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High-performance liquid chromatographic analysis of staurosporine in vivo
Its translocation and pharmacokinetics in rats

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Abstract

The protein kinase inhibitor staurosporine (Stsp) has been used extensively to study physiological functions, biochemical mechanisms, and cancer therapy. Using an HPLC assay for Stsp developed in our laboratory, we find that only 0.7% of Stsp remains in circulating blood of rats 5 min after injection. In vitro, Stsp is adsorbed to red blood cells (RBC) weakly and reversibly. In vivo, all but 1.2–2.5% of Stsp injected is adsorbed by the heart and lungs in one passage through them, indicating that the endothelium acts as a major Stsp sink. Following initial adsorption, pharmacokinetic studies demonstrated that Stsp had a half-life of 51.6 min in plasma and 75.3 min in RBC. Thus, plasma Stsp was in the cancer therapy range of 1–10 ng/ml for 2.7 h following a bolus injection. This data indicates that a bolus injection of Stsp must be followed by a continuous infusion of low Stsp concentration for several days to produce the G1 arrest in cells necessary to stop cell proliferation. Published by Elsevier Science B.V.

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1. Introduction

Staurosporine (Stsp), a microbial alkaloid [1], has been used extensively to study physiological functions, biochemical mechanisms, and cancer therapy. For example, Stsp has been shown to inhibit platelet aggregation [2], lower blood pressure [3,4] and inhibit the plasma membrane Ca^{2+} pump [5]. These physiologic responses are attributed to the fact that Stsp is a potent protein kinase inhibitor [6,7] that is particularly active in inhibiting protein kinase C and myosin light-chain kinase [8].

In biochemical investigations Stsp has been used to study the role of protein kinase C in the regulation of prostaglandin synthesis [9] and the role of histone cyclin-dependent kinases in cell proliferation [10–14]. These uses have led to investigations of Stsp as a possible cancer therapy agent. For example, topical application of Stsp has been shown to inhibit tumor promotion in mice [15] and Stsp has been shown to inhibit cell proliferation in cultured tumor cells such as mouse mammary carcinoma cells, tumorigenic WCHE Chinese hamster cells, and human leukemia cells [10] as well as in human lung carcinoma cells [16,17]. From these studies, strategies for using Stsp in cancer therapy have been suggested [10,17].

To use Stsp in vivo to study physiological functions, biochemical mechanisms or cancer therapy, the

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dose levels of Stsp in the circulating plasma must be kept low (in the nanogram/ml range) to avoid the physiological side effects mentioned at the beginning. Also, work with cultured cells in vitro indicate cancer therapy studies should be conducted in the 1–100-ng/ml range of Stsp in the blood stream in vivo [10,17].

From these preliminary studies, it was clear that it would be necessary to analyze the level of Stsp in the blood in order to determine the dose levels and time schedules for administering the drug for in vivo studies. Thus, we developed an HPLC analysis procedure for quantifying Stsp in blood samples [18,19]. In this paper, we report the use of that HPLC procedure to demonstrate the translocation of Stsp from the plasma to red blood cells and to other tissue compartments in rats. In addition, we have modified the HPLC procedure to perform nanogram-level Stsp blood analysis. This modification involves the concentration of Stsp from blood samples onto an HPLC column and the use of a common multiwavelength UV absorption flow detector (Waters Model 490) in conjunction with a computer-amplified chromatogram analysis to quantify the nanogram levels of Stsp in the blood of rats. We have used this procedure to determine the pharmacokinetics of Stsp in vivo. This data lays the foundation for designing treatment strategies for the use of Stsp in physiological, biochemical and cancer therapy studies in vivo.

2. Experimental

2.1. Staurosporine

Stsp was obtained from Kamiya (Thousand Oaks, CA, USA) and dissolved in poly(ethylene glycol)-400 (PEG) (J.T. Baker) at a concentration of 1 μg/μl. This stock solution was than diluted to appropriate concentrations in various solvents, plasma, or whole blood samples for experiments.

2.2. Animals, blood collection and blood processing

Adult male Fisher-344 rats, specific-pathogen-free, were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). Whole blood to be used for plasma and red blood cell (RBC) preparations were collected from rats anesthetized with an i.p. injection of 50 mg of sodium pentobarbital. The blood was removed by cardiac puncture, using an unheparinized 10-ml syringe. Generally, 5–7 ml of blood was collected per rat.

To prepare plasma and RBC, the blood was immediately placed in a 7-ml sterile blood collection tube (Vacutainer) containing 10.5 mg of disodium EDTA (Bacon Dickinson, Rutherford, NJ, USA) to prevent clotting. The blood was then centrifuged at 800*g* in a clinical centrifuge for 6.5 min. The supernatant plasma fluid was removed with a positive-displacement 250-μl micropipetter (Rainin, Emeryville, CA, USA) and placed in a separate tube. The hematocrit of rat blood at our altitude is ~50%. Thus, the volumes of plasma and RBC were approximately equal.

To inject Stsp into the rats, we anesthetized the animals using enfluorane in air (5% vol/vol, 2 l/min) and inserted a sterile catheter fitted with a 3-cm silastic tubing tip into the right jugular vein, advancing it so that the tip was placed just above the right atrium of the heart. The tubing was prefilled with a 10% heparinized saline solution to prevent clotting at the tip. The catheter was attached to a 1-ml syringe and a single bolus injection of 0.2 mg Stsp, dissolved in a 1:1 PEG–10% glucose/H_2O solution, was administered. A chase solution of 0.5 ml of 10% glucose/H_2O was then injected to clear the catheter dead space (0.2 ml). At 5 min or 15 min after injection of Stsp, blood was collected from the rats by withdrawing it from just above the right atrium using the i.v. catheter inserted in the jugular vein. To prevent clotting, we used a 10-ml syringe prerinsed with heparin solution. The blood was immediately placed in a blood collection tube containing disodium EDTA. Plasma was obtained from these samples as described above.

To measure Stsp adsorbed by only the heart and lungs, we gave rats bolus injections of 500, 250 or 100 μg of Stsp dissolved in 100 μl as described above. These injections were made into the superior vena cava leading to the heart. Just before injection, a needle fitted with a withdrawal syringe was inserted into the abdominal aorta and tied off so that no blood could flow past it. The Stsp was immedi-

ately injected when the abdominal aorta was tied off and blood was collected in 1- to 2-ml aliquots in EDTA-treated vacuum vials in rapid succession from the abdominal aorta until no more was available. Usually 3–5 samples were obtained per rat, totaling 5–6 ml of blood per rat. The blood was mixed with the EDTA in the vacuum tubes and then transferred to centrifuge tubes. Its volume was recorded and it was centrifuged to separate plasma from RBC as described above. These samples were then frozen for storage.

To measure the Stsp uptake by RBC, blood was collected in tubes containing disodium EDTA. Stsp dissolved in PEG was added to the whole blood in vitro and the blood was incubated for 15 min in a 37°C water bath. The blood was then centrifuged at room temperature and the plasma was removed from the RBC as described above. The plasma and RBC samples were frozen for storage until they could be analyzed for Stsp content.

