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The binding of thio-TEPA in human serum and to isolated serum protein fractions*

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Summary. Binding of triethylenethiophosphoramide (thio-TEPA) in serum from healthy individuals and from cancer patients and its binding to isolated serum protein fractions were studied by equilibrium dialysis. A drug-protein binding in the order of 10% was demonstrated in serum, with little interindividual variation. The protein binding of thio-TEPA seemed to be restricted to albumin and lipoproteins, with the greatest affinity to albumin. The previously reported selective binding of thio-TEPA to gamma globulin was contrary to our findings. The almost insignificant degree of serum protein binding of thio-TEPA indicates that the drug is well suited for use in cancer drug combinations.

Introduction

The recognition of a narrow therapeutic range for cancer drugs, combined with wide variation among cancer patients as to their ability to handle drugs in the body, has made cancer drug pharmacokinetics an expanding field of interest. The alkylating agent thio-TEPA has been used in cancer therapy for more than 30 years and is one of the oldest alkylating agents still in clinical use. In a previous article we reported on the pharmacokinetics of thio-TEPA in human patients [7].

For a complete understanding of the clinical pharmacology of a drug, knowledge of its extent of binding to the serum proteins is needed. In view of the little knowledge about the clinical pharmacology of thio-TEPA until recently, the information available on the protein binding of the drug is scant and rather contradictory. Mellett and Woods [12] studied the binding of thio-TEPA to plasma proteins in dogs. With a drug concentration of 2 μg/ml, which is comparable to achievable peak drug concentrations in human patients after conventional dosing, they reported drug binding in the range of 0–10%.

In the search for possible alterations with the serum proteins in response to cancer drug treatment, Bateman et al. [1] examined human sera after in vitro incubation with isotopically labeled thio-TEPA at concentrations assumed to be 100 and 200 times the therapeutic serum concentrations of the drug. Gas-flow counting after protein electrophoresis revealed the radioactivity to be restricted to the protein bands, and it was concluded that thio-TEPA was attached in some way to the serum proteins while in the blood stream. Maxwell [10] performed detailed studies on the protein binding of thio-TEPA. Patients were given isotopically labeled drug, and serum electrophoresis revealed that the isotope migrated in a similar way to the gamma globulins. This formed the basis for the hypothesis that thio-TEPA in humans was selectively bound to the gamma globulins. Human albumin and beta and gamma globulin were then evaluated by serial dialysis with reduced pressure ultrafiltration, and the hypothesis of binding confined to gamma globulin was claimed to be confirmed. Recently, however, McDermott et al. [11] reported a low degree of binding to several serum proteins, including the gamma globulins.

In order to obtain a complete characterization of the serum protein binding of thio-TEPA, we have determined the binding in vitro by equilibrium dialysis in whole sera from healthy individuals and from cancer patients and the binding to isolated protein fractions known to be associated with drug binding.

Material and methods

Determination of serum protein binding. The binding of thio-TEPA in serum or to serum protein fractions was determined by equilibrium dialysis using plexiglass cells with two compartments each 1 ml in volume, separated by a semipermeable membrane [Medicell International size 8–32/32′ (UK)]. Individual whole sera from patients and healthy individuals were dialysed against Krebs Ringer bicarbonate buffer at pH 7.40 under an atmosphere of 5% CO_2 in air without predialysis of the sera. Apart from individual sera, the remaining dialysis experiments with pooled serum and isolated protein fractions were performed against a Krebs-Ringer phosphate buffer at pH 7.40. Prior to equilibrium dialysis, the protein solutions were predialysed against 1000 ml buffer at 4°C for 24 h. The equilibrium dialysis was run with 500μl serum or protein solution against 500μl buffer with ^{14}C-labeled thio-TEPA added in the protein compartment. The cells were gently shaken according to a standard procedure. When equilibrium had been obtained, 100μl aliquots were taken from each compartment and added to 5 ml scintilla-

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tion fluid. Counting was performed in a Packard Tri Carb liquid scintillation spectrophotometer with a counting efficiency, for ^{14}C, of 65%. The binding of thio-TEPA was calculated from the distribution of the isotope in the two compartments of the cell according to the relationship:

$$\% \text{ bound drug} = \frac{(A-B) \times 100}{A}$$

where A and B are the radioactive counts in the protein and buffer compartments, respectively.

