well as its structural and functional role with respect to tRNA has been extensively reviewed (6). The importance of the intact allylic double bond moiety of the side chain of the purine, in addition to the ribose group of the nucleoside I, is generally recognized as being essential for antileukemic activity.

Table I—Preliminary Determination of Optimum Concentration of N^6-(Δ^2-Isopentenyl)adenosine (without Silicone Polymer) Required to Inhibit the Growth of Cultured L-1210 Mouse Leukemic Cells

Experimental Series[a]	Time after Addition of I to Culture Medium: Concentration of I in Culture Medium[b], μg/ml (4 hr)	Change in Total Cell Number[c], % (4 hr)	Concentration of I in Culture Medium, μg/ml (24 hr)	Change in Total Cell Number, % (24 hr)
A (control)	0	+24.3	0	+141.3
B (182 μg I/ml)	143	−25.1	140	−67.2
C (320 μg I/ml)	253	−61.3	250	−81.2

[a] Each T-flask contained 5 ml of growth medium containing RPMI 1640 plus 10% FCS, L-1210 cells and initial concentrations of I as indicated; cultures were conducted in triplicate. [b] Performed using HPLC. [c] Initial numbers of cells in control and treated cultures were $2 \times 10^5 \pm 6\%$ per ml of growth medium.

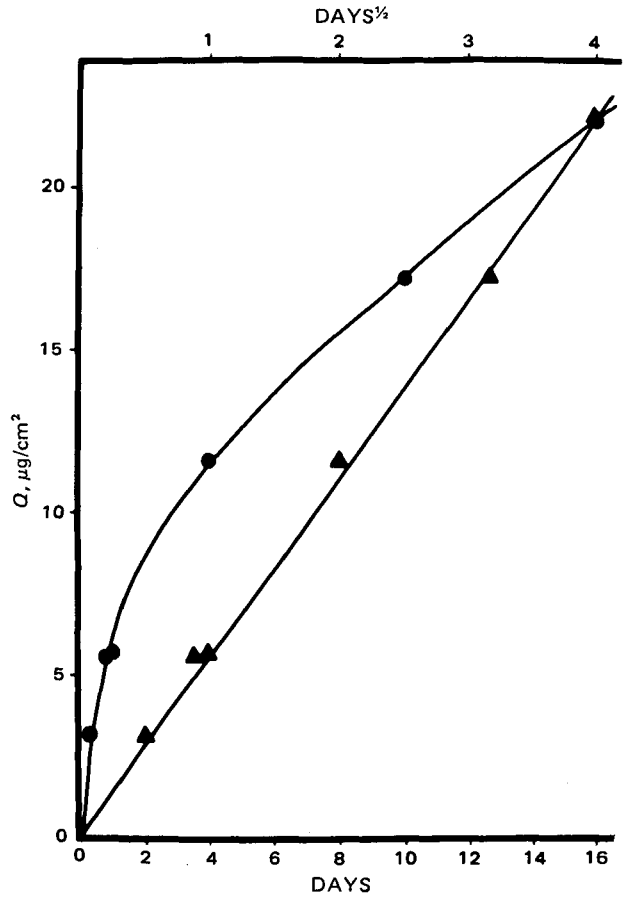

Figure 2—*Release of I in phosphate buffered saline solution from silicone polymer sheet containing 3% (w/w) I (points indicate the average of two experiments). Key:* ●, Q versus t; *and* ▲, Q versus $t^{1/2}$.

Although I has antineoplastic and cytotoxic effects against leukemic cells when administered to humans, it is known to be susceptible to enzyme degradation. Chheda *et al.* (7) showed that the biological half-life of I after intravenous administration was 4 hr. Other results (8) indicated that adenosine deaminase catalyzes conversion of I to inosine. It was also reported (9) that elevated adenosine deaminase activity in human blood significantly facilitates I degradation.

The present study developed a polymeric delivery system capable of releasing the optimum amount of I in a given interval of time required for most effective antileukemic activity.

EXPERIMENTAL

Preparation of N^6-(Δ^2-Isopentenyl)adenosine—I was prepared according to procedures described previously (2, 4, 5). Samples for analytical comparison using enzymatic and chromatographic techniques were described earlier (5).

Isolation and Propagation of L-1210 Mouse Leukemic Cells—Lymphocytic mouse leukemias were first described by Law *et al.* (10). Drug-sensitive and certain resistant sublines were propagated and cryopreserved (11) for *in vivo* and cell culture experiments. Parent lines were initially provided by other institutes[2,3]. Parent L-1210 mouse leukemic cells were grown to various densities as suspension cultures in tissue

[2] Dr. A. E. Bogden, Mason Research Inst., Worcester, Mass.
[3] Dr. H. Harder, Oral Roberts University Medical Center, Tulsa, Okla.

