Biochemical Pharmacology, Vol. 21, pp. 1609–1624. Pergamon Press, 1972. Printed in Great Britain.

STUDIES ON THE BINDING OF [^{3}H]VINBLASTINE BY RAT BLOOD PLATELETS *IN VITRO*

EFFECTS OF COLCHICINE AND VINCRISTINE*

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(*Received* 7 *November* 1971; *accepted* 17 *December* 1971)

Abstract—Tritiated vinblastine (^{3}H-VLB) of high specific activity was prepared by proton exchange with tritio-trifluoroacetic acid. The compound was used to study the binding *in vitro* of vinblastine by rat blood platelets in citrated platelet rich plasma. At 37°, ^{3}H-VLB was initially taken up very rapidly by platelets; after ca. 90 min the amount bound reached a plateau which was maintained for at least 4 hr. The compound was bound as such and was not metabolized to any significant extent by platelets. The binding process was apparently fully reversible. A plot of the amount of ^{3}H-VLB bound at equilibrium vs. the concentration in the plasma gave a sigmoidal curve. A Scatchard plot of the data indicated that at vinblastine plasma concentrations up to 0·06 μg/ml, when the platelets were less than one-quarter saturated, there was a "co-operative" (possibly allosteric) interaction between the alkaloid and platelet receptors; at higher vinblastine concentrations, the binding followed the simple Langmuir isotherm. Association and rate constants for the simple mechanism—

$$\text{vinblastine} + \text{platelets} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{vinblastine–platelet complex}$$

—were derived from the Scatchard plot and from an analog computer analysis of the binding kinetics. The platelet saturation value at 37° was 0·3 μg vinblastine per 10^9 platelets. Non-radioactive vinblastine rapidly displaced (and equilibrated with) platelet-bound ^{3}H-VLB. Vincristine, in terms of its ability to displace ^{3}H-VLB, interacted much more slowly than vinblastine with platelets. Colchicine, even at high concentrations, did not displace ^{3}H-VLB; instead it enhanced significantly the vinblastine-binding capacity of platelets. The results suggest that platelet preparations may be suitable model systems for studying the binding of certain antimitotic drugs to cell receptors. The marked ability of platelets to concentrate vinblastine from plasma and the ready reversibility of the process indicate that platelets could have an important role in the distribution of the drug *in vivo*.

THE DIMERIC indole alkaloid, vinblastine (Fig. 1), is used in the treatment of certain cancers. In studying the metabolism and distribution of the tritiated alkaloid in the rat, we have recently found that after administering sub-acutely toxic doses, a major proportion of the circulating alkaloid was bound to the blood platelets.[1] It was suggested that the platelets could have a function in transporting the alkaloid to "sensitive" tissues such as bone-marrow or tumors and also to the liver where it

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* This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada.

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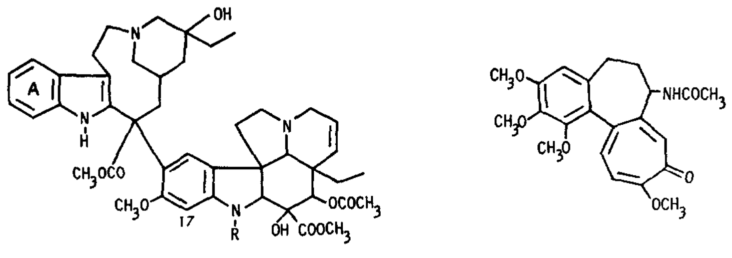

VINBLASTINE $R = CH_3$

VINCRISTINE $R = CHO$

COLCHICINE

FIG. 1. Alkaloids used in the present study.

appears to be metabolized.[2] The pharmacological and therapeutic properties of the drug might thereby be mediated to some extent by the platelets.

It has also been shown in a preliminary experiment that when platelet-rich plasma was incubated *in vitro* with tritiated vinblastine, a substantial amount of the radioactivity was taken up by the platelets.[1] The degree of platelet labeling *in vitro* was in good agreement with the amount of alkaloid bound to the platelets in the animal, suggesting that certain of the pharmacological features of the interaction of platelets with vinblastine could validly be studied *in vitro* using platelet preparations. The present communication, in part, describes a more detailed investigation of the labeling of platelets when platelet-rich plasma is incubated *in vitro* with tritiated vinblastine. The preparation of the labeled alkaloid is described and the chemical nature of the platelet bound radioactivity has been determined. The reversibility of the platelet–vinblastine interaction has been studied and estimates made of some of the binding constants.

Vinblastine, at appropriately low concentrations (e.g. similar to those in the blood of rats receiving the drug),[1] can arrest the replication of mammalian cells by interfering, apparently rather specifically, with the assembly and/or function of the microtubules in the mitotic "spindle". It seems very likely that the toxicity and anti-cancer activity of vinblastine are due, at least partially, to its anti-mitotic properties. Similarly vincristine, a vinca alkaloid structurally related to vinblastine, and colchicine (Fig. 1), a tropolone alkaloid, are both anti-mitotic compounds which are also able to inhibit the growth of certain tumors. It appears to be significant that all three alkaloids induce similar structural changes in platelets, particularly the loss of their discoidal shape, and this change in shape has in fact been related to the disruption of the circumferential band of microtubules present in the cells.[1,3,4] In view of these correlations, it is proposed that platelet preparations may provide comparatively simple and convenient model systems for studying the interactions of mitotic inhibitors with cell receptors, and give information on the biochemical and biophysical mechanisms of uptake and binding of these compounds which might be relevant not only to platelets but also to other cell types. Some preliminary results on the effects of vincristine and colchicine on the binding of vinblastine by platelets *in vitro* are included in this communication.

MATERIALS AND METHODS

Materials. Vinblastine sulphate and vincristine sulphate were gifts from Eli Lilly & Company, Indianapolis, Ind. Colchicine was purchased from the British Drug Houses, Poole, England. Tritio-trifluoroacetic acid was obtained from the New England Nuclear Corp., Boston, Mass., and consisted of CF_3COOH (10 mg) to which 1 c of carrier-free ^3H_2O had been added.