2.3. Extraction of Stsp from blood samples

The procedure for preparing Stsp samples for analysis has been previously described [19]. Briefly, a positive-displacement 250-μl micropipetter was used to withdraw 0.1-ml samples of either whole blood, plasma or RBC. These samples were placed in a 12-ml glass centrifuge tube and 1 ml of acetone was added, which precipitated the proteins immediately. The mixture was stirred with a glass rod and centrifuged at 800 *g* for 5 min. The supernatant acetone containing the Stsp was withdrawn and placed in another tube. The precipitate was washed twice with 0.5 ml of acetone, and the wash acetone was recovered and added to the original extract. The acetone extract was diluted with 4.5 ml of aqueous 0.2% trifluoroacetic acid (TFA). This 6.6-ml sample contained the Stsp ready for analysis by HPLC.

2.4. Analysis of Stsp by HPLC

The HPLC system used for Stsp analysis has been previously described in detail [19]. The system consisted of three Waters solvent-delivery pumps whose flow-rates and solvent gradients were controlled by a Digital Model 350 computer. The pumps were connected to a Waters μBondapak C_{18} Radial-PAK reversed-phase column. The chromatographic separation of Stsp was accomplished by elution from the column with a gradient of acetonitrile in aqueous 0.2% TFA. The Stsp eluting from the column at ca. 37 min was detected by monitoring the UV absorption of the effluent at 292 nm using a Waters Model 490 multiwavelength flow detector. The detector was interfaced with the Digital Model 350 computer that collected the 292 nm absorption data.

Before each HPLC run, the column was equilibrated for 20 min with H_2O containing 0.2% TFA (hereafter referred to as H_2O–TFA or aqueous 0.2% TFA) at a flow-rate of 1 ml/min. Most samples were larger than the standard 2-ml sample injector attached to the HPLC system, so the samples were loaded on the column by dipping the intake line of the pump into the sample tube and pumping the sample through the column at 1 ml/min. To ensure quantitative transfer of the entire sample into the column, 5 ml of H_2O–TFA was added to the empty sample tube after the sample was delivered and this was pumped through the column to rinse the tube, pump and pump lines. Following this sample loading, the pumping of H_2O–TFA was continued until the column effluent absorbance at 292 nm was less than 1. These large sample volumes resulted from the necessity to dilute the acetone extracts of Stsp with sufficient water to make the sample solvent polar so that the Stsp would all be transferred quantitatively to the C_{18} apolar column [19]. For example, the samples from the in vitro blood-treatment experiments in this report were 6.6 ml as described above.

The absorption data of the Stsp peaks from the HPLC were collected by the computer as mV. The area of this peak was quantified as $mV/peak \times 10^{-6}$ using the Data-Process program of a Waters 840 Data and Chromatography Control Station [20]. The area of the peak was then converted to μg of Stsp using a standard curve [19].

2.5. Detection of Stsp in the nanogram range

Measuring Stsp in vivo usually required working with blood samples containing nanogram quantities of Stsp. During the course of this work, we discovered that nanogram-size Stsp peaks were detected at 292 nm by the Waters Model 490 flow detector even

though they were too small to be recorded above background during the chromatography. The data for these peaks was stored in the Digital Model 350 computer and could be accessed using a post-chromatography data processing program called 'Scanner' [20] in the Waters 840 Data and Chromatography Control Station. To do this, we specified the time region where Stsp was expected (36–38 min) and greatly expanded the absorbance scale in that region until the peak could be visualized. No significant background noise was detected. The exact time-range of the peak was then specified to the computer and the area of the peak was quantified using the 'Scanner' program [20]. The area was then converted to nanograms Stsp using a standard curve.

3. Results

3.1. Staurosporine in plasma of rats given Stsp in vivo

In order to determine the concentration levels of Stsp circulating in the blood stream following an i.v. injection of Stsp, an HPLC assay for Stsp was developed [18]. This method produces a Stsp peak at 37 min that can be quantified using a linear calibration curve which has a limit of detection of 0.1 μg of Stsp per sample loaded on the column [19]. For example, when a plasma sample spiked with 2 μg of Stsp in vitro was subjected to this analysis, an HPLC Stsp peak was easily detectable and quantified (Fig. 1A). From this, it was calculated that a 0.2 mg i.v. injection of Stsp into a rat should be easily detectable in the circulating blood unless it was destroyed, eliminated, or absorbed into the body tissues very rapidly.

Thus, 0.2 mg of Stsp dissolved in 0.2 ml of PEG was injected into the jugular vein of a rat. If this amount of Stsp was uniformly distributed throughout the 10-ml blood volume of a rat and if the Stsp was distributed by passive diffusion throughout the volume of both the RBC and the plasma, the initial concentration of Stsp in the plasma would be expected to be 20 μg/ml. Acetone extraction of a 0.1-ml aliquot of this plasma would result in a 2-μg Stsp load for HPLC analysis identical to the in vitro analysis shown in Fig. 1A. If the Stsp did not

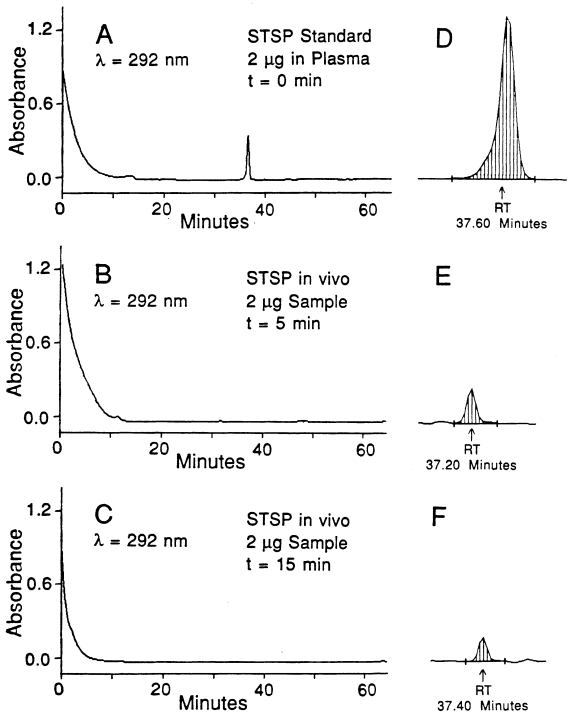

Fig. 1. HPLC analysis of staurosporine in the plasma of rats injected intravenously with staurosporine. (A) Analysis of a plasma sample spiked in vitro with 2 μg of Stsp. (B) Analysis of a 0.1-ml plasma sample withdrawn from a rat 5 min after administering a 200-μg bolus injection of Stsp. (C) Analysis of a 0.1-ml plasma sample withdrawn from a rat 15 min after administering a 200-μg bolus injection of Stsp. (D) 'Scanner'-amplified Stsp peak seen at 37.6 min in Fig. 1A. (E) 'Scanner'-amplified Stsp peak undetected at 37.2 min in Fig. 1B. (F) 'Scanner'-amplified Stsp peak undetected at 37.4 min in Fig. 1C.

penetrate the red blood cells and was thus confined to the 5-ml fluid plasma compartment, the concentration of Stsp in the plasma would be expected to be 40 μg/ml, resulting in an HPLC load of 4 μg Stsp (twice that shown in Fig. 1A). From these considerations, the analysis of Stsp in the in vivo samples of this experiment was expected to be within the operational range of the assay.