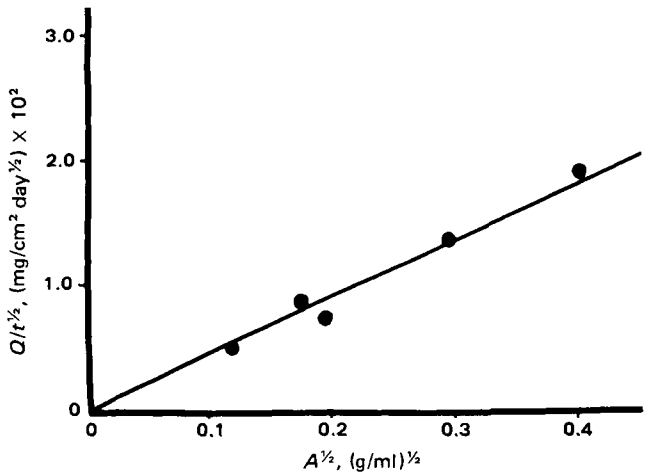

Figure 3—*Effect of the square root loading dose ($A^{1/2}$) in the silicone polymer sheet on the release rate of I ($Q/t^{1/2}$).*

culture flasks[4] containing 5.0 ml of growth medium[5]. Incubations were done at 37° under controlled conditions using a digital incubator[6] containing a 5% carbon dioxide–air atmosphere with a 98% relative humidity. Cells were visually monitored daily to assess general growth characteristics and to ensure the absence of contaminants using an inverted microscope–video system designed and interfaced in this laboratory and described elsewhere (12). Cell number and viability values were determined using Turk's solution and trypan blue exclusion methods, respectively, in hemocytometer cells (11).

Preparation of Isopentenyl Adenosine–Silicone Polymer Monolithic Sheets—N^6-(Δ^2-Isopentenyl)adenosine–silicone polymer sheets were prepared by mixing the required amounts of I into the silicone polymer and polymerizing with catalyst[7]. The mixture was then spread in a thin film on a glass plate and left overnight before the release study. The concentration of I in the polymeric delivery devices was calculated based on the weight-ratio of the drug and polymer used. Sheets of I–silicone for the cell culture media were sterilized using ethylene oxide sterilization[8]. Before use, each preparation was left at room temperature at atmospheric conditions overnight to eliminate residual ethylene oxide effects on the culture medium. Preliminary experiments indicated that equivalent quantities of the silicone polymer without I were not cytotoxic to normal growth of L-1210 cells.

Determination of Antineoplastic Activity of I against L-1210 Lymphocytic Leukemic Cells—Mouse L-1210 leukemic cells were grown in supplemented growth medium at several initial cell concentrations. Aliquots were removed using aseptic techniques for the determinations of cell number, and for centrifugation–filtration to obtain filtrates for assessing I concentration by high-pressure liquid chromatography (HPLC). Cell numbers in each culture flask were determined initially and after the introduction of I–silicone preparations or sterile-filtered solutions of I. These solutions were prepared by dissolving various quantities of solid I in 0.1–0.5-ml volumes of growth medium, and sterile-filtered using individual microunits[9]. After introducing small-volume aliquots of I solutions or I–silicone, aliquots of cell suspensions were removed during continuous incubation conditions and subjected to centrifugation–filtration. The latter consisted of using a centrifugal microfilter device fitted with 0.2-μm regenerated cellulose filters[10] to centrifuge and filter cell suspensions to get a cell-free filtrate.

Determination of I Released from the Polymer Sheet—Polymer sheets of known sizes were suspended on a chrome wire in 100-ml portions of phosphate-buffered saline solution, pH 7.4. The solution was covered to minimize evaporation. Throughout the diffusion experiment, the solution was stirred constantly to reduce boundary-layer effects. Fresh portions of phosphate-buffered saline solution replaced spent medium at each sampling time. For the quantitation of I released, aliquots (30 μl) of the eluting solution were applied onto a high-pressure liquid chromatograph[11]. All determinations of release rates were performed at least in duplicate.

