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In vitro interactions of TNO_6 with human plasma

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Summary. *The ability of TNO_6 to react with human plasma was investigated by in vitro incubation of plasma or plasma fractions with injectable TNO_6. HPLC, ultrafiltration and flameless atomic absorption spectrophotometry were used to separate the platinum-containing chemical species and to measure the platinum content. The initial concentration of TNO_6 in plasma declines very rapidly. The kinetics of the loss of initial TNO_6 is very different from that of cisplatin loss. Most of the TNO_6 is bound more quickly to proteins, while a little is transformed to less reactive or nonreactive platinum species. The level of final nonreactive platinum species depends on the particular plasma concerned. In addition, the reactivity of TNO_6 towards proteins is very sensitive to Cl^- concentration. The usefulness of HPLC for the study of TNO_6 kinetics is demonstrated.*

Introduction

TNO_6 (1,1-diamino-methylcyclohexane-sulfate-platinum II) is a new neoplastic platinum compound tested in clinical trials. TNO_6 differs from cisplatin structurally and belongs to the 'second-generation' metal complex with expected equivalent or better antitumor activity and reduced toxicity. While the pharmacodynamics of cisplatin are well documented [3–6], the pharmacodynamic properties of TNO_6 are still not well established.

Reactions of cisplatin with human plasma, especially protein binding, are important factors for the behavior of cisplatin in the body [1, 2, 7], and it can be expected that TNO_6 also reacts with plasma components.

The purpose of the present study was investigation of the TNO_6 reactions with human plasma. To eliminate other pharmacokinetic parameters, only in vitro TNO_6 reactions with plasma collected from patients untreated with platinum compounds were investigated.

Materials and methods

All chemicals used were analytical-grade reagents. Cisplatin and TNO_6 was obtained from Bristol Laboratories (New York, USA) as injectable solutions.

TNO_6 and cisplatin reactions were studied at 37° C with plasma from patients with different neoplasms. None of the patients had received chemotherapy in the weeks prior to the blood collection. Aliquots (2–3 ml) of the reaction solutions were sampled at appropriate time intervals.

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The samples were ultrafiltered by centrifugal ultrafiltration using centriflo CF 50 filters (Amicon Corp) to separate ultrafilterable and non-filterable platinum.

HPLC was used to separate components of the ultrafiltrates. The HPLC system consisted of Waters Associates model 6000 A and model M 45 pumps, model 660 solvent programmer and model U6K injector. Separations were accomplished on a Waters C18 reverse phase radial compression column in a Waters Z module radial compression separation system. Ultrafiltrates were eluted by application of a solvent gradient. Solvent A was 0.1 *M* KH_2PO_4 (adjusted to pH 2.55 with H_3PO_4). Solvent B was acetonitrile-solvent A (3/2, v/v). After elution with solvent A for 2 min, the proportion of the solvent B in the mobile phase was increased from 0 to 100% over a period of 20 min (linear gradient). The flow rate was 1.5 ml/min and the retention times were about 3 min for cisplatin and 11 min for TNO_6. The injection volumes were 20 µl.

Quantification of platinum in the ultrafiltrates or in the fractions of HPLC eluate was accomplished by flameless atomic absorption spectrophotometry (FAAS). A Perkin Elmer model 2280 absorption spectrophotometer equipped with a model HGA 400 graphite furnace was used for these analyses, with a 3-stage heating program consisting of drying at 120° C for 30 s, ashing at 1,400° C for 30 s, and atomizing at 2,700° C for 5 s. The inert gas was argon.

Results

Binding of TNO_6 and cisplatin to proteins (molec. wt > 50,000)

TNO_6 and cisplatin were incubated with two samples of the same plasma at 37° C. Aliquots were ultrafiltered after several different incubation periods, using Amicon filters with a molecular weight cut-off value of 50,000.

The rate of loss of platinum in ultrafiltrates is very different for cisplatin and TNO_6 (Fig. 1). The observed binding of TNO_6 to proteins with molecular weights up to 50,000 is faster than the binding of cisplatin.