Preparation of tritiated vinblastine. Vinblastine was labeled with tritium by the proton exchange reaction which takes place between strong acids and aromatic compounds. Tritio-trifluoroacetic acid was used as the strong acid since it not only provides the labeled atom, but is also a good solvent for vinblastine, giving solutions in which there is minimal degradation of the alkaloid during the exchange process. A simple vacuum line was used to transfer the [^{3}H]trifluoroacetic acid to the alkaloid and also to remove it at the end of the reaction.

Vinblastine sulphate (5·8 mg) was weighed into a small reaction tube which was then attached to the vacuum line and heated at $\sim$ 70° for 2 hr at 10^{-5} torr in order to remove traces of moisture. [^{3}H]trifluoroacetic acid (10 mg) was then transferred *in vacuo* to the reaction tube by immersing the latter in a dry ice/MeOH bath. When the transfer was complete the reaction tube was filled with dry nitrogen to atmospheric pressure. The alkaloid dissolved to give a viscous solution which was allowed to stand for 2 days at room temperature in the dark. The excess trifluoroacetic acid was then pumped off and the semi-crystalline residue of vinblastine sulphate dissolved in 0·2 ml methanol and diluted with 2 ml hot ethanol. Crystallization began as the solution cooled and was completed by storing overnight at −25°C. The crystals were collected by centrifugation and recrystallized twice more from methanol–ethanol as described above. The yield of the recrystallized tritiated vinblastine sulphate was estimated from the u.v. absorption at 262 nm and the radioactivity by liquid scintillation counting; 2·2 mg tritiated vinblastine sulphate was obtained—specific activity $6{\cdot}5 \times 10^9$ dis./min/mg (2·6 c/m-mole). The u.v. spectrum of the compound was identical with that of authentic vinblastine sulphate. When the labeled alkaloid was chromatographed on thin-layer sheets of alumina or Silica (Eastman Kodak 6063 and 6060, respectively), its R_f values were identical with those of authentic vinblastine. Its radiochemical purity was determined by isotopic dilution analysis using procedures already described.[1,5] An aliquot of the labeled compound was added to a large excess of carrier vinblastine, and the mixture then chromatographed on columns of either carboxymethyl cellulose[5] or cellulose phosphate.[1] The profile of the radioactivity in the eluted fractions coincided with the optical density of the carrier vinblastine (Fig. 2). It was calculated from the specific activity of the alkaloid in the peak tubes that 96 ± 3 per cent of the radioactivity applied to the column was present as tritiated vinblastine.

The tritiated vinblastine sulphate was stored at −25° in methanol; the degradation of the compound was minimal under these conditions ($\ngtr$ 10 per cent a year). When necessary, the compound was repurified either by thin-layer chromatography or on ion-exchange columns as described above.

Other studies in this laboratory have shown that the tritiated alkaloid was as effective biologically as authentic (non-radioactive) vinblastine. For example, the replication of L5178Y mouse lymphoma cells in culture was inhibited by the labeled alkaloid at a concentration of 0·01 μg/ml.

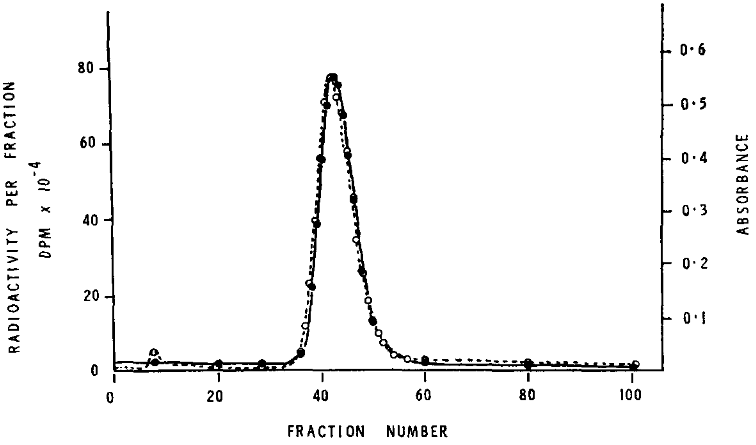

FIG. 2. Chromatography of a mixture of purified tritiated vinblastine and non-radioactive carrier vinblastine on a carboxymethyl cellulose column. $[^3H]$vinblastine, 1·1 μg, 7·15 × 10^6 dis./min; carrier vinblastine, 1·43 mg. Column (8 g) was eluted with 0·04 M phosphate buffer, pH 3·7;[5] fraction vol., 4·5 ml. ○– – – –○, radioactivity; ●———●, absorbance of effluent at 262 nm.

Nuclear magnetic resonance studies on deutero-vinblastine prepared from deutero-trifluoroacetic acid following the procedure described above have shown that proton exchange occurs rapidly in the vindoline moiety at position 17 and more slowly in the aromatic ring of the velbanamine portion (Fig. 1A).

Preparation of platelet rich plasma. Platelet rich plasma was prepared from the heart blood of lightly etherized male Hooded rats (350 ± 50 g), using a slight modification of a method already described.[1] The blood (8 ml) was drawn into a 10-ml plastic syringe which already contained 1·6 ml of 0·15 M citrate as anticoagulant. The syringe was inverted several times to mix the contents which were then slowly expelled into a siliconized centrifuge tube. A solution of dextran (mol. wt. 100,000–200,000, Mann Research Laboratories, Inc., N.Y.) in 0·9% NaCl solution (6% w/v) was then gently mixed in to give a final dextran concentration of 10 mg/ml citrated blood. After centrifuging the mixture at 80 *g* for 20 min at room temperature, the platelet-rich supernatant was removed with a siliconized pipette. The average platelet count was 1 × 10^6/mm^3; red cell contamination was negligible and the luekocyte count was usually $\ngtr$ 400/mm^3.