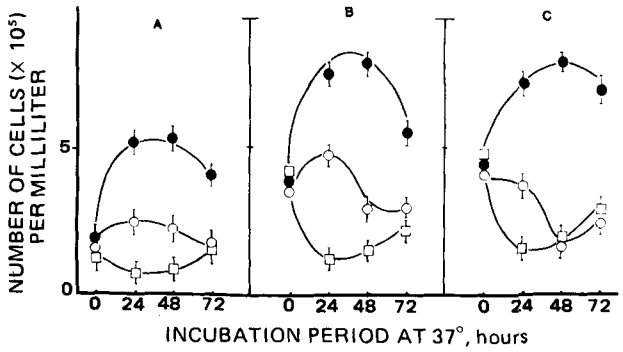

Figure 4—*The antileukemic effect of I using a silicone polymeric delivery system and varying numbers of cultured L-1210 mouse leukemia cells (A, 0.01 ml; B, 0.02 ml; C, 0.03 ml of 24-hr cell stock). Key: ●, control cultures; ○, I–silicone (5 mg I per 200-mg silicone polymer sheet); □, I (40 μg/ml).*

High-Pressure Liquid Chromatographic Procedure—The HPLC separation was performed using a reversed-phase column[12] with a flow rate of 1.0 ml/min using methanol[13]–water (3:2) as the mobile phase. Column effluent was pumped through a variable wavelength detector[14] monitored at 269 nm at ambient temperature. The detector sensitivity for phosphate buffer saline sample and cell culture sample was set at 0.05 and 0.5 aufs respectively. All quantitative determinations were made based on the standard curve obtained by plotting the peak height or electronic integrator response against the concentration of I.

RESULTS AND DISCUSSION

Monolithic sheet devices of I–silicone polymer were prepared that release I at the concentration range for >95% destruction of leukemic cells in the culture medium within 24 hr. The pattern of antileukemic activities of intact I and the I–silicone polymer system were compared.

High-Pressure Liquid Chromatography System—HPLC analysis of I in diffusion samples of phosphate buffered saline solution or cell culture medium was sensitive enough for the diffusion study and provided rapid, quantitative results. The results shown in Fig. 1 show a typical HPLC chromatogram of I in diffusion sample. Retention time of I was 5.0 min. Injection with several possible degradation products such as inosine, xanthine, or hypoxanthine showed no interference with I. These possible degradation products had retention times <5.0 min.

The released concentration of I in 100 ml of phosphate buffered saline solution was ~1–3 μg/ml. The standard curve for I was linear over the concentration range of 0.2–30 μg/ml. The correlation coefficient for the standard curve was 0.99 (n = 7). Standard solution injected at each time of diffusion sample assay showed no significant deviation from the standard curve during a set of experiments.

Since the total volume of cell culture medium was 5.0 ml, the concentration of I in the sample was high enough to change the detector sensitivity 0.5 aufs. The cell culture sample was filtered through cellulose filters before the injection on the HPLC column. The loss of I due to the filtration procedure was found to be negligible. The residual culture base peaks appeared close to the solvent.

Release of I from Silicone Polymer Matrix—Release time profiles of I were obtained with different loading doses on the same size silicone polymer sheets, each having an exposed surface area of 12.0 cm² and 0.35 ± 0.05 mm thick. All the experimental data were analyzed according to Eq. 1 (13, 14) on the basis of the diffusion controlled transport in a polymer matrix:

$$Q = [D(2A - C_s)C_s t]^{1/2} \qquad \text{(Eq. 1)}$$

where Q is the cumulative amount of drug released per unit area of devices (g/cm²) at time t, A is the amount of drug dispersed in a unit volume of device (g/cm³), D is the diffusivity of the drug in the matrix, and C_s is the solubility of the drug in the matrix phase. Based on Eq. 1, the cumulative amount of drug released (Q) from unit area should increase linearly with the square root of time.

[4] Falcon Plastics, 25 cm², No. 3012.
[5] RPMI-1640 medium including L-glutamine, supplemented with 10% fetal bovine serum (all products of Grand Island Biological Co., Grand Island, N.Y.).
[6] Model 3029, Forma Scientific, Marrietta, Ohio.
[7] Dow Corning Catalyst M., Dow Corning Corp., Midland, Mich.
[8] Steri-Vac Gas Sterilizer, 3M Co., St. Paul, Minn.
[9] Nalgene filter unit (cat. no. 120-0020;0.12μm), Sybron-Nalge Co., Rochester, N.Y.
[10] Bioanalytical System, West Lafayette, Ind.; Schleicher-Schuell filters.
[11] Varian 5020, Varian Instrument Co., Palo Alto, Calif.
[12] Micropak MCH-10(30 × 0.4 cm i.d.), Varian Instrument Co., Palo Alto, Calif.
[13] HPLC grade, Fisher Scientific Co., Fair Lawn, N.J.
[14] UV-50, Varian Instrument Co., Palo Alto, Calif.