The loss of cisplatin in the ultrafiltrate follows a familiar apparent first-order kinetic, while the loss of TNO_6 is more complex (Fig. 2). The loss of TNO_6 is very significant in the first minutes, then decreases to a constant concentration of platinum in the ultrafiltrate. The value of the constant concentration raised after 4 h depends on the plasma (Fig. 3). In no case is the value ever equal to zero. The binding of TNO_6

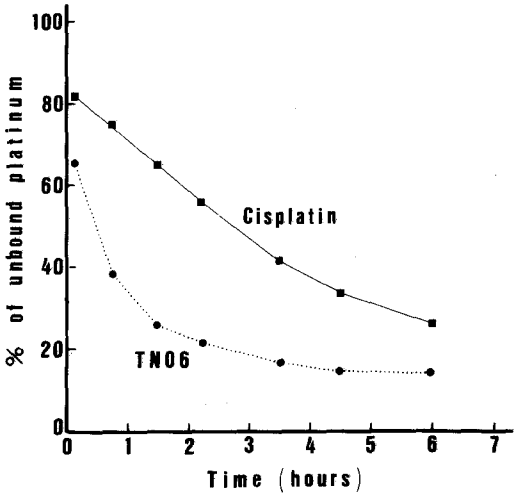

Fig. 1. The loss of platinum in ultrafiltrates of a plasma incubated at 37° C with TNO_6 2.9 μg/ml (●) and cisplatin 2.9 μg/ml (■)

Table 1. Loss of platinum in ultrafiltrates of plasma incubated with TNO_6 and cisplatin (25 μg/ml)

Time (min)	Platinum in ultrafiltrate			
	TNO_6 incubated alone	Cisplatin incubated alone	TNO_6 and cisplatin incubated together (theorical)	TNO_6 and cisplatin incubated together (experimental)
15	14.5	4.3	18.8	19.4
60	11.4	1.8	13.2	13
120	7.4	1.2	8.6	8.5
180	5.4	0.9	6.3	5.9
255	3.7	0.7	4.4	4
300	2.8	0.7	3.5	3.5
360	2.2	0.7	2.9	2.7

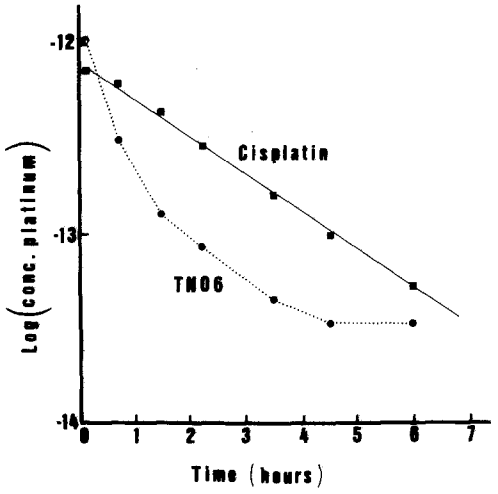

Fig. 2. Analysis of the hypothesis of a first-order loss of platinum for TNO_6 (●) and cisplatin (■) incubated at 37° C in a plasma. Log (conc. platinum) = f(t) must be a straight line

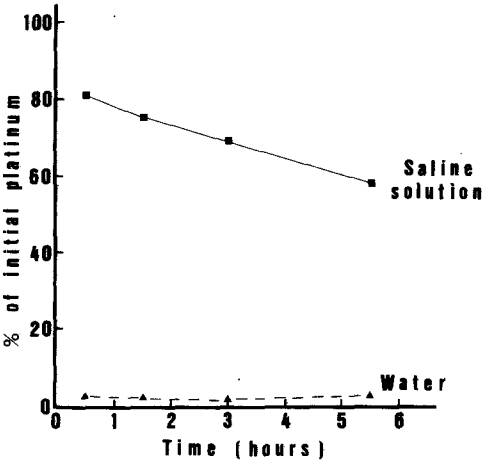

Fig. 4. Loss of platinum in ultrafiltrate of a TNO_6 (10 μg/ml) and a BSA (40 mg/ml) solution in water (▲) and in NaCl/water (9 g/l) (■) at 37° C

