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ORIGINAL ARTICLE

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Measurement of nitrobenzylthioinosine in plasma and erythrocytes: a pharmacokinetic study in mice

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Abstract *Purpose*: Nitrobenzylthioinosine (NBMPR), a potent inhibitor of nucleoside transport in many cell types, modulates the in vivo disposition of several cytotoxic nucleoside analogs. In this study, a radioligand binding assay was developed for measurement of the NBMPR content of plasma and erythrocytes. *Methods*: The assay was based on the competition between NBMPR and [^{3}H]NBMPR for high-affinity sites on human erythrocyte membranes. With this assay, we followed in mice changes in the NBMPR content of blood plasma and erythrocytes, following the intraperitoneal injection of the disodium salt of NBMPR 5′-monophosphate (NBMPR-P), a prodrug form of NBMPR. *Results*: The radioligand binding assay was able to measure precisely as little as 2.5 pmol of NBMPR, allowing the direct determination of NBMPR concentrations in plasma as low as 16 n*M*. As few as 8×10^3 molecules of NBMPR per cell could be determined in erythrocytes. The NBMPR content of plasma from mice injected with NBMPR-P was maximal at about 20 min after injection and declined to $< 0.2\%$ of the peak value by 10 h. Erythrocyte-associated NBMPR was also maximal at 20 min, and declined to 11% of the peak value by 10 h after injection. Time courses for the disappearance of NBMPR from plasma and erythrocytes were monoexponential and yielded half-life values of 0.39 h and 0.68 h, respectively, an apparent volume of distribution of 0.61 l/kg, and a clearance of 1.1 l/h per kg. *Conclusions*: The radioligand binding assay is a sensitive and facile method for monitoring NBMPR concentrations in mammalian plasma and tissue extracts.

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Introduction

Nitrobenzylthioinosine (NBMPR) is a potent inhibitor of the "*es*" equilibrative nucleoside transporter, which is one of several nucleoside-specific transporters expressed in animal cells (see references 3, 4 and 8 for nomenclature of nucleoside transport processes). The currently recognized, diverse array of nucleoside transport processes comprises seven transporter subtypes, which are facilitated diffusion processes or sodium-dependent, secondary active transport processes, and which differ in sensitivity to NBMPR [4]. NBMPR has been employed as a host-protecting agent during treatment of mice with certain cytotoxic nucleosides [1, 18, 19]. Coadministration of NBMPR with potentially lethal doses of these agents alters their toxicology, apparently by limiting drug entry into dose-limiting host tissues [1]. Difficulties in the in vivo use of NBMPR arising from its limited water solubility (about 50 μ*M* at 37 °C) appear to have been overcome by the availability of NBMPR 5′-monophosphate (NBMPR-P), a readily soluble prodrug form of the inhibitor [1, 18, 20]. NBMPR-P *per se* is not transport inhibitory [22], but is rapidly converted in the circulation to NBMPR.

In mice implanted with L1210 leukemia cells, administration of potentially lethal doses of nebularine and host-protective doses of NBMPR-P resulted in substantial leukemic cell kill with long-term survivors [20]. Similarly, mice with L1210 leukemia, when treated with neurotoxic doses of fludarabine phosphate coadministered with NBMPR-P, were protected against the toxic agent and "cures" (long-term survivors) were obtained [1]. The host protection phenomenon has also been shown with diverse toxic nucleosides and in rats. For example, Jakobs and Paterson [14] showed that when rats bearing implants of Walker 256 carcinosarcoma

were treated with NBMPR-P at high toxic doses of tubercidin, long-term survivals were achieved. Under the conditions of those experiments, therapeutic effects were achieved because the neoplastic cells were not protected by NBMPR-P against the toxic nucleoside, whereas dose-limiting host tissues were protected. Leukemia L1210 cells and Walker 256 carcinosarcoma cells have components of nucleoside transport that are of low sensitivity to NBMPR [8, 9]. Recently, the potential of NBMPR as a retentive agent in the chemotherapy of leukemia with nucleoside analogs has been demonstrated in experiments that have shown the reduction by NBMPR of transporter-mediated efflux of 2-chlorodeoxyadenosine in cells from patients with chronic lymphocytic leukemia [2]. Together, these studies illustrate the tactics in the modulation of the in vivo availability of nucleosides by nucleoside transport inhibitors such as NBMPR. These tactics apparently have a basis in the cell type-specific expression of nucleoside transporters that differ in sensitivity to NBMPR, and may have therapeutic applicability.