Incubation of platelet-rich plasma with alkaloids. Since the vinca alkaloids are light sensitive, all incubations were conducted in the dark with only intermittent exposure to dim light for sampling. The uptake and release of $[^3H]$vinblastine by platelets was measured by incubating 2-ml aliquots of platelet rich plasma with the alkaloid in siliconized tubes in an atmosphere of air. The tubes were either rocked slowly (~ 20 times/min) or rotated end over end at 3 rev/min. Unless otherwise stated, the temperature was 37°. The labeled alkaloid was added at the start of the incubation in amounts indicated in the Results. In most experiments, non-radioactive vinblastine, vincristine or colchicine were also added either at zero time or after 2 hr, depending on whether direct competition or displacement effects were being studied. The alkaloids were added dissolved in small volumes (5–50 μl) of 0·9% NaCl solution. At intervals throughout the incubation, samples of the platelet-rich plasma (100 ± 10 mg) were withdrawn into preweighed 7-cm lengths of polyethylene tubing, 0·066 in. i.d. (Intramedic, Clay Adams). The tubes were reweighed, sealed at one end and centrifuged for 7 min at 4300 *g* (Hematocrit head No. 952; International Equipment Company, Boston, Mass.). The sealed end of the tube containing the platelet pellet was cut off with a razor blade as nearly as possible 2 mm above the seal. The platelet-free plasma in the upper portion of the tube was centrifuged directly into a scintillation vial for radioassay.

The radioactivity of the platelet pellet and the platelet-free plasma was determined in a liquid scintillation counter (Packard TriCarb, Model 3003), using solubilization procedures previously described.[1] All samples were counted to an accuracy of ± 3 per cent at the 95 per cent confidence level, and the data corrected for quenching. The platelet radioactivity was corrected for contamination by the small amount of plasma associated with the pellet. This correction was *ca.* 5 per cent of the radioactivity present in the platelet-free plasma.

RESULTS

Low vinblastine concentrations—effect of temperature, nature of bound radioactivity. The labeling of platelets when platelet-rich plasma was incubated at 37° and 4° with a low concentration of tritiated vinblastine (0·05 μg/ml) is shown in Fig. 3. The uptake of radioactivity at 37° was initially very rapid; it reached a plateau after about 90 min, when approximately 70 per cent of the total isotope was bound by the platelets. The radioactivity in the platelets is loosely bound since it could be readily released by extraction at pH 7–8 with benzene or by extraction with dilute acid. Isotopic dilution analysis showed that after incubating [^{3}H]vinblastine with platelet-rich plasma for 1 and 3 hr at 37°, 98 and 92 per cent, respectively, of the platelet-bound radioactivity was still due to vinblastine. These figures indicate that the compound was not metabolized or degraded to any significant degree under the conditions employed.

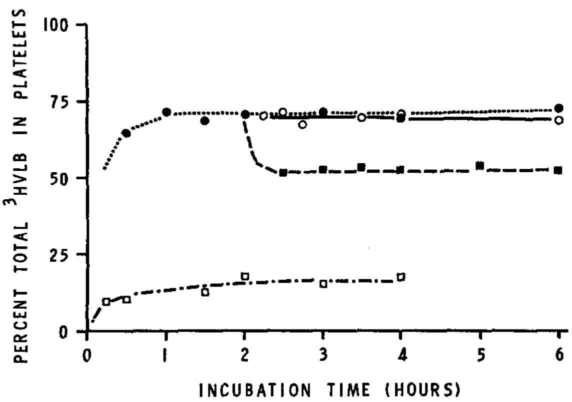

FIG. 3. Effect of temperature and of resuspension in fresh plasma on the binding of [^{3}H]vinblastine by rat platelets *in vitro*. [^{3}H]vinblastine conc: 0·05 μg/ml; 10^9 platelets/ml plasma. ●– – – –●, incubated at 37°; □ – — – □, incubated at 4°; ○———○, incubated at 4° after 2 hr at 37°; ■ — ■, platelets prelabeled by incubating for 2 hr at 37°, then collected by centrifugation and resuspended in non-radioactive platelet-free plasma equal in volume to the original sample.

At 4° the uptake of radioactivity was very much slower and tended to a plateau after 1·5 hr at which time only approximately 17 per cent of the radioactivity was bound by the platelets. On the other hand, platelets which had been labeled by pre-incubation with [^{3}H]vinblastine for 2 hr at 37° released the bound radioactivity to only a very limited extent when the incubation temperature was lowered to 4° (Fig. 3). These results suggest the initial uptake is temperature dependent, but that once the alkaloid is bound, the degree of retention is not affected by the change in temperature.

When platelets which had been labeled by incubating platelet-rich plasma with [^{3}H]vinblastine (0·05 μg/ml, 2 hr, 37°) were separated by centrifugation and re-incubated in non-radioactive platelet-free plasma, some of the bound alkaloid was released into the plasma (Fig. 3). Equilibrium between the bound and free alkaloid was established rapidly ($<$ 30 min) with $\sim$ 70 per cent of the redistributed radioactivity once more being retained by the platelets or, as is shown in Fig. 3,70 $\times$ 70 per cent $\simeq$ 50 per cent of the total radioactivity originally present.

Effect of alkaloid concentration on platelet labeling. Platelet-rich plasma was incubated with [^{3}H]vinblastine in concentrations ranging from 0·025 to 4·5 μg/ml; the labeling of the platelets was monitored over a period of several hours. The platelet-bound radioactivity is expressed in Fig. 4 as a percentage of the total radioactivity present in the sample. Throughout the concentration range 0·025–0·18 μg/ml, the percentage of the radioactivity in the platelets at equilibrium was essentially constant and only some of the values in this range have therefore been plotted. At higher vinblastine concentrations, the percentage of bound radioactivity progressively declined as the alkaloid concentration was increased. At all alkaloid concentrations, the rate of uptake was initially very rapid and eventually reached a plateau after approximately 90 min incubation.

A plot of the amount of [^{3}H]vinblastine bound at equilibrium by the platelets vs. the concentration of free alkaloid in the plasma is shown in Fig. 5. The amounts of bound vinblastine and the plasma concentrations were calculated from the per cent uptake values at 2 hr in Fig. 4 and from the per cent uptake data at the additional concentrations referred to above in the range 0·025–0·18 μg/ml. Up to a point, as the concentration of vinblastine in the plasma was increased, the amount of platelet-bound alkaloid also increased until, when the plasma concentration was $\sim$ 0·7 μg/ml, approximately 0·3 μg vinblastine was bound per 10^9 platelets. The platelets then appeared to be essentially saturated with vinblastine since there were only minor increases in the amount of alkaloid bound even if the concentration in the plasma was markedly increased. From the results in Fig. 5 and previous data,[1] it may be concluded that the amount of vinblastine bound by platelets (i) in rats given small doses of the alkaloid, or (ii) when platelet-rich plasma is incubated with 0·05 μg VLB/ml, is about one-tenth of the amount bound by the platelets at saturation.