Radioactive recovery was evaluated at each experiment by comparing the total radioactivity added to the dialysis cell with the sum of the radioactivity in the two compartments after the dialysis had been performed. The radioactive recovery throughout all experiments was 97% ± 9% (mean ± SD) with no dependence on time after the start of dialysis.

Dilution of the protein solution during equilibrium dialysis due to osmotic effect was evaluated by determination of the protein concentration before and after the dialysis. Dilution up to 10% was encountered with whole serum dialysed against Krebs-Ringer phosphate buffer, whereas with the isolated protein fractions no measurable dilution occurred during equilibrium dialysis. The equilibrium dialysis was routinely performed at room temperature (22 ± 1°C), while control experiment was performed in serum at 37°C.

The effect of pH was evaluated by controlling the pH in serum and buffer prior to and after equilibrium dialysis and by performing control experiments with pH 7.0, 7.2, 7,4, 7.6 and 7.8 in the buffer at the start of equilibrium dialysis.

Experiments with individual sera from patients and healthy individuals were performed in duplicate, while experiments at different pH levels and at different concentrations of protein fractions were performed in quadruplicate.

Serum. Serum was chosen instead of plasma to avoid any possible interaction with the protein binding assay of the content of heparin or EDTA in plasma. Blood samples were obtained from ten healthy individuals of both sexes, with age ranging from 25 to 41 years. The blood was allowed to coagulate at room temperature for 45 min. Serum was separated after centrifugation (1100 g for 10 min at room temperature) and immediately thereafter stored at −20°C. In addition to the determination of thio-TEPA protein binding in individual sera, a serum pool was made and used for temperature and pH experiments and for experiments designed to establish the time required to reach equilibrium and the stability of the equilibrium.

Serum was obtained, and treated as described above, from ten patients with ovarian cancer ranging in age from 34 to 84 years. All had histologically proven epithelial carcinoma, the distribution of clinical stages (FIGO) being: three with stage I; one with stage II; five with stage III; and one with stage IV. At the time of blood sampling four patients had progressive disease, whereas six patients had no evidence of active disease. Three patients had never received thio-TEPA treatment, three patients had previously received thio-TEPA treatment, and four patients were currently undergoing thio-TEPA treatment. In order to avoid any interference from administered drug, in vitro protein binding assays were carried out at least 24 h after the thio-TEPA had been given. Serum protein binding of thio-TEPA was determined in sera from individual patients.

Albumin. Human albumin (A 1653) was purchased from Sigma Chemical Co. (USA). During predialysis of the stock solution a twofold increase of the initial volume occurred, corresponding to a final albumin concentration of 125 g/l. Dilutions were made from this solution, and thio-TEPA binding was determined at five different albumin concentrations. All determinations of albumin concentration were performed using a commercial kit for colorimetry with bromcresol green [Beckman Instruments International S.A. (Switzerland)].

Alpha$_1$-acid glycoprotein. Human alpha$_1$-acid glycoprotein (AAG) was purchased from Behring Werke (FRG). The concentration was determined before and after predialysis of the stock solution by radial immunodiffusion [Nor – Partigen, Hoechst (FRG)]. Only minor dilution occurred during predialysis. The binding of thio-TEPA to AAG was determined at five different concentrations of the protein.

Gamma globulin. Human gamma globulin (G 4386) was purchased from Sigma Chemical Co. Determination of the concentration of the stock solution before and after predialysis was performed by agarose gel electrophoresis (kit from Beckman International S.A.) and revealed that no change had occurred in the concentration. The binding of thio-TEPA to five different concentrations of gamma globulin was determined.

Lipoproteins. Lipoproteins were separated from a fresh serum pool from ten healthy individuals (see above) by ultracentrifugation. Potassium bromide (293.9 mg/ml) was added to serum to give a density of 1.195 g/ml. Serum was then centrifuged at 40 000 rpm for 48 h at 4°C in a Kontron TGA 50 Ultracentrifuge with a TY 40 rotor. The floating lipoproteins were then gently collected. An indirect measure of the lipoprotein concentration was obtained by determination of the triglyceride concentrations in the serum pool and in the lipoprotein fraction after ultracentrifugation. A standard enzymatic triglyceride assay was used [Greiner Diagnostica (Switzerland)]. Provided that both triglycerides and lipoproteins were restricted to the floating fraction after the ultracentrifugation, a 5.6-fold concentration had occurred. A minor dilution of the lipoprotein solution occurred during the predialysis, after which a repeated triglyceride determination revealed a 5.0-fold concentration relative to serum. From this stock solution dilutions were made and thio-TEPA binding was determined at five different lipoprotein concentrations.