The present report describes a sensitive radioligand binding assay for measurement of NBMPR concentrations in both plasma and erythrocytes. The assay obviates the need for sample preparation procedures that are associated with analysis of plasma samples by HPLC. We illustrate its use in a study of the blood kinetics of NBMPR in mice, a species widely used in the preclinical testing of therapeutic strategies that employ nucleoside drugs. Clanachan and Serignese [7] have described a similar assay.

Materials and methods

Chemicals

[G-^{3}H]NBMPR (16 Ci/mmol) was purchased from Moravek Biochemicals, Brea, Calif. NBMPR was prepared in this laboratory [23]. NBMPR-P, disodium salt, was prepared by the Research Laboratory, Yamasa Shoyu Co., Chosi, Chiba, Japan, and 6-thioinosine was purchased from Pharma-Waldhof, Dusseldorf, Germany. Heparin, sodium salt (153 USP units/mg), and nitrobenzylthioguanosine (NBTGR) were from the Sigma Chemical Co., St. Louis, Mo.

Drug administration and blood fraction recovery

NBMPR-P (1.5 mg/ml in 0.15 *M* NaCl) was administered intraperitoneally (i.p.) at 15 mg/kg body weight to female B6D2F$_1$ (C57BL/6J × DBA/2J, F$_1$) mice, 18–25 g in weight, obtained from the Health Sciences Animal Service, University of Alberta. To follow changes with time in the NBMPR content of blood plasma and erythrocytes, mice were killed by decapitation to obtain blood samples at specified times after NBMPR-P injection. About 15 min before blood collection, mice were injected i.p. with heparin (77 U/ml in 0.15 *M* NaCl solution; 765 U/kg body weight). Blood samples were immediately centrifuged (16 000 *g*, 1 min) to separate cells from plasma, which was stored at −20 °C. Erythrocyte pellets were washed six times with ice-cold 0.15 *M* NaCl, and stored at −20 °C in tared microcentrifuge tubes. In assays of NBMPR content, pellet weights were determined and their volumes calculated using a predetermined pellet density of 1.08 g/ml. The erythrocyte pellets were suspended in 2.3 volumes of ice-cold "reagent alcohol" (90% ethanol/5% methanol/5% isopropanol), and after 30 min at 4 °C, the NBMPR-containing extracts were recovered by centrifugation (16 000 *g*, 2 min).

Erythrocyte membranes

Outdated human erythrocytes (Canadian Red Cross Society Blood Transfusion Service), were washed in 20 m*M* Tris-HCl buffer (pH 7.4, 22 °C) containing 140 m*M* NaCl, 5 m*M* KCl, 2 m*M* $MgCl_2$, and 0.1 m*M* Na_2EDTA, before the preparation of membranes [10], which were stored under liquid nitrogen.