However, when blood samples were collected by cardiac puncture 5 min or 15 min after injection of Stsp, no Stsp could be detected in the plasma (Fig. 1B and C, respectively). Thus, the levels of Stsp in the plasma were <0.1 μg per 0.1 ml of sample, which is the detection limit of this assay [19]. This reduction of plasma Stsp from 40 μg/ml to <1

Table 1
Uptake of Stsp in red blood cells

Rat number	RBC		Plasma		RBC:plasma ratio
	μg/0.1 ml	μg/0.5 ml	μg/0.1 ml	μg/0.5 ml	
1	2.19	10.95	0.86	4.30	2.5:1
2	1.50	7.50	1.20	6.00	1.3:1
3	2.60	13.00	1.04	5.20	2.5:1
4	2.18	10.90	1.03	5.15	2.1:1
5	2.05	10.25	1.04	5.20	2.0:1
Average					2.1:1

μg/ml in less than 5 min indicated that the Stsp was either being rapidly destroyed or transferred from the plasma compartment to some other compartment in vivo.

3.2. Staurosporine distribution in blood

If Stsp was being removed from the plasma and sequestered in some other compartment in vivo, the most likely candidate for this sequestration was the red blood cells that are suspended in the plasma. To determine if this was happening, 1 ml of whole rat blood was spiked with 20 μg of Stsp and incubated at 37°C for 15 min. The RBC were then separated from the plasma by centrifugation and 100-μl samples of both RBC and plasma were extracted with acetone and the acetone was diluted and subjected to HPLC analysis as described above. The analysis of blood from five different rats indicated that Stsp was removed from the plasma and sequestered in RBC in an RBC:plasma ratio of 2.1:1 (Table 1).

There was concern that the Stsp might not be efficiently extracted from intact RBC by acetone. However, when the RBC were lysed by two freeze–thaw cycles followed by sonication prior to acetone extraction, HPLC analysis still indicated that Stsp was removed from the plasma and sequestered in RBC in a ratio of 2.1:1 (Table 2). These experiments suggested that the RBC sequester Stsp administered in vivo.

To determine the kinetics of Stsp uptake by RBC, 500 μl of whole blood was incubated with 10 μg of Stsp for various periods of time. Following incubation, the RBC were removed from the plasma by centrifugation. The shortest centrifugation time required to effectively sediment the RBC was 2 min (see insert in Fig. 2A). Thus, our shortest incubation time was the 2-min exposure of RBC to Stsp during this centrifugation step. The plasma was removed from the RBC pellet immediately after centrifugation and the Stsp was measured in both RBC and plasma. This data indicated that the RBC:plasma Stsp distribution reached an equilibrium ratio of 2:1 in less than 2 min (Fig. 2A).

The dose dependency of the RBC:plasma Stsp distribution was measured on similar whole blood samples incubated 15 min at 37°C. It was found that a concentration of 10 μg Stsp/500 μl of whole blood

Table 2
Effect of RBC lysis on Stsp extraction

Rat number	RBC		Plasma		RBC:plasma ratio
	μg/0.1 ml	μg/0.5 ml	μg/0.1 ml	μg/0.5 ml	
1	2.57	12.85	1.06	5.30	2.4:1
2	2.15	10.75	1.03	5.15	2.1:1
3	2.59	12.95	1.17	5.85	2.2:1
4	1.99	9.95	1.04	5.20	2.0:1
5	2.06	10.30	1.04	5.20	2.0:1
Average					2.1:1

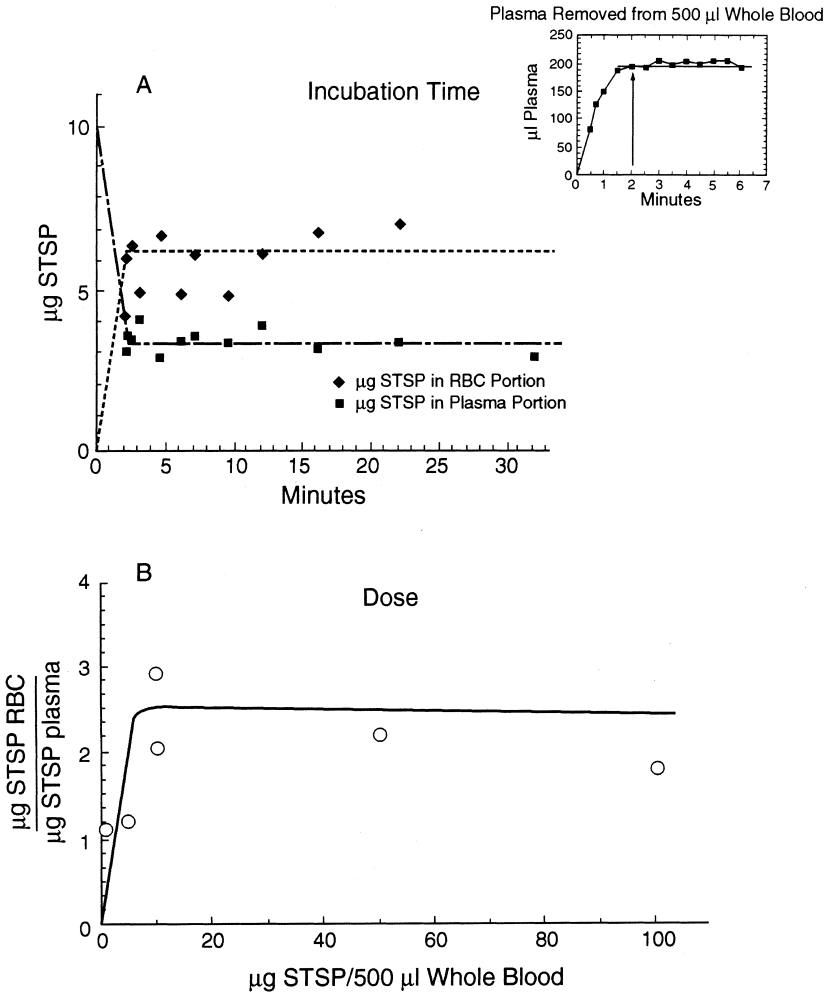

Fig. 2. Kinetics and dose dependency of staurosporine uptake by red blood cells. (A) Kinetics of Stsp uptake: 500 µl of whole blood was incubated with 10 µg of Stsp at 37°C for various periods of time. Following this, the blood was centrifuged for 2 min which is the shortest time required for sedimentation of RBC (figure insert, upper right). The supernatant plasma and RBC pallet were separated and each was analyzed for Stsp by HPLC. (B) Dose dependency of Stsp uptake by RBC. A volume of 500 µl of whole blood was incubated at 37°C for 15 min with various doses of Stsp. Following centrifugation, the plasma and RBC were each analyzed for Stsp by HPLC.