Labeled drug. ^{14}C-Labeled thio-TEPA was obtained from Amersham (UK). A routine purity control using thin-layer chromatography (TLC) with silica gel plates in chloroform: acetone (80:20) revealed radiochemical impurities of the batch obtained. Only 85% of the isotope was migrating with a R_f value equal to that of thio-TEPA. In addition, the solubility of the compound was far lower than originally described. It was obvious that chemical decomposition had taken place. When the 85% fraction representing thio-TEPA was removed from the TLC plate, extracted with ethylacetate and run through a second TLC, more than 99% of the radioactivity migrated in an identical manner

to unlabeled pure thio-TEPA [Lederle Labs. (USA)]. This purity was further confirmed by a specific gas chromatographic assay for thio-TEPA [6]. This double TLC procedure was utilized for the purification of labeled drug, yielding 99% radiochemical purity and a specific activity of 5,5 μCu/mg. The purified drug isotope was stable when stored at −20°C and during equilibrium dialysis. An initial drug concentration of 1000 ng/ml in the equilibrium cell was used throughout all binding experiments.

Results

The relation between time and distribution of the drug in the equilibrium cell was evaluated with whole serum against Krebs-Ringer phosphate buffer. Equilibrium was reached after about 4 h and was stable for at least 26 h. The relation between time and drug distribution in the equilibrium cell when the drug was added to the protein or buffer compartment is shown in Fig. 1 (A and B, respectively). The time needed for the achievement of equilibrium was independent of which compartment the drug was added to. The remaining experiments were performed with an equilibration time of 12 h, and thio-TEPA was routinely added to the protein compartment.

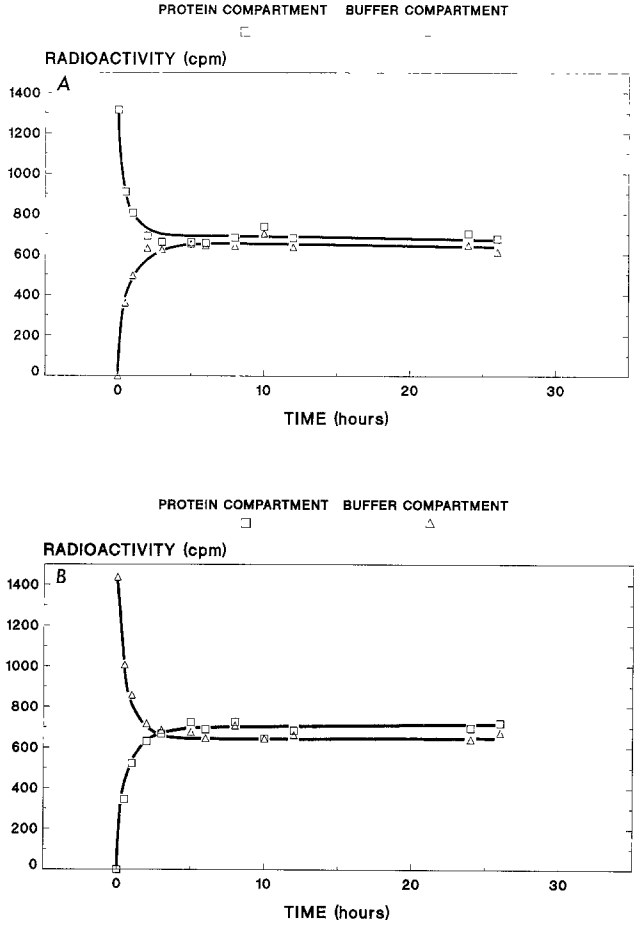

Fig. 1A, B. Distribution of ^{14}C-labeled thio-TEPA between the protein and buffer compartments of the equilibrium cell as a function of time after the start of the dialysis. **A** Addition of the drug to the protein compartment; **B** addition of the drug to the buffer compartment

Ten parallel experiments with determination of thio-TEPA protein binding in whole serum at 37°C revealed a protein binding of 10% ±2% (mean ±SD). This is within the range found at room temperature, justifying the use of room temperature in the experimental design.