Radioligand binding assay

This assay measured changes in the amount of a standard quantity of [^{3}H]NBMPR bound by *es* sites on a standard quantity of erythrocytes under fixed conditions, the changes being the consequence of reduction in the specific activity of the [^{3}H]NBMPR by nonisotopic NBMPR (the analyte) in samples of plasma or erythrocyte extracts added to assay mixtures. Assay mixtures were prepared in 50 m*M* KH_2PO_4 buffer (pH 7.4), and contained 0.5 pmol [^{3}H]NBMPR, erythrocyte membranes containing about 1 pmol binding sites (assuming an NBMPR site density of 10^{14} sites/ml packed membranes), and either plasma or alcoholic erythrocyte extract, in a total volume of 1.25 ml. For the preparation of calibration curves, graded amounts of nonradioactive NBMPR in phosphate buffer, or phosphate buffer alone (control) were added in place of erythrocyte extract or plasma. Samples were held for 30 min at 22 °C (to achieve equilibrium between ligand and binding sites) prior to filtration of the assay mixtures through Whatman GF/C filters that had been pretreated with 0.5 ml of an ice-cold solution of 5 μ*M* NBMPR and two 5-ml rinses of ice-cold phosphate buffer (50 m*M*, pH 7.4). Membrane samples on the filters were washed with two 5-ml portions of ice-cold phosphate buffer. The filters were assayed for ^{3}H-content by liquid scintillation counting. Assays were performed in triplicate. Bound radioactivity in samples containing graded amounts of nonradioactive NBMPR was expressed as percent of bound radioactivity in control samples, and plotted against the amounts of added nonradioactive NBMPR to obtain calibration curves from which concentrations of NBMPR in plasma and erythrocyte extracts were determined by multiplying the NBMPR concentration determined from the calibration curve by a factor that accounted for the volume of undiluted plasma or erythrocyte extract present in the assay mixture.

The dissociation constant (K_D) for NBMPR bound to human erythrocyte membrane sites was determined under equilibrium conditions. Replicate portions of membrane preparations containing about 0.2 pmol of NBMPR binding sites were incubated for 30 min at 22 °C with graded concentrations of [^{3}H]NBMPR (0.125–4.0 n*M*) in 50 m*M* KH_2PO_4 buffer (pH 7.4) in final volumes of 1.25 ml. Nonspecific binding of [^{3}H]NBMPR was determined in the presence of 10 μ*M* NBTGR. After incubation, the membranes were collected on filters and assayed for radioactivity. Equilibrium concentrations of free [^{3}H]NBMPR were determined by measurement of radioactivity in the medium. The values of K_D and B_{max}, determined by linear regression analysis of Scatchard plots, were $K_D = 1.3 \pm 0.4$ n*M* and $B_{max} = 3.3 \times 10^{14} \pm 1.4 \times 10^{14}$ sites/ml packed membranes (mean ± SEM of four determinations).

Data analysis

Polynomial equations describing monoexponential and biexponential decay models were fitted to concentration versus time data for the disappearance of NBMPR from plasma and erythrocytes by nonlinear regression analysis, using Origin 3.5 software (Microcal Software, Northampton, Mass.). In the curve-fitting procedure, data points were inversely weighted according to the variance of the replicates for each point. The fitted curves yielded rate constants, *k*,

from which values for the half-lives, $t_{1/2}$, were calculated ($k = 0.693/t_{1/2}$). V, the apparent volume of distribution, was calculated as V = dose/extrapolated plasma concentration at $t = 0$, and CL, the clearance of NBMPR, was calculated as $CL = kV$[12].

Results

Figure 1 shows a calibration curve for the determination of NBMPR by the radioligand binding assay. These data illustrate the effect on membrane binding of [^{3}H]NBMPR of the addition of graded amounts of non-isotopic NBMPR to the assay system. NBMPR amounts from 2.5 pmol to 12.5 pmol could be measured in the assay, values that were equivalent to 2 nM and 10 nM concentrations, respectively, in assay mixtures; the low SEM of individual calibration points in that range of the curve (< 4% of absolute values) and high correlation coefficient ($r = 0.99$) of the logistic curve indicate a high degree of assay precision. Nonspecific binding of [^{3}H]NBMPR to the membranes (determined in the presence of 10 μM NBTGR) was less than 2% of total binding and was not considered in the assays.