(20 µg/ml) was required to produce the maximum 2:1 RBC:plasma distribution of Stsp. Below this concentration, the distribution was reduced to 1:1 (Fig. 2B). This data demonstrated that the in vivo experiment in Fig. 1B was conducted for a sufficient time (5 min) and at a sufficient dose (20 µg/ml) to facilitate maximum uptake of Stsp by the RBC in vivo.

The 2:1 RBC:plasma distribution of Stsp existed over a 10-fold range (Fig. 2B). This indicates that the Stsp affinity for RBC is weak, resulting in an equilibrium being established between these two blood compartments. If the RBC were the only sink for Stsp in vivo, the 200-µg bolus injection given rats in Fig. 1 should have resulted in a 2:1 distribution of Stsp between RBC and plasma, or a concentration of 2.7 µg of Stsp/100 µl of RBC and 1.3 µg of Stsp/100 µl of plasma. This would have been easily detectable by our analytical procedure. Since no Stsp was detectable under these conditions

(Fig. 1, B and C), it must be concluded that some other compartment in addition to RBC is sequestering the Stsp, perhaps the endothelium of the heart and lungs.

3.3. Weak binding of staurosporine to red blood cells

The 0.2-ml volume of Stsp injected into a rat would be exposed to a constantly changing set of RBC due to turbulence in the vascular system in vivo. To determine how many sets of RBC it would take to reduce the plasma Stsp concentration to nondetectable levels, plasma containing Stsp was exposed to a series of fresh RBC. After each exposure the plasma and RBC were analyzed for Stsp by HPLC. Similarly, RBC containing Stsp were exposed to a series of fresh plasma samples to determine if Stsp would be redistributed from the RBC to the plasma. This experiment and its results are diagrammed in Fig. 3.

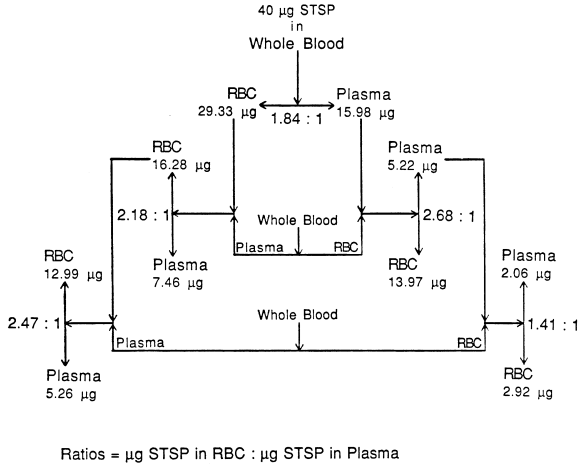

Fig. 3. Diagram of an experiment demonstrating the removal of staurosporine from the plasma by red blood cells. Whole rat blood spiked with 40 µg of Stsp was separated into plasma and RBC. The Stsp-containing plasma was then exposed to Stsp-free RBC and the Stsp-containing RBC were exposed to Stsp-free plasma which was separated from untreated whole blood in the second step. This procedure was repeated in a third step. The Stsp recovered in the plasma and RBC was determined by HPLC analysis of 0.1-ml aliquots at each stage of the experiment and is shown beneath the plasma and RBC. The RBC: plasma Stsp ratio is shown between the RBC and plasma at each stage of the experiment.

In this experiment, 40 µg of Stsp was added to 2 ml of whole rat blood and incubated for 15 min at 37°C. The RBC and plasma were then separated by centrifugation. At this step, the Stsp was partitioned between the RBC and plasma in a ratio of 1.84:1. When the plasma from this sample was exposed to fresh RBC containing no Stsp, the Stsp was again partitioned with a RBC:plasma ratio of 2.68:1. When the plasma from that sample was exposed a third time to fresh RBC, the Stsp was partitioned with an RBC:plasma ratio of 1.41:1. When the concentration of Stsp in the plasma after each exposure was subjected to a semilog plot, it was found that the Stsp was removed from the plasma exponentially with continuous exposure to fresh RBC (Fig. 4). From this plot, it was also determined that the Stsp in the plasma would be reduced to below detectable levels in less than five exposures to fresh RBC (0.1 µg of Stsp/100 µl intercept in Fig. 4).

When the RBC from the initial Stsp-exposed whole blood sample in Fig. 3 were exposed to fresh plasma, part of the Stsp in the RBC was redistributed to the plasma, but the RBC:plasma ratio remained

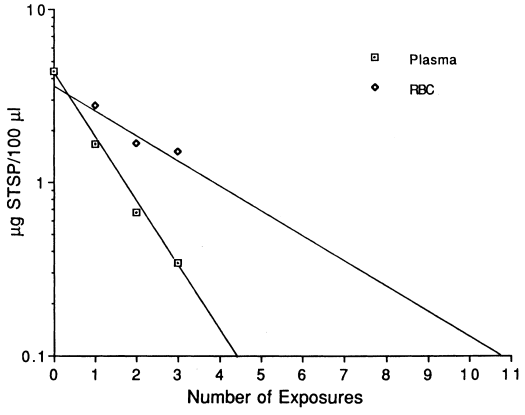

Fig. 4. Removal of staurosporine from plasma and RBC by repeated exposures to plasma and red blood cells containing no Stsp. The amount of Stsp in plasma (□) and RBC (◇) was plotted on a semilog plot after each exposure to untreated RBC and plasma, respectively (i.e., the data in Fig. 3). When this data was extrapolated to 0.1 µg of Stsp/100 µl of plasma (the limit of Stsp detection by the HPLC assay) the intercept with the abscissa indicated that <5 exposures of plasma to RBC were required to reduce plasma Stsp to a nondetectable level (□) and <11 exposures of RBC to plasma were required to reduce RBC Stsp to a nondetectable level (◇).

2.18:1 (Fig. 3). When those RBC were again exposed to fresh plasma, the Stsp was again redistributed to the plasma, but the RBC:plasma ratio remained 2.47:1. When the concentration of Stsp in the RBC after each exposure was subjected to a semilog plot, the RBC concentration of Stsp was rapidly reduced, but at a slower rate than that in the plasma exposed repeatedly to fresh RBC (Fig. 4). Assuming an exponential loss of Stsp from the RBC, extrapolation of the data to the 0.1 μg of Stsp/100 μl intercept indicated that RBC levels of Stsp would be reduced to below detectable levels in only 11 repeated exposures to fresh plasma in vitro.

This data confirms that the affinity of Stsp for RBC is weak and that in vivo the RBC would be expected to rapidly unload their Stsp to Stsp-free plasma, resulting in a steady-state equilibrium being rapidly established between plasma and RBC. This data also reinforces our previous conclusion that, since no Stsp was detectable in vivo (Fig. 1, B and C), there must be some other compartment in addition to the RBC that sequesters the Stsp in the whole animal.