The results in Fig. 5 show that when the amount of bound vinblastine is plotted against the concentration of VLB in the plasma, the points do not fall on a hyperbolic curve as would be expected if the binding followed the simple Langmuir isotherm; instead a sigmoidal curve is obtained. Certain of the binding characteristics are more clearly shown when the ratio, platelet-bound VLB/plasma VLB concentration, is plotted against the "platelet-bound VLB", according to the method of Scatchard.[6] A plot of this type (Fig. 6) expresses a hyperbolic type binding curve as a straight line, and this condition is satisfied quite closely over the higher plasma concentration

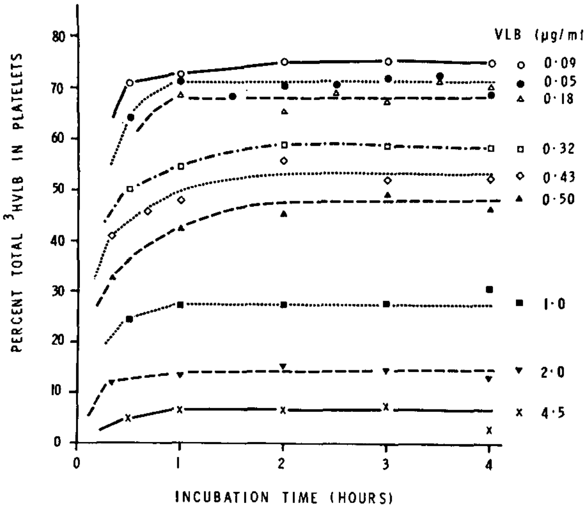

FIG. 4. Percentage of total radioactivity bound by platelets when platelet-rich plasma (10^9 platelets/ml) was incubated at 37° with various concentrations of [^{3}H]vinblastine. Other incubation conditions are as in Methods.

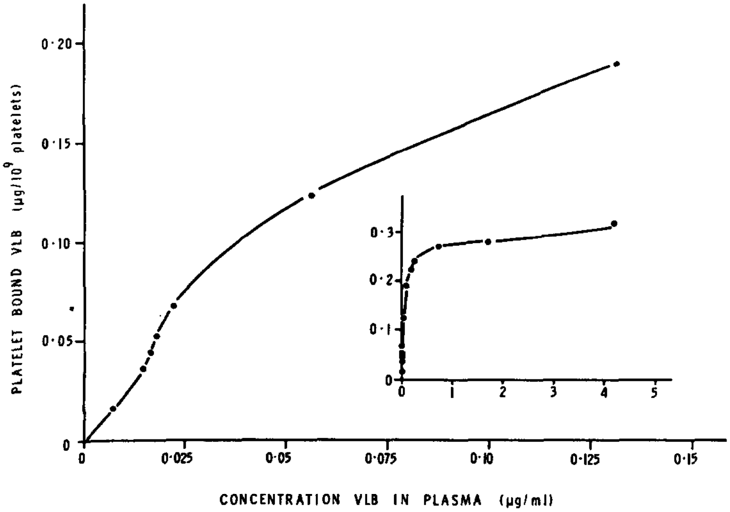

FIG. 5. Plot of the total amount of vinblastine bound by platelets vs. the concentration of vinblastine in the plasma. The values for the platelet-bound alkaloid (µg/10^9 platelets) and the concentrations of free alkaloid (µg/ml plasma) were calculated from the distribution of radioactivity between platelets and plasma after a 2-hr incubation with [^{3}H]vinblastine (i.e. when the platelet-bound radioactivity had reached a plateau). The points on the curves are derived from the results shown in Fig. 4 and from other similar experiments. The units on the inset graph are the same as in the larger graph which shows an expanded version of the rising portion of the smaller curve.

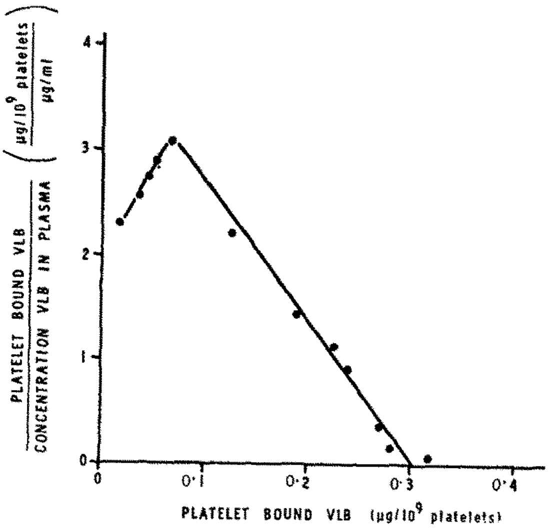

Fig. 6. Scatchard plot of the binding of vinblastine by platelets. The values for the bound vinblastine (μg/10^9 platelets) and the concentrations of free vinblastine (μg/ml plasma) were obtained from the data plotted in Fig. 5.

range (0·06–1·7 μg/ml). At the lower concentrations, however, the points no longer fall on or near the linear portion of the plot. The increase in the ratio, platelet-bound VLB/plasma VLB concentration, following small increases in concentration in the lower range indicates that the initially bound alkaloid significantly enhances the binding capacity of the platelets. This effect occurs when the latter are still less than one-quarter saturated with the alkaloid.

The intercept of the Scatchard plot on the abscissa gives the vinblastine-binding capacity of the platelets at saturation as 0·3 μg vinblastine/10^9 platelets. This agrees with the maximum uptake of vinblastine shown in Fig. 5. The slope of the Scatchard plot (13·5 ml/μg) when converted into molar quantities is the association constant, $K_A = 1{\cdot}23 \times 10^7$ l. moles^{-1} (or $K_D = 0{\cdot}82 \times 10^{-7}$ moles l.$^{-1}$).