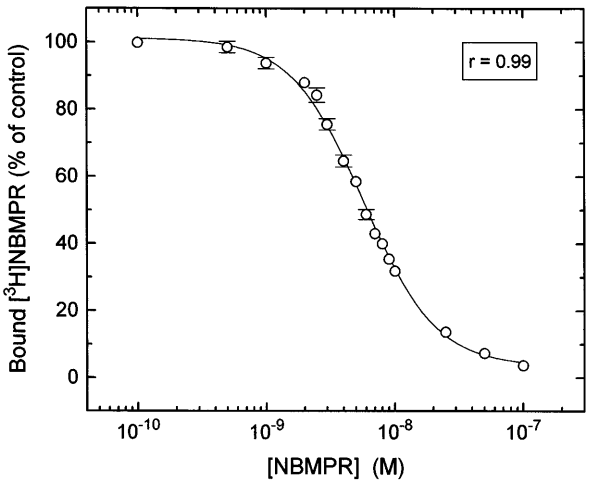

Fig. 1 Aggregate calibration curve for the determination of NBMPR using the radioligand binding assay. For each assay of NBMPR, a calibration curve was prepared to define the relationship between graded amounts of nonradioactive NBMPR added to assay mixtures and the equilibrium binding of [^{3}H]NBMPR to the fixed content of erythrocyte membranes in the replicate assay mixtures. The data are means ± SEM from 14 experiments, each performed in triplicate

The binding of NBMPR to erythrocyte membrane sites was not affected by the presence of mouse plasma in the assay if the volume of plasma did not exceed 10% of the assay volume. Since as little as 2.5 pmol NBMPR could be determined in the assay, NBMPR concentrations as low as 16 nM could be measured directly in plasma. This limit might be further lowered if concentrated sample extracts, prepared from larger volumes of plasma, were assayed. Since reagent alcohol volumes of at least 45 μl were tolerated in the assay, erythrocyte-associated NBMPR could be determined at levels as low as 130 pmol/10^{10} cells, equivalent to 8×10^3 molecules NBMPR per cell.

The application of the assay to the determination of the NBMPR content of mouse plasma and erythrocytes following i.p. administration of NBMPR-P is shown in Fig. 2. The assay system does not recognize the presence of NBMPR-P (the 5′-monophosphate derivative of NBMPR), since this compound does not interact with erythrocytic NBMPR binding sites [22]. Although NBMPR-P is rapidly dephosphorylated in vivo, its presence in early plasma samples cannot be excluded. Plasma levels of NBMPR (Fig. 2A) reached a maximum of 27 μM at 20 min. The concentration of NBMPR in plasma had declined to 28 nM, < 0.2% of the peak value, 10 h after injection of NBMPR-P. The