3.4. Computer-amplified detection of Stsp

At this point in the course of these experiments, we discovered that the UV monitor on the HPLC (a Waters Model 490 flow detector) could detect Stsp peaks eluting from the column even though the peak was too small to normally be recorded above background. By using a postchromatography computer program called 'Scanner' as described in the Section 2, the region of the chromatogram where Stsp was expected (36–38 min) could be specified to the computer and the scale of the UV absorbance in that region could be greatly expanded. For example, when this expansion operation was performed on the chromatograms in Fig. 1, a Stsp peak could be found (Fig. 1, E and F) even though no Stsp peak was recorded above background during the chromatography (Fig. 1, B and C). A calibration curve was constructed using plasma samples containing 0.01 to 2.0 μg of Stsp (Fig. 5). The calibration of this computer-amplified detection system was linear with respect to Stsp loaded on the column, and the correlation coefficient (r^2) of the calibration curve

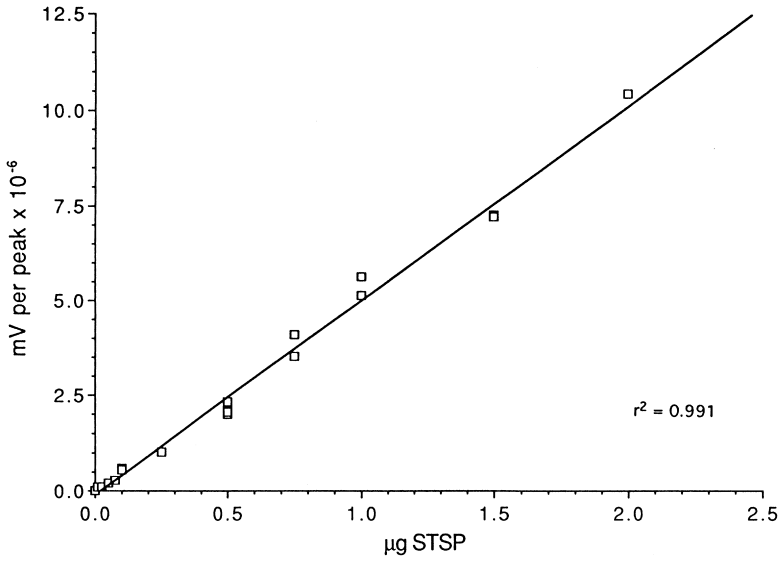

Fig. 5. Calibration curve for nanogram quantities of staurosporine in plasma. Plasma samples containing 10–2000 ng of Stsp/100 μl were extracted with acetone and the extract was subjected to HPLC. Following the chromatography, the scale of the UV absorbance at 292 nm in the 35–38-min region of the chromatogram was expanded until the Stsp peak was visible using the 'scanner' computer program. The area of the peak was then quantified as the accumulated mV per peak and plotted vs. ng of Stsp in the 100-μl plasma sample to use as a calibration curve for blood Stsp analysis. The least-squares linear fit of the data had a correlation coefficient (r^2) of 0.991.

was 0.991. The limit of detection was 20 ng loaded on the column. Thus, this method can be used to measure ng quantities of Stsp in blood, if the volume of the sample is large enough to contain a total load greater than 20 ng.

3.5. Pharmacokinetics of staurosporine in vivo

Using this computer scale expansion method for measuring low levels of Stsp in blood, we were able to measure the in vivo kinetics of Stsp loss in the blood of rats. To do this, Stsp was dissolved in PEG at a concentration of 1 mg/ml and diluted 1:1 with 5% glucose to reduce the viscosity of the solution. This solution was injected into the jugular vein of a rat to administer a bolus dose of 250 μg of Stsp. Blood samples were then taken by cardiac puncture from different rats at different times ranging from 5 to 240 min after injection. A blood sample was also taken from a rat injected with the same solution containing no Stsp to use as a baseline control.

The blood was fractionated into plasma and RBC and both fractions were subjected to HPLC analysis. The Stsp was quantified by postchromoatography measurements of the computer-amplified peaks (Fig. 6, A and B). A semilog plot of this data was linear, indicating that Stsp disappeared from both plasma and RBC with first-order kinetics (Fig. 7, A and B). From the least squares fit of that data, it was found that, in vivo, the Stsp in plasma had a half-life of 51.6 min and the Stsp in RBC had a half-life of 75.3 min.

The shorter in vivo half-life of Stsp in the plasma compared to that in the RBC resulted in an increase in the RBC:plasma ratio with time (Fig. 7C). Since the blood concentration of Stsp is decreasing with time (Fig. 7, A and B), the RBC:plasma Stsp ratio is increasing with decreasing blood Stsp concentration. This observation is opposite that made in vitro where the RBC:plasma ratio decreases with decreasing Stsp concentration (Fig. 2B). This indicates that, in vivo, the Stsp is being removed from the plasma to some tissue compartment faster than the RBC can replenish it in the plasma thus driving the RBC:plasma ratio up.

Work with cultured normal and tumor cells in vitro [10,17] has indicated that cancer therapy studies with Stsp should be conducted in the 1–10 ng/ml range in vivo. Extrapolation of the data in Fig. 7 to 1 ng Stsp/ml indicated that Stsp in plasma was in the 1–10 ng/ml range for only 2.7 h and was in this range in RBC for only 4.2 h. From this data, it is concluded that bolus injections of small doses of Stsp alone are not likely to accomplish the arrest of cells in G1 in vivo due to the short duration of the time Stsp is in this concentration range.

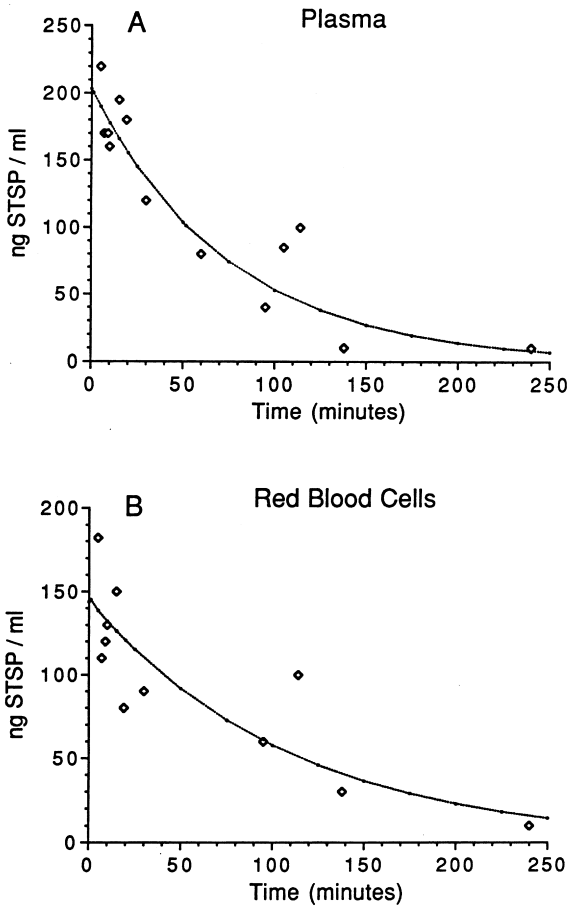

Fig. 6. Blood staurosporine levels following injections of a bolus dose of 250 μg of Stsp into the jugular veins of rats. (A) Plasma Stsp levels. (B) Red blood cell Stsp levels.