Table II—Antileukemic Effect of N^6-(Δ^2-Isopentenyl)adenosine against Cultured L-1210 Leukemic Cells within 24 hr Using an Optimized Silicone Polymeric Delivery System

Experimental Series[a]	Time after Addition of I–Silicone Polymer Device: Concentration of I in Culture Medium[b], μg/ml (4 hr)	Change in Total Cell Number[c], % (4 hr)	Concentration of I in Culture Medium, μg/ml (24 hr)	Change in Total Cell Number, % (24 hr)
A (control)	0	+22.4[b]	0	+130.6
B (46 mg I/235-mg silicone device; 8 cm^2)	230	−37.2	350	−83.6
C (92 mg I./470-mg silicone device; 16 cm^2)	325	−56.1	456	−98.1

[a] Each T-flask contained 5 ml of growth medium containing RPMI 1640 plus 10% FCS, L-1210 cells, and I–silicone polymer; cultures were conducted in duplicate. [b] Performed using HPLC. [c] Initial numbers of cells were $2 \times 10^5 \pm 5\%$ per ml of growth medium.

Figure 2 shows the plots of Q *versus* t and Q *versus* $t^{1/2}$ obtained from I–silicone sheet containing 3% I. The release rate of drug decreased with time. The linear relationship between Q and $t^{1/2}$ was observed as expected. All other systems showed this type of release pattern. However, at the high loading dose, the Q *versus* $t^{1/2}$ plot showed intercept values on Q axis. The intercept value increased with increased loading dose. This may be due to the rapid release of drug deposited on the surface of the devices. Such a rapid initial drug release was reported previously (15).

The prediction of optimum loading dose in silicone polymer devices to get the effective minimum concentration of I in culture medium was attempted. The slope of Q *versus* $t^{1/2}$ is defined as:

$$Q/t^{1/2} = [D(2A - C_s)C_s]^{1/2} \quad \text{(Eq. 2)}$$

In the case where $2A \gg C_s$, Eq. 2 may be written as:

$$Q/t^{1/2} = (2D\,C_s)^{1/2}\,A^{1/2} \quad \text{(Eq. 3)}$$

indicating the linear relationship of $Q/t^{1/2}$ *versus* $A^{1/2}$. Experimentally, this linear relationship was observed as shown in Fig. 3. The slope, $(2D\,C_s)^{1/2}$ was calculated as 0.0482 with a linear coefficient of 0.986. The ratio of the matrix device volume to its weight was 0.85 ± 0.05. By relating this factor to Fig. 3, the approximate loading dose for the optimum concentrations of I to be released from a device was calculated.

Antineoplastic Effects of I Against L-1210 Mouse Lymphocytic Leukemic Cells—In preliminary experiments to assess the effectiveness of the silicone polymeric delivery system for releasing low concentrations of I certain salient features were uncovered. The I–silicone device initially containing 5 mg of I gave approximately the same concentration after 24 hr as adding I directly; the I–silicone system showed ~38% inhibition compared to ~65% for the direct addition method. On the other hand, the cytotoxic effects of the I–silicone system against L-1210 cells was comparable within 48–72 hr. At 72 hr, at the highest concentration of cells used (Fig. 4C), the pattern was reversed, with the I–silicone system becoming the more inhibitory of the two. In these experiments (and those depicted in Tables I and II), inhibition of cells in fresh medium appeared to be more permanent or irreversible for cells in cultures where I was introduced in the form of I–silicone. In contrast, those cells surviving the direct addition of I allowed for the recovery of viable cells when propagated in fresh medium. This suggested that the exposure time of L-1210 cells to I, which is limited directly to the timing of its release, is important with respect to the permanency of inhibition. This conclusion was supported by data presented in Tables I and II where at higher I–silicone concentration (92 mg/470 mg), inhibition was virtually complete (98.1%). The few surviving cells were deformed, did not exclude trypan blue dye, and did not transfer successfully to fresh medium. This inhibition level was not achievable with any concentration of I using the direct addition method. Recently, the National Cancer Institute group concluded that the pharmacokinetics of drug release and exposure time is important for another adenosine analog, sangivamycin, against Sarcoma-180 (16).

These data suggest that although comparable concentrations of I may be achieved using direct addition, the I–silicone delivery technique appears to produce a timed, controllable, and sustained release which optimizes inhibition by I against L-1210 lymphocytic leukemia. The use of the polymeric delivery systems with other antitumor agents, especially where enzyme degradation or inactivation of the drug is possible, may offer a better means of administering labile drug agents.

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