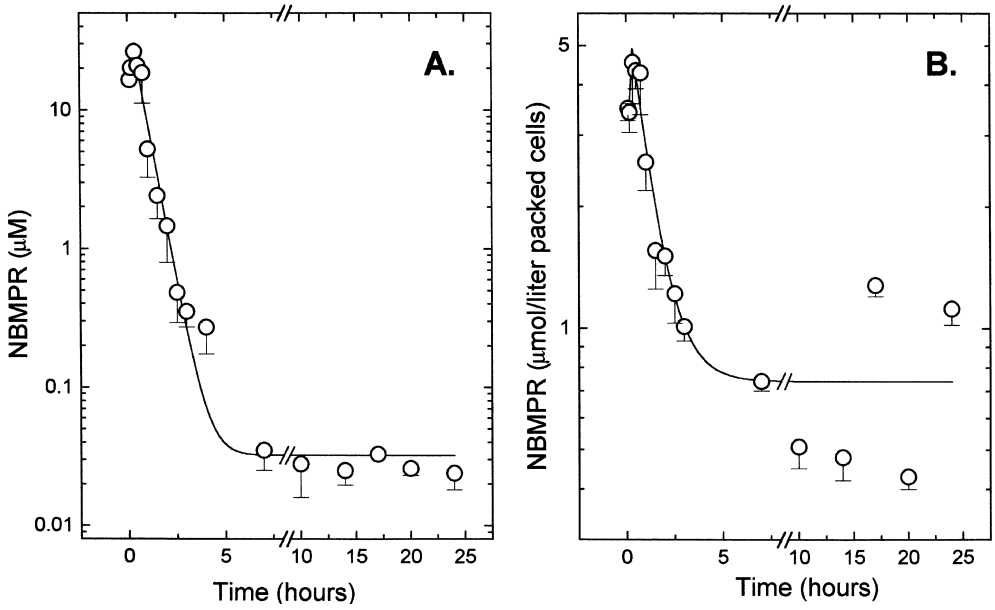

Fig. 2A,B NBMPR concentrations in plasma and erythrocytes in mice following a single i.p. dose of NBMPR-P (15 mg/kg). Data points are means ± SD of measurements made on four or five animals (**A** plasma, **B** alcoholic extracts of washed erythrocytes)

concentration profile of NBMPR in plasma best fitted an equation for monoexponential decay, yielding a half-life of 0.39 h. The data of Fig. 2A yielded values for V and CL of 0.61 l/kg and 1.1 l/h per kg, respectively, for NBMPR. The time course of decay of erythrocyte-associated NBMPR during the same period of time (Fig. 2B) showed a peak in the erythrocyte NBMPR content of 4.6 μmol/l packed cells at 20 min, and a decline to 11% of this value after 10 h. The decay of the NBMPR content of erythrocytes was monoexponential, with a half-life of 0.68 h.

Discussion

This study followed the time course of changes in the NBMPR content of blood plasma and erythrocytes in mice after a single i.p. dose of NBMPR-P. NBMPR was determined by an assay in which retention of [^{3}H]NBMPR by high-affinity binding sites on a standard quantity of stored erythrocyte membranes was proportional to the specific radioactivity of [^{3}H]NBMPR in the assay mixtures. In the assay, nonisotopic NBMPR in the analysed samples changed the specific radioactivity (dpm per pmol) of the constant quantity of [^{3}H]NBMPR present in the assay mixtures. Calibration curves, prepared for each assay, defined relationships between added nonisotopic NBMPR and ^{3}H bound by the standard quantity of membranes. Although NBMPR may also be determined by reversed phase liquid chromatography, such procedures with conventional HPLC systems are about 50-fold less sensitive than the present assay, unless sample extracts are concentrated prior to chromatography. The use of human erythrocyte membranes in the present assay allowed the preparation of membrane stocks that may be stored frozen for several months.

As Clanachan and Serignese [7] have shown, the radioligand binding assay may be adapted to the determination of other ligands with high affinity for the erythrocyte NBMPR binding protein, including other S^6-substituted thiopurine nucleosides [5, 13], as well as dilazep and dipyridamole [13]. While the K_D for site-bound NBMPR on human erythrocyte membranes is about 1 nM, K_i values for the inhibition of NBMPR binding by adenosine and uridine are about 100-fold higher [16, 17]. For this reason, and because of their low concentrations in plasma (less than 10 μM [15, 21, 25]), the physiological nucleosides in plasma samples are not likely to interfere in the determination of NBMPR by the radioligand binding assay. In the present investigation, 6-thioinosine, a possible metabolite of NBMPR, at concentrations as high as 10^{-4} M did not reduce binding of [^{3}H]NBMPR at erythrocyte membrane sites (data not shown).

Plasma concentrations of NBMPR in mice peaked about 20 min after the i.p. injection of NBMPR-P and 10 h later, plasma levels of NBMPR were 20–30 nM, less than 0.2% of the peak concentration, but sufficient to inhibit the *es* nucleoside transport process [6, 24]. The NBMPR content of erythrocytes, 24 h after NBMPR-P administration, expressed as a concentration in those cells, was more than 10-fold higher than the NBMPR concentration of plasma, indicating that erythrocyte binding sites represent a major compartment from which dissociation of the ligand is slow. The K_D value for NBMPR binding at *es* sites on mouse erythrocytes (0.12 nM [11]) indicates that the slow dissociation of NBMPR from erythrocytic sites, which are a reservoir of NBMPR in the circulation, may account for the presence of NBMPR in both erythrocyte and plasma samples at times exceeding 10 h after the administration of NBMPR-P.

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