3.6. Tissue adsorption of staurosporine

The levels of Stsp in the blood at the earliest time measured in vivo (5 min in Fig. 6) was 200 ng/ml for plasma and 150 ng/ml for RBC. Assuming a rat

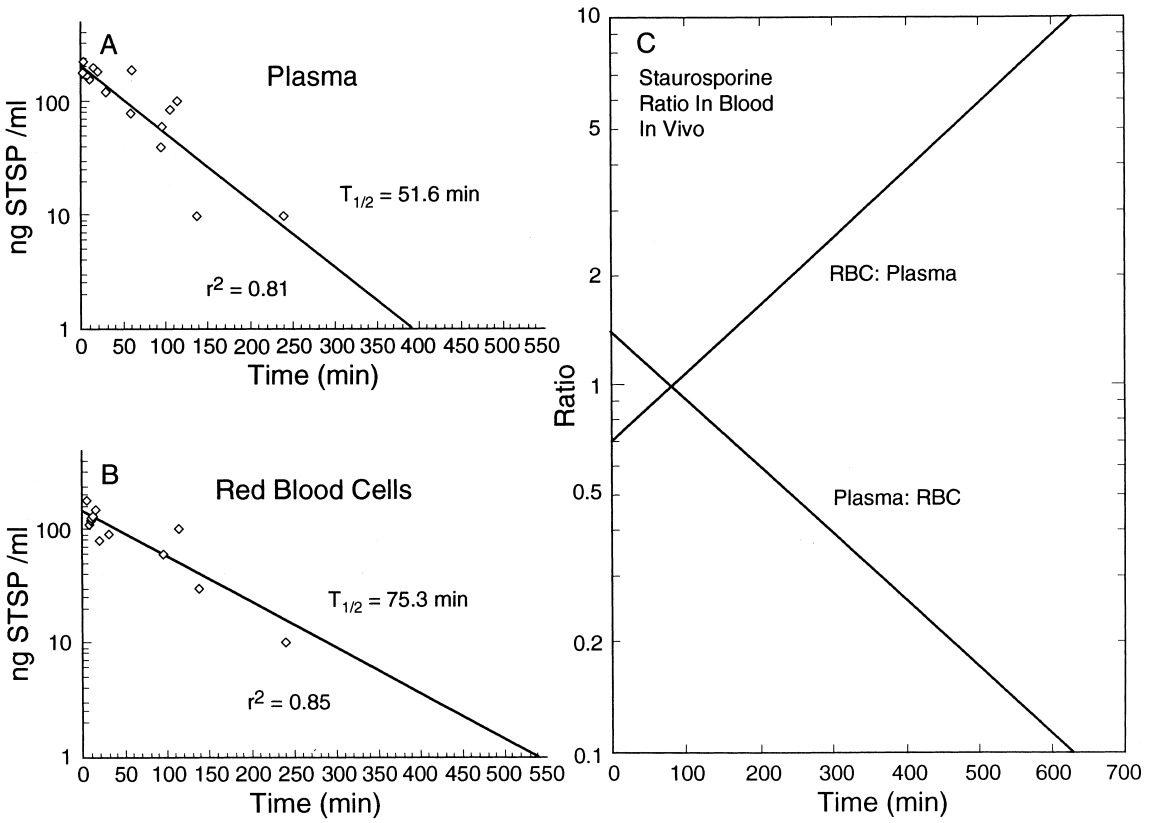

Fig. 7. Pharmacokinetics of a 250-μg bolus injection of staurosporine in rats. Semilog plots of the data in Fig. 6 were linear indicating that the kinetics of Stsp lost from both plasma (A) and red blood cells (B) was first order. The least-squares fit of the data had a correlation coefficient (r^2) of 0.81 for plasma and 0.85 for red blood cells. Extrapolation of the data indicated that it took 395 min to reduce plasma Stsp to 1 ng/ml and 540 min to reduce red blood cell Stsp to the same level. (C) The ratio of Stsp in blood. Plasma:RBC ratio decreases with time, or RBC:plasma ratio increases with time as the Stsp blood concentration decreases with time.

contains 5 ml of plasma and 5 ml of RBC, only 1750 ng of the initial 250 000 ng injected (0.7%) was found in the circulating blood 5 min after injection (Fig. 6). This means that over 99% of the Stsp is rapidly adsorbed by the tissues of the body. The data in Fig. 7C indicated that this tissue adsorption continues to some lesser extent following the initial large losses from the plasma.

To measure this tissue absorption directly, 100 μg of Stsp was injected into rats through a catheter into the superior vena cava leading to the heart. Just before injection, a needle fitted with a withdrawal syringe was inserted into the abdominal aorta and tied off so that no blood could flow past it. The Stsp was immediately injected and blood was collected immediately, as fast as the heart could pump it through the heart and lungs. Five 1-ml samples of blood were collected in sequence using vacuum vials, it taking approximately 10 s to collect each of the first three samples, 20 s for the fourth, and 30 s for the fifth. Using this surgical procedure, the Stsp made only one pass through the heart and lungs and was exposed to no other tissues. The blood samples were immediately centrifuged, the plasma and RBC were separated, and each was frozen until it could be subjected to Stsp analysis. We have previously demonstrated that these samples are stable up to 90 days when frozen [19].

Analysis of these samples indicated that the heart and lungs adsorbed almost all the Stsp from the blood stream as it passed through them one time (Fig. 8). The total Stsp recovered from rat #1 was

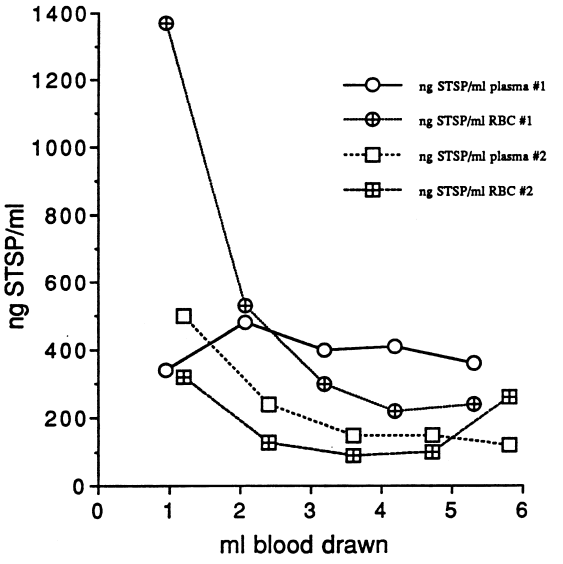

Fig. 8. Staurosporine in blood after one pass through the heart and lungs. Two rats (≠1 and ≠2) were injected with 100 μg of Stsp into the superior vena cava leading to the heart. Five sequential blood samples were immediately withdrawn from the abdominal aorta producing samples of blood that had passed through heart and lungs only once. Then ~1 ml blood samples were centrifuged into plasma and RBC and analyzed for Stsp. The concentration of Stsp is plotted vs. the total ml of blood collected.