Reversibility of platelet–vinblastine binding. The results in Fig. 3 indicate that a portion of the platelet-bound vinblastine was released when the labeled platelets were re-incubated at 37° in fresh alkaloid free plasma. When the new plateau had been reached, the percent distribution of the tritiated alkaloid between the plasma and the platelets was quite close to that found in the original platelet-rich plasma. This result suggested that the platelet bound and plasma alkaloid might be freely in equilibrium. This particular method for studying the release of platelet-bound vinblastine was not, however, entirely satisfactory, since the platelets were subjected to the trauma of high speed centrifugation and resuspension (with a possible effect on their binding properties) and the proportion of bound vinblastine released into the plasma was inconveniently low for precise assay. The release of platelet-bound vinblastine was therefore studied by measuring the extent to which platelet-bound tritiated vinblastine was displaced following the addition of non-radioactive carrier vinblastine. The platelets were first pre-labeled by incubating platelet-rich plasma with tritiated vinblastine (0·05 μg/ml) for 2 hr, and carrier alkaloid was then added to give final concentrations ranging from 0·05 (control) to 9·4 μg/ml. Figure 7a shows that following the addition

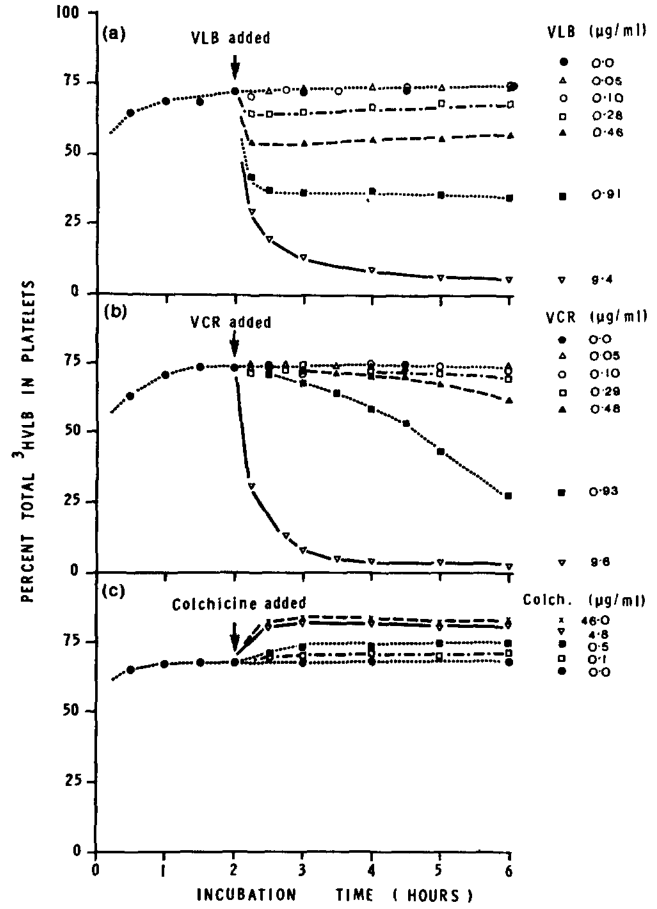

FIG. 7. Effect of non-radioactive vinblastine, vincristine and colchicine on the retention of radioactivity by platelets which had been pre-labeled with [^{3}H]vinblastine. Platelet-rich plasma was incubated for 2 hr at 37° with [^{3}H]vinblastine at a concentration of 0·05 μg/ml and the non-radioactive alkaloids were then added at the concentrations indicated above.

of the carrier alkaloid there was a rapid release of some of the platelet-bound alkaloid and the establishment of new plateau levels. The amounts released were related to the concentrations of the diluent alkaloid added. At the highest concentration (9·4 μg VLB/ml), over 90 per cent of the initially bound radioactivity was displaced. When calculated on the basis that the added carrier alkaloid equilibrated with its radioactive counterpart, the actual amounts of vinblastine bound by the platelets after adding the carrier were very close to those taken up by the platelets when they were incubated from the start with the same total concentration of alkaloid. For example, at the new plateau established after increasing the vinblastine concentration to 0·51 μg/ml (Fig. 7a) the amount bound was 0·235 μg/10^9 platelets (assuming the tritiated alkaloid was uniformly diluted with the carrier); when the alkaloid was present from the start of the incubation at a concentration of 0·5 μg/ml (Fig. 4), the amount bound was 0·233 μg/10^9 platelets. The results show that the tritiated alkaloid taken up by the platelets during the initial 2-hr incubation period was reversibly bound and appeared to equilibrate rapidly with the plasma alkaloid. It may be noted that when the lower concentrations of diluent vinblastine ($\leqslant$ 0·1 μg/ml) were added to the labeled platelets there was no significant displacement of the radioactivity. This may appear to conflict with the conclusion that equilibrium is readily established between the bound and the plasma alkaloid. However, at the lower concentrations, the platelets are far from saturated with the alkaloid and, although the platelet [^{3}H]vinblastine may rapidly equilibrate with and be diluted by, the non-radioactive compound, the increase in the total amount taken up by the platelets compensates for the dilution. At the higher concentrations ($\geqslant$ 0·28 μg/ml), the additional amounts of vinblastine bound by the platelets do not compensate for the concurrent isotopic dilution of the labeled alkaloid.

Effect of vincristine and colchicine on platelet labeling. In view of the structural similarity between vinblastine and vincristine, the ability of vincristine to displace platelet-bound tritiated vinblastine was examined. Following the procedure just described, platelets were labeled by incubating platelet-rich plasma for 2 hr with [^{3}H]vinblastine (0·05 μg/ml). Non-radioactive vincristine was then added in concentrations ranging from 0·05 to 9·6 μg/ml. The effect of vincristine on the release of platelet-bound vinblastine is shown in Fig. 7b. In contrast to the rapidity with which platelets released the labeled vinblastine when incubated with its non-radioactive counterpart (Fig. 7a), radioactivity was released only slowly in the presence of vincristine except at the highest concentration. The absence of a true plateau indicates that, even after 4-hr incubation, the re-distribution of [^{3}H]vinblastine between the platelets and plasma had still not reached a new equilibrium.