The results of protein-binding experiments at different pH values in the buffer compartment are shown in Fig. 2. No significant variation in the the extent of drug-protein binding was demonstrated with pH in the range between 7.2 and 7.8. However, at pH 7.0 the radioactivity in the buffer compartment exceeded that in the protein compartment in each of four parallel experiments, demonstrating that no drug-protein binding, but rather an expulsion of drug from protein-binding sites occurred at this low pH. In each experiment with individual sera against Krebs-Ringer bicarbonate buffer, pH in serum and buffer was controlled at the end of dialysis. When experiments were started with pH in serum and buffer in the range 7.30–7.35, corresponding pH values in the serum and buffer compartments after dialysis were 7.43 ±0.02 (mean ±SD) and 7.36 ±0.01 in ten healthy individuals and 7.43 ±0.03 and 7.37 ±0.01 in ten cancer patients.

The drug-protein binding in whole sera from ten healthy individuals and ten patients with epithelial ovarian cancer was 8% ±2% and 13% ±3% (mean ±SD), respectively. The difference is statistically significant ($P<0.0005$, Student's t-test). Among the healthy individuals no difference in protein binding existed between the sexes. Among the cancer patients the extent of protein binding did not seem to be correlated to the disease status or to whether or not the patient had already received thio-TEPA treatment.

The results of experiments with thio-TEPA binding to isolated protein fractions, given in Table 1, correlated with the results obtained with whole serum. In common with most other drugs, thio-TEPA seemed to have the greatest affinity for the albumin fraction. At physiological concentrations of the proteins our results indicate that the binding of the drug to serum proteins is restricted to albumin and lipoproteins. With alpha$_1$ acid glycoprotein, an almost 8-fold concentration of the protein relative to physiological concentrations was needed before any binding of thio-TEPA was demonstrated. With gamma globulins, which were previously reported to be the main thio-TEPA binding protein in human serum, no binding was demonstrated

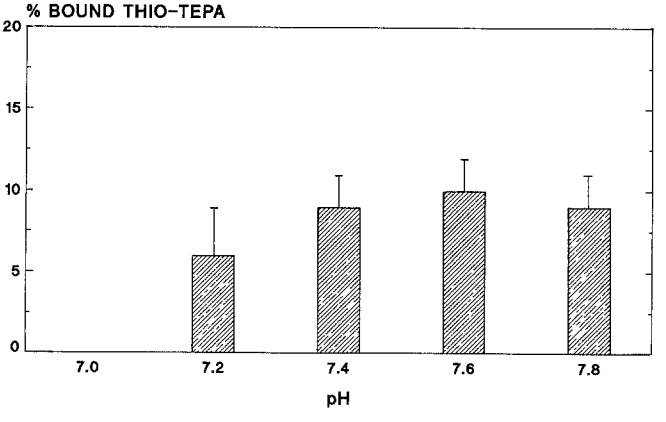

Fig. 2. Serum protein binding of thio-TEPA as a function of pH at the start of equilibrium dialysis. Data *points* represent means + SD ($n = 4$)

Table 1. Binding of thio-TEPA in whole sera from healthy control individuals[a] and cancer patients[a] and to different concentrations of serum protein fractions

Control sera % bound	Patient sera % bound	Albumin		Lipoprotein		Gamma globulin		Alpha$_1$ acid glycoprotein	
		Conc.[b]	% bound	Conc.[c]	% bound	Conc.[b]	% bound	Conc.[b]	% bound
8±2	13±3	121	17	×5	2	110	0	4.70	6
		60.5	7	×2	3	44	0	3.60	4
		40	9	×1	4	22	0	1.20	0
		24	4	×0.7	5	15	0	0.94	0
		20	4	×0.5	2	11	0	0.47	0

[a] Figures shown represent means ± SD (n = 10)
[b] Concentrations given as grams per litre
[c] Concentrations given as multiples of assumed normal human serum concentration. Boxed-in figures represent the binding at the anticipated normal serum concentrations of the protein fractions

in the wide range of concentrations studied. A positive correlation between drug binding and the concentration of albumin was indicated. This was not the case with the lipoproteins, however.