2455 ng in 5.3 ml and that recovered from rat ≠2 was 1192 ng in 5.8 ml. This amounted to a Stsp recovery of 1.2–2.5% of that injected. This data confirmed the conclusions drawn from Figs. 6 and 7 that the body tissues adsorbed most of the Stsp. Furthermore, it demonstrated that this process is extremely fast, occurring in a matter of seconds after injection.

Another conclusion drawn from this experiment is that the plasma levels from a 100-μg Stsp injection can vary in individual rats from 100 to 400 ng/ml after one pass through the heart and lungs, a variability confirmed in another experiment (Fig. 9C to be presented below). This individual variability may account for the variability of the data points in the pharmacokinetic data in Figs. 6 and 7.

As the blood left the heart and lungs for the first time after injection, the Stsp levels in the RBC fluctuated somewhat probably due to the dynamics of the tissue adsorption, RBC adsorption and blood flow-rate (Fig. 8). However, it appears that the level of Stsp in the RBC was quickly moving to a concentration less than that seen in the plasma (Fig. 8). This reduced level in RBC compared to plasma was also seen after a steady state was achieved 5 min later (Fig. 6). In earlier experiments, when Stsp was added to blood in vitro where no tissue compartment was involved, the RBC:plasma ratio was observed to be 2:1 at high Stsp concentrations (Fig. 2B). However, at concentrations below 20 μg/ml, this concentration of Stsp into RBC was reduced in vitro (Fig. 2B). The data in Fig. 8 indicates that, in vivo, tissue adsorption reduces the Step levels in the circulating blood so low that the RBC:plasma ratio is initially reduced to less than 1. This ratio then gradually rises during the next 10 h (Fig. 7C).

3.7. Dose dependency of tissue adsorption of staurosporine

To determine if there was a dose dependency on the plasma Stsp concentration leaving the heart and lungs, bolus injections of 500 μg, 250 μg and 100 μg Stsp were made into the superior vena cava and blood samples were taken from the abdominal aorta as described in the previous experiment. Stsp analysis indicated that there was some dose dependency, but the individual animal variability overlapped the dose dependency (Fig. 9, A–C). This suggests that the tissue capacity to adsorb Stsp is greater than any of these levels of Stsp injected. This data also confirms the conclusion reached earlier that there is a wide animal variability of plasma Stsp levels after a single pass through the heart and lungs.

Blood samples were also taken 15 min after a 250-μg Stsp injection (Fig. 9D). By this time, the equilibrium between Stsp in the plasma and Stsp in body tissues and RBC had ample time to reach a steady state as indicated by the identical analysis on four successive blood samples. The plasma Stsp level at 15 min (Fig. 9D) was within the variability range of samples taken after one pass through the heart and lungs (Fig. 9B). This suggests that either the other body tissues are not adsorbing any Stsp or that a Stsp steady-state equilibrium is established between plasma, RBC and body tissues which maintains the plasma levels for the short term. This latter case would seem the most logical. For the long term, the plasma Stsp has a half-life of 51.6 min (Fig. 7A) indicating that it is either being metabolically de-

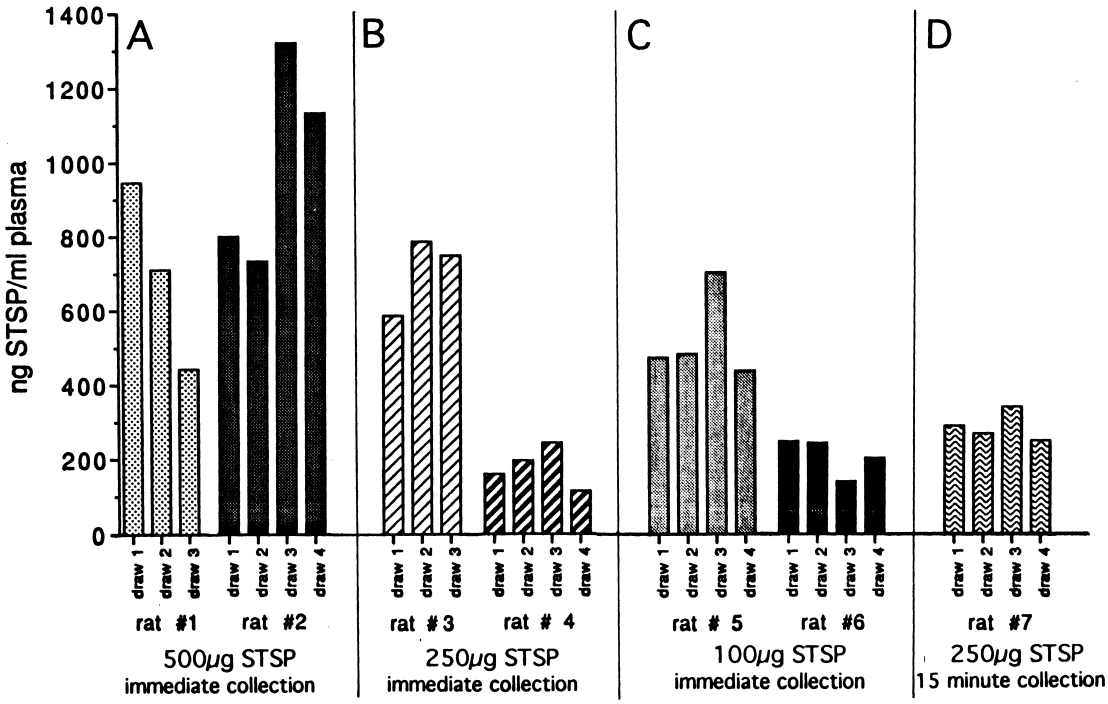

Fig. 9. Dose dependency of Stsp concentration in plasma after one pass through heart and lungs. Rats were injected with (A) 500 μg, (B) 250 μg and (C) 100 μg Stsp into the superior vena cava leading to the heart. Sequential blood samples were immediately withdrawn from the abdominal aorta producing samples of blood that had passed through heart and lungs only once. Plasma was obtained by centrifugation and analyzed for Stsp. Each bar represents the Stsp concentration in a 1–2-ml sequential blood collection designated by draw number. (D) This rat was injected with 250 μg of Stsp as the others but sequential blood samples were not collected until 15 min after injection.

stroyed, physiologically eliminated, or irreversibly deposited in some tissue such as fat.