The effect of colchicine on the labeling of platelets by tritiated vinblastine was studied in an experiment similar to those just described for vinblastine and vincristine, i.e. the colchicine was added at various concentrations after the platelet radioactivity had reached its plateau value. In contrast to the release of platelet radioactivity which followed the addition of vinblastine and vincristine, colchicine actually enhanced the vinblastine-binding capacity of the platelets (Fig. 7c). There was a detectable effect even at the low colchicine concentration of 0·1 μg/ml; in the presence of colchicine concentrations $\geqslant$ 5·0 μg/ml, the percentage of the total tritiated vinblastine bound by the platelets increased from 68 per cent in the control to ca. 83 per cent. The colchicine-induced enhancement of the vinblastine binding was maintained at a steady level throughout the 4-hr period the platelets were incubated with the alkaloid.

As shown in Fig. 7b, platelets pre-labeled with [^{3}H]vinblastine slowly released the alkaloid following the subsequent addition of vincristine. The effect of vincristine on the uptake as well as on the retention of [^{3}H]vinblastine was studied more directly by incubating platelet-rich plasma with both alkaloids present from the start of the incubation period (Fig. 8). At the concentration of [^{3}H]vinblastine used in this experiment (0·5 μg/ml), the amount of alkaloid available was in excess of that needed to saturate the platelets; vincristine was present in concentrations up to 20-fold that of the vinblastine. Vincristine concentrations up to twice that of vinblastine had only a minor effect on the initial rate at which platelets bound the tritiated vinblastine. For example, the amounts of the latter bound at 30 min were only a few per cent less than in the control (VCR concentration = 0). However, in the period corresponding to that in which the rate of platelet labeling in the control declined markedly ($\sim$ 60 min), the inhibitory effect of the lower concentrations of vincristine on vinblastine

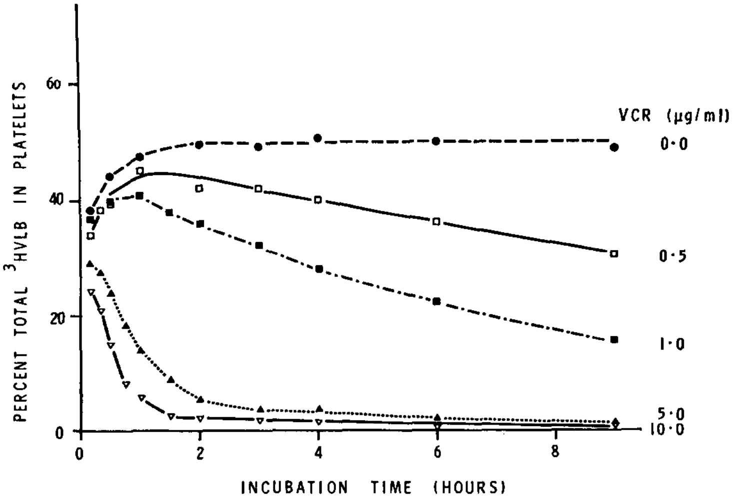

FIG. 8. Effect of vincristine on the binding of [^{3}H]vinblastine by platelets at 37°. [^{3}H]vinblastine concentration 0·5 µg/ml; 10^9 platelets/ml of platelet-rich plasma. The vincristine was added concurrently with the vinblastine.

binding became more obvious. Thus as incubation continued, the amounts of vinblastine bound by the platelets passed through a maximum and then decreased slowly at a rate which was proportional to the vincristine concentration. In the presence of vincristine concentrations 10 to 20-fold that of the vinblastine, the amounts of the latter taken up by the platelets in the initial incubation period were much less and the subsequent release of the bound alkaloid more rapid. A qualitative interpretation of the results in Fig. 8 is that vinblastine is bound much more rapidly than vincristine to vinblastine acceptor sites; vincristine then slowly occupies and/or inactivates these sites in terms of their vinblastine-binding ability. A simple quantitative model based on this proposal is considered in the Discussion.

DISCUSSION

In a previous study[1] it was shown that when rats were given sub-acutely toxic doses of tritiated vinblastine, a high proportion (two-thirds) of the circulating radioactivity was concentrated in the blood platelets, mainly as the unchanged alkaloid. A preliminary experiment also showed that when platelet-rich plasma was incubated *in vitro* with tritiated vinblastine at a concentration similar to that found in the blood of the rats, three-quarters of the isotope was bound by the platelets. This earlier study suggested that an investigation of the interaction of vinblastine with platelets *in vitro* might yield information relevant to the pharmacology and mode of action of vinblastine *in vivo*. In the present study, conditions *in vivo* have been partially simulated by incubating the alkaloid with platelet-rich plasma at 37° rather than with suspensions of washed cells in buffer. The results (Fig. 3) show that tritiated vinblastine was taken up and concentrated very readily by the platelets. The latter do not appear to metabolize the alkaloid to any significant extent since even after several hours incubation there was no loss of radioactivity from the platelets and essentially all the platelet-bound radioactivity was still due to vinblastine. When the results in Fig. 5 are considered in relation to the levels of vinblastine in the blood of rats given sub-acutely toxic doses,[1,5] it is clear that although the platelets were carrying a major proportion of the circulating alkaloid, they were still far from being saturated with it. For instance, at the higher vinblastine concentrations, up to 0·3 μg of the alkaloid were bound per 10^9 platelets, which is about 10-fold the amount present in the circulating platelets (0·032 μg VLB/10^9 platelets).[1]

The platelet-bound vinblastine exchanged freely with the plasma alkaloid and readily reached equilibrium with it (Figs. 3 and 7). The process was apparently fully reversible with equilibrium markedly in favor of the platelet–alkaloid complex ($K_{\text{Assoc}} = 1{\cdot}23 \times 10^7$ l. moles^{-1}). Both the high VLB-binding capacity of the platelets and the readiness with which the alkaloid was released support the suggestion that platelets may play an important role in transporting the drug *in vivo* and in mediating its pharmacological activity. It may be anticipated that the platelet-bound vinblastine will be released when the concentration of the alkaloid in the plasma falls to low levels, for example when the blood passes through tissues which are able to remove the alkaloid either by binding it as such or by metabolizing it to other compounds.