Discussion

Cancer patients are highly predisposed to disease-induced alterations in the serum proteins, and they are often subjected to multiple-drug therapy. Thus, with drugs that are highly protein-bound, the possibility of interindividual variation in pharmacokinetics and pharmacodynamics exists, due to both serum protein variation and drug interactions [15]. Generally, therefore, detailed knowledge of the serum protein binding of cancer drugs is desirable.

Equilibrium dialysis together with ultrafiltration is the reference method for in vitro studies of drug protein binding [13]. The applicability of small sample volumes using equilibrium dialysis made this method suitable both because of the limited amount of sample obtainable from the cancer patients and because of a relative shortage of purified labeled drug. The demonstrated stability of the equilibrium indicated that no protein denaturation or bacterial contamination, factors known to be possible pitfalls with equilibrium dialysis, occurred [4].

pH has been shown to influence the binding to serum proteins of several drugs [2]. The results of our experiments with various pH values in the buffer indicated that a low pH of about 7.0 was associated with a substantial reduction in the binding of thio-TEPA, demonstrating the importance of controlling pH in protein-binding experiments with this drug too. However, the binding was constant in the pH range 7.2–7.8, including that encountered physiologically and the small variations obtained by our equilibrium dialysis.

A thio-TEPA concentration of 1000 ng/ml was selected for determination of the serum protein binding, because this concentration is equal to that achieved in the blood soon after a conventional dose of the drug given as a bolus injection [3, 7]; however high-dose thio-TEPA and autologous bone marrow transplantation are currently undergoing investigation, and proportionally higher peak levels have been reported [8].

This study has demonstrated a total binding of thio-TEPA to human serum in the order of 10%. In contrast to an earlier investigation, which suggested that gamma globulin was the only thio-TEPA-binding protein in human serum [10], albumin seemed to be the dominant protein with respect to thio-TEPA binding. However, an interaction with the lipoproteins was also demonstrated, which could be due to a dissolution phenomenon of thio-TEPA in the lipid phase of the protein complex. In the study by McDermott et al. [11], human serum albumin and alpha, beta, and gamma globulins were examined for binding of thio-TEPA at a concentration of 100 ng/ml. Apart from an apparently higher binding between drug and protein in this study, which can be attributed to the low concentration of thio-TEPA and differences in the experimental set up, the significance of the results correlates well with ours: a low degree of binding, which is mainly associated with the albumin fraction. With our experimental design we were not able to detect any binding between thio-TEPA and gamma globulins, which was also in agreement with the results obtained by McDermott et al.

Although statistically significant, the difference in thio-TEPA-protein binding between sera from healthy individuals and sera from cancer patients is not thought to be of clinical importance. The drug binding in sera from the cancer patients never exceeded 20%.

The purpose of examining serum protein fractions at different concentrations was to evaluate whether the substantial alterations in serum proteins that can be encountered in cancer patients [16] could possibly lead to clinically relevant alterations in thio-TEPA-protein binding. Our data, with respect to both the low interindividual variation in the total drug protein binding among cancer patients and the apparent lack of dependence on lipoprotein concentration for its binding to thio-TEPA, make this possibility seem unlikely. Similarly, the interindividual variation in thio-TEPA pharmacokinetics that we have recently reported [7] is not likely to be explained by different serum protein binding. The desirability of measuring the free fraction, as for highly protein-bound drugs, does not seem to apply to thio-TEPA, owing to a low degree of binding (<20%) and small interindividual variations in patients with highly different protein patterns in serum.

During the rapid development of cancer drug therapy, multidrug combinations have become widely used, and although the alkylating agents have been replaced to some extent by newer drugs, they are still used, one alkylating agent being included in most drug combinations. From a theoretical point of view, the individual drugs in a combi-

nation should preferably not be highly bound to the same serum proteins, because the consequences of drug-drug displacement interactions are difficult to predict [14]. Although a common mode of action is assumed for the alkylating agents, drugs in this class are structurally different, forming the basis of different pharmacological properties. Chlorambucil, which is a widely used alkylating agent, has recently been reported to be extensively (>99%) bound to serum proteins in mice [9]. Furthermore, the serum protein binding of melphalan has been shown to be concentration-dependent in humans and to vary over the range 40%–75% [5]. From this point of view, the almost insignificant degree and small interindividual variation of serum protein binding of thio-TEPA in humans indicate that this drug is well suited for use as an alkylating agent in cancer drug combinations.

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