The individual animal variability of plasma Stsp levels is not great enough to be viewed as a problem at this point. Stsp is effective as a normal G1-blocking agent within the range of 1–10 ng/ml [10]. The data in Figs. 8 and 9 indicate that the individual variation is less than 10-fold, so it should be possible to determine a dose that would generate plasma Stsp levels within that 10-fold range.

Extrapolation of the data from the 100-μg Stsp injections (Figs. 8 and 9) which produced plasma levels of 200–700 ng/ml suggests that 1-μg Stsp injections should produce a 2–7-ng/ml Stsp plasma level which is in the effective range. The short half-life of Stsp in plasma (51.6 min in Fig. 7) indicates that in order to maintain the Stsp level in plasma at 1–10 ng/ml for the 2–3 day period necessary to achieve the in vivo G1 arrest necessary to begin cancer therapy, it will be necessary to infuse the Stsp at some low level following the initial bolus injection.

4. Discussion

Previous work in our laboratory using cultured cells has demonstrated that Stsp arrests normal proliferating cells in the G1 phase of the cell cycle, while permitting transformed cells to continue to enter S phase and synthesize DNA [10]. This lead to suggestions that Stsp might be useful in cancer-treatment strategies by arresting normally proliferating bone morrow and intestinal mucosa cells in G1 thereby sparing them from the toxic effects of chemotherapeutic drugs that kill cancer cells undergoing DNA synthesis in S phase [11]. Other laboratories have suggested using Stsp directly as a cancer chemotherapueutic drug [15–17] and as a biochemical inhibitor in physiological and biochemi-

cal studies [2–9]. To determine if the Stsp effects observed in vitro could be expressed in vivo, we injected rats with Stsp, but could not detect any G1 arrest in bone marrow cells using flow cytometry [21]. Those studies indicated that the pharmacokinetics of Stsp must be determined before proceeding further with such studies of the use of this drug in vivo. Therefore, we initiated a program to determine the fate of Stsp in vivo. First we developed an HPLC analysis method for measuring Stsp levels in blood [18,19]. Now, in this report we have used that Stsp assay to determine the propensity of cells and tissues for sequestering the drug and to measure the pharmacokinetics of Stsp in vivo.

Measurements of Stsp in the blood of rats given a bolus i.v. injection of 250 μg of Stsp indicated that only 0.4% of that Stsp remained in the circulating plasma 5 min after injection. Such rapid removal of Stsp suggested that the RBC (which are suspended in the plasma) or the body tissues (especially the endothelium of the heart and lungs) probably were sequestering the Stsp and preventing it from reaching the bone marrow target in our preliminary experiments [21].

In vitro experiments in which whole blood was spiked with Stsp revealed that RBC adsorbed Stsp from the plasma immediately (in less than the 2 min it took to do the experiment) and concentrated it at a RBC:plasma ratio of 2:1. However, further experiments in which RBC loaded with Stsp were exposed to Stsp-free plasma demonstrated that the RBC readily released the Stsp back to the plasma. Thus, it is concluded that the binding of Stsp in the RBC is weak and reversible. Dose dependency studies demonstrated that a threshold of 20 μg of Stsp/ml of whole blood was necessary for the 2:1 concentration of Stsp in RBC. Below this concentration the RBC:plasma Stsp ratio was 1:1 or less indicating that the Stsp in the RBC was in equilibrium with that in the plasma suggesting it freely passes in and out of the RBC by passive diffusion. This data indicates that at the low plasma concentrations of Stsp anticipated for cancer therapy strategies (1–10 ng/ml), sequestration of Stsp in RBC should not be a serious problem.

The above experiments suggested that the rapid loss of Stsp from the circulating plasma must be due to adsorption by the body tissues. To determine the rate at which this occurs, pharmacokinetic studies on Stsp in vivo were performed using a modification of Stsp analysis system that quantified nanogram concentrations in blood. In these experiments, 250 μg of Stsp was injected into the jugular veins of rats, and blood samples were taken by cardiac puncture at various times thereafter. It was found that Stsp disappeared rapidly from both plasma and RBC with first-order kinetics. The plasma Stsp had a half-life of 51.6 min and the RBC Stsp had a half-life of 75.3 min. From this data, it was determined that Stsp in the plasma was in the 1–10 ng/ml range in vivo for only 2.7 h. This was not long enough to create a G1 synchrony in the proliferating bone marrow cells. This data clearly indicates that a single bolus injection of Stsp alone would not accomplish the G1 arrest of cells needed in a therapeutic regimen of chemotherapy.

From that data, it was also determined that only 0.7% of the Stsp injected remained in the blood stream (in plasma and RBC) after 5 min, which meant that over 99% of the Stsp was rapidly adsorbed by the tissues of the body. We suspected that much of this adsorption occurred on the endothelium of the heart and lungs which was the tissue first exposed to Stsp after injection. This was confirmed in experiments in which Stsp was injected into the blood stream just before it entered the heart and which was withdrawn immediately after leaving the lungs. Analysis of this blood demonstrated that only one pass of the Stsp through the heart and lungs was necessary for those tissues to adsorb all but 1.2%–2.5% of the initial Stsp injected. These measurements confirm, in vivo, conclusions drawn from in vitro experiments where saturable binding sites, which bound [^{3}H]Stsp with high affinity and in a reversible manner, have been found on the surface of cultured capillary endothelial cells [22]. Our experiments also demonstrated that the RBC:plasma Stsp ratio was less than 1 as the blood left the lungs confirming in vivo our earlier conclusion drawn from in vitro experiments that at low plasma Stsp concentrations, the RBC are not a major sequestering sink for Stsp.

The fact that the RBC can transport Stsp and readily release it as plasma concentrations decrease is probably advantageous since it will prevent some of the drug from being adsorbed as it passes through the heart and lungs and, thus, make it available for

target tissues downstream. That this process is occurring is demonstrated by the fact that Stsp disappears from both plasma and RBC with first-order kinetics but the RBC Stsp has a longer half-life than the plasma Stsp.

In conclusion, the data of this report support an in vivo model of Stsp kinetics in which 99% of the injected Stsp is rapidly adsorbed by the endothelium cells of the circulatory system, thus greatly reducing the initial plasma levels of Stsp. The remaining 1% is circulated throughout the body, the Stsp being partitioned between plasma and RBC with slightly more Stsp in plasma than in the RBC. The circulating levels of Stsp then decay by a first-order kinetic process, in which the plasma Stsp concentration is reduced with a half-life of 52 min. Extrapolation of the data of these experiments suggests that a 1-μg Stsp bolus injection should produce an initial Stsp plasma level of 2–7 ng/ml, which is in the effective range for G1 arrest of normal cells. Because of the short plasma half-life of Stsp in vivo, however, it will be necessary to then continuously infuse Stsp for several days to maintain this level and produce a G1 arrest in tissues such as bone marrow and intestinal mucosa [10,11]. Experiments will now be necessary to determine if these concentrations will produce life-threatening side effects on physiological functions such as low blood pressure, platelet aggregation and membrane Ca^{2+} pump functions mentioned earlier.

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