The biochemical and cytological nature of the platelet receptors to which vinblastine binds is not known. Platelets are able to bind certain aromatic amines, including some which are normally present in the body such as the biogenic catecholamines[7,8] and serotonin[9,10] and also various drugs such as reserpine[7,11] and dopamine.[12] There is evidence that some of these amines are concentrated in specific storage granules from which they may be released in response to various stimuli.[7,13] It is possible that since vinblastine, like serotonin and reserpine, is an indole compound, it too may be similarly bound in platelets. On the other hand the alkaloid may be bound instead by the microtubule protein present in platelets. Vinblastine appears to complex with microtubule protein and has been used as a rather specific precipitant for isolating the latter from a variety of cell types.[14–16] Moreover, vinblastine, in common with vincristine and colchicine, has the property of disrupting the organized systems of protein microtubules found in various cell types—including the mitotic spindle in dividing cells,[17–21], the neurotubules in neurones[21–23] and the circumferential band of microtubules in platelets.[1,3,4,24,25] While there is a substantial amount of indirect evidence implicating microtubule protein, further work is needed to establish unambiguously the true identity of the vinblastine receptors in platelets with due regard to the possibility that, since the alkaloid is loosely bound, it might translocate during the isolation of organelles or sub-cellular fractions.

Although the specific biochemical nature of the vinblastine receptors is still uncertain, some preliminary conclusions on the properties of these receptors can be drawn from the quantitative relationship between bound and free vinblastine as shown by the sigmoidal binding curve (Fig. 5) and particularly by the Scatchard plot (Fig. 6). A Scatchard plot relates the ratio, amount bound ligand/free ligand concentration, to the amount of bound ligand and gives a stright line when the ligand binds to receptor sites which are equivalent and independent of each other—"non-cooperative binding."[6,26] This requirement of linearity for non-cooperative binding of vinblastine was satisfied at vinblastine plasma concentrations ranging from 0·06 to 1·7 μg/ml, a concentration at which the platelets were essentially saturated with the alkaloid (Fig. 5). On the other hand, at the lower vinblastine plasma concentrations (below

0·06 μg/ml), when the platelets were less than one-quarter saturated with the alkaloid, the Scatchard plot deviates from the linearity found at the higher concentrations. This departure from linearity shows that the capacity of the platelet receptors to bind vinblastine is significantly enhanced following the occupation by the alkaloid of a rather low proportion of the total binding sites. Sigmoidal binding curves and deviations in Scatchard plots of the type found here are characteristic of allosteric systems in which the receptors mediate cooperative interactions between identical ligand molecules.[27,28] At the higher VLB concentrations—when the Scatchard plot is essentially linear—the binding data satisfy the simple Mass Law formulation of the binding equation 1 shown in Table 1:

$$V + P_c \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} V{\cdot}P_c.$$

The K_{Assoc} values (k_{+1}/k_{-1}) calculated from the distribution at equilibrium of the alkaloid between platelets and plasma at various concentrations in the range 0·06–1·7 μg/ml (Fig. 5) are all very similar as is shown by the closeness of the individual points to the straight line in the Scatchard plot (Fig. 6). Values for the rate constants k_{+1} and k_{-1} have been determined by modelling the expression 1 in Table 1 on an analog computer* and matching the computed curves for $V{\cdot}P_c$ vs. time against the experimental curve shown in Fig. 8 (initial vinblastine conc., 0·5 μg/ml). A good fit was obtained when the k values for expression (1) in Table 1 were: k_{+1}, $0{\cdot}99 \times 10^7$ l. moles^{-1} hr^{-1}; k_{-1}, 0·75 hr^{-1}. The resulting K_{Assoc} value (k_{+1}/k_{-1}) of $1{\cdot}32 \times 10^7$ l. moles^{-1} is very close to that obtained from the Scatchard plot ($1{\cdot}23 \times 10^7$ l. moles^{-1}).

It may be significant that the cooperative effect occurs at a vinblastine concentration (0·017–0·06 μg/ml) which can also induce marked changes in the organization and structure of microtubules in platelets[1] and in the "spindle" of dividing cells which are thereby arrested at mitosis.[17,29] If the cooperative binding reflects conformational changes in the microtubule protein, these changes might also promote the dissociation of the organized tubules into protein sub-units. In this connection, it may be noted (Fig. 7c), that the binding of vinblastine by platelets was significantly enhanced by colchicine, an alkaloid which binds rather specifically to microtubule protein[30–33] and which disrupts the organization of microtubules in platelets.[3,4,24,34] It may therefore be tentatively proposed that vinblastine and colchicine are bound to topographically distinct sites on a common platelet receptor—possibly microtubule protein—in which conformational changes induced by colchicine favour the binding of vinblastine. Creasey and Chou[35] have described what appears to be a related effect in which vinblastine promoted the binding of radioactive colchicine by a supernatant fraction from Sarcoma 180 cells. As a corollary, the results in Fig. 7c also indicate that, under conditions in which platelets readily take up vinblastine, colchicine, even in great excess, did not compete for the same sites as vinblastine, a result which does not seem unreasonable in view of the unrelated structures of vinblastine and colchicine.

On the other hand, vinblastine and vincristine are structurally almost identical, yet in spite of this similarity the two alkaloids appear to differ significantly in the way

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* Analog computer programmed and run by Dr. K. L. Pinder, Dept. of Chemical Engineering, University of British Columbia.

they interact with platelets. Thus, whereas platelet-bound [^{3}H]vinblastine rapidly equilibrated with non-radioactive vinblastine when the latter was added to the platelet suspension, the labeled alkaloid was released only slowly from the platelets when non-radioactive vincristine was added (Fig. 7b). In addition, vincristine concentrations several times that of vinblastine had only a minor effect on the initial rapid uptake of vinblastine by platelets (Fig. 8). However, the results indicate that vincristine

TABLE 1. MODELS CONSIDERED FOR THE BINDING OF VINBLASTINE AND VINCRISTINE BY PLATELETS*

Model 1: Platelets with common binding sites (P_c) for vinblastine and vincristine.

$$V + P_c \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} V{\cdot}P_c \qquad (1)$$

$$C + P_c \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} C{\cdot}P_c \qquad (2)$$

Model 2: Platelets with separate binding sites (P_s) for vinblastine and vincristine.

$$V + P_s \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} V{\cdot}P_s \qquad (3)$$

$$C + P_s \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} C{\cdot}P_s \qquad (4)$$

$$C + V{\cdot}P_s \xrightarrow{k_3} C{\cdot}P_s + V \qquad (5)$$

Rate constants for computed curves (Model 1)† which closely matched the experimentally derived curves in Fig. 8.

Alkaloid conc. (μg/ml)					
V	C	k_{+1} (l. moles^{-1} hr^{-1})	k_{-1} (hr^{-1})	k_{+2} (l. moles^{-1} hr^{-1})	k_{-2} (hr^{-1})
0·5	0·0	0·99 × 10^7	0·75		
0·5	0·5	0·99 × 10^7	0·75	0·15 × 10^7	0·19
0·5	1·0	0·99 × 10^7	0·75	0·15 × 10^7	0·024

* See text for fuller description. V = vinblastine; C = vincristine; $V{\cdot}P_c$, $V{\cdot}P_s$, $C{\cdot}P_c$, $C{\cdot}P_s$ = alkaloid-platelet complexes.

† The *k* values were derived from analog computer models of the above expressions, programmed and run by Dr. K. L. Pinder, Dept. of Chemical Engineering, University of British Columbia. The molarity of the vinblastine binding sites used in the analog computations (0·33 × 10^{-6} moles l.$^{-1}$) was calculated from the amount of vinblastine bound by 10^9 platelets at saturation as given by the Scatchard plot in Fig. 6. Mol. wt. vinblastine sulphate = 909.

eventually diminishes the ability of platelets to bind and/or retain vinblastine. In the absence of direct information on the mechanism of interaction of vincristine with platelets, only tentative and preliminary interpretations of the results can be made at this stage. Two very simple models have been considered: one in which vinblastine and vincristine compete directly for the same site(s) on a given receptor; in the second model the receptor has sites which are specific for each alkaloid but the binding of vincristine indirectly modifies the binding characteristics of the vinblastine site(s), perhaps through conformational changes in the molecule. Expressions for the competitive model are given in Table 1: (1) and (2) and for a simple version of the alternative indirect model, in Table 1: (3), (4) and (5). In the latter it is postulated that, as vincristine is bound to its specific sites, the vinblastine sites are inactivated and, if occupied, vacated.

The formulations in Table 1 have been modelled on an analog computer to give curves in which the amount of platelet-bound vinblastine ($V{\cdot}P_c$ or $V{\cdot}P_s$) was plotted against the incubation time. The vincristine rate constants (k_{+2}, k_{-2}, k_{+3}) were adjusted until the computed curves gave the best fit with the experimentally derived curves shown in Fig. 8. The curves from the competitive model, Table 1: (1) and (2), could be matched much more closely to the experimental curves than those from the model based on "indirect" inhibition. A very good fit for the competitive model was obtained with the rate constants as given in Table 1. These constants indicate that if the two alkaloids compete for the same sites, then vincristine binds much more slowly than does vinblastine, e.g. at less than one-sixth the rate when both compounds are present in the same molar concentrations. The agreement between the observed and computed curves should be considered only as providing tentative support for the proposed mechanism which in reality is undoubtedly substantially more complicated than the simple model implies. For example if, as seems likely, the alkaloids induce conformational changes in a common receptor, then the k values, represented in Table 1 as constants, may in fact vary in a complex manner as the compounds are bound.

The effects of vincristine on platelet structure are essentially identical with those produced by vinblastine at similar concentrations.[3] As a mitotic inhibitor, vincristine is at least as effective as vinblastine.[18,36,37] Therefore the slow rate at which vincristine displaces vinblastine from platelets may appear to be inconsistent with the relative biological activities of the compounds. However, in the present study the parameter for the interaction of vincristine with platelets is the displacement of tritiated vinblastine, and it cannot therefore be excluded that vincristine may still be bound readily but at sites (on microtubule protein?) which are independent of the vinblastine sites. A method for preparing tritiated vincristine is under development in this laboratory; when the labeled alkaloid is available, it is hoped to obtain direct information on its binding characteristics in platelets and other cell types and to relate this to the cytotoxicity of the alkaloid.

It has recently been reported by Creasey *et al.*[14] that vinblastine and other antimitotic drugs are bound *in vitro* by preparations of human leukocytes. In view of the different conditions used for isolating and incubating the cells, it is difficult to compare directly the binding of vinblastine by human leukocytes in their work and by rat platelets in the present study. However, previous work in our laboratory,[1] using both autoradiographic and cell separation procedures, has shown that, at least in the rat, platelets bind vinblastine much more readily than do leukocytes and red cells.*

Results in the present study indicate that platelet-rich plasma preparations are suitable for investigating *in vitro* some of the pharmacological properties of vinblastine and biologically related compounds. They also suggest that platelets can be used as

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* Preliminary experiments have shown that the distribution of radioactivity in human blood after incubation with [^{3}H]vinblastine *in vitro* resembles that in rat blood.

comparatively simple models for studying the mechanisms of interaction of this type of drug with cell receptors, microtubule or other, in a physiological milieu. In this regard it may be noted that although vinblastine, vincristine and colchicine are all potent anti-mitotic compounds, individually they differ markedly in their interaction with platelets—as shown here— and also in their ability to arrest the growth of various tumors and in the symptoms of toxicity they elicit.[38] An understanding of the pharmacological and biochemical reasons for these differences could have important implications in the clinical use of this type of drug.

Acknowledgements—The authors wish to thank Dr. K. L. Pinder, Dept. of Chemical Engineering, Univ. of British Columbia, for programming and running the analog computer models and for helpful discussions and Dr. R. L. Noble, Director of the Cancer Research Centre, Univ. of British Columbia, for providing space and facilities.

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