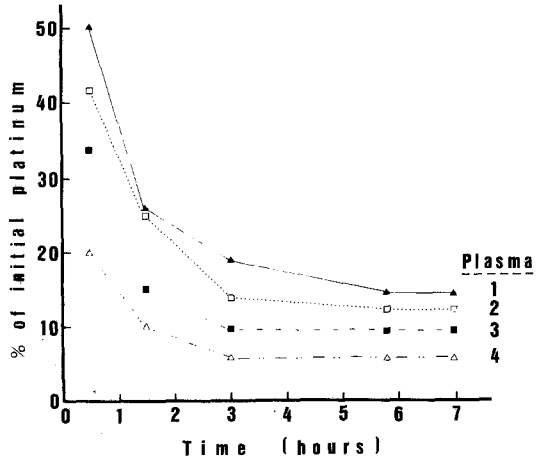

Fig. 3. Variation of the kinetics of TNO_6 (2.9 μg/ml) binding in different plasmas at 37° C

to proteins is not complete and the protein-binding capacity of plasma differs with patients.

These results do not depend on the plasma concentration of platinum compound, at least in the range of 2 μg/ml to 50 μg/ml.

The loss of platinum in the ultrafiltrate of a plasma when TNO_6 and cisplatin were incubated together was next examined (Table 1). The total loss of platinum is about equal to the sum of the losses for each compound incubated alone in the same plasma. Cisplatin and TNO_6 do not seem to compete for protein binding, at least in pseudophysiological concentrations.

To allow a better understanding of the TNO_6 protein reaction, the loss of TNO_6 (initial concentration 10 μg/ml) in the ultrafiltrate of a 40-mg/ml serum albumin (BSA) solution in water was examined (Fig. 4). The observed rates of reaction do not agree with the findings in plasma, since after 30 min the concentration of TNO_6 in ultrafiltrate was less than 2% of the initial concentration. TNO_6 binding to protein alone in water is

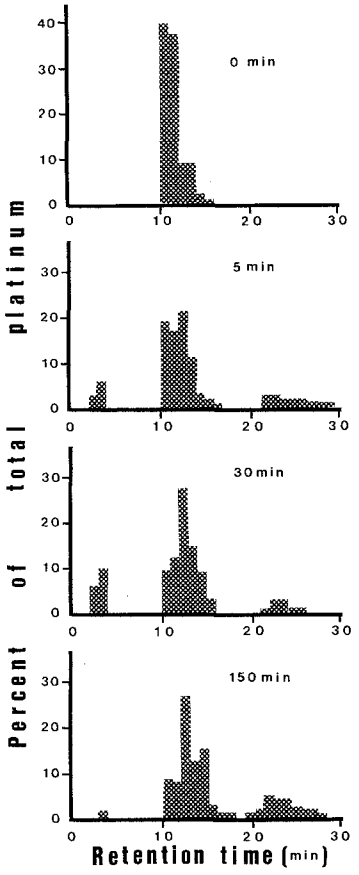

Fig. 5. Platinum contents of HPLC eluate from plasma ultrafiltrate incubated at 37° C several times with TNO_6 (43 μg/ml)

very much faster than to proteins in plasma. In contrast, the loss of TNO_6 incubated with BSA in isotonic solution (NaCl 9 g/l) was very slow (Fig. 4), since 60% of initial platinum concentration remained in the ultrafiltrate after 5 h. These data illustrate the role of the milieu on the kinetics of the protein binding of TNO_6. Therefore we studied the reaction of TNO_6 with ultrafiltrates alone.

Reaction of TNO_6 with plasma ultrafiltrates

TNO_6 reactions with low-molecular-weight (< 50,000) compounds were first conducted with ultrafiltrates from a pool of plasma. TNO_6 (40 μg/ml) was incubated with the ultrafiltrates, and an aliquot of the solution (20 μl) was then injected into a HPLC chromatograph as described in *Materials and methods*. The platinum content of each fraction of the eluate was measured by atomic absorption.

Figure 5 shows the HPLC chromatograms of TNO_6 alone and TNO_6 in ultrafiltrates after several different incubation periods. With increasing time the initial peak of TNO_6 quickly disappeared and it was observed that several other platinum-containing peaks appeared, associated with the loss of TNO_6. TNO_6 is not stable in the ultrafiltrate, and reacts either with chemical degradation or with association to low-molecular-weight elements. TNO_6 is also unstable in a solution of NaCl (9 g/l). In an NaCl solution with a low concentration of TNO_6 (43 μg/ml) the initial peak in HPLC disappears (Fig. 6). There is evidence that a physiologic concentration of NaCl can induce chemical degradation of TNO_6. Such a degradation can occur in the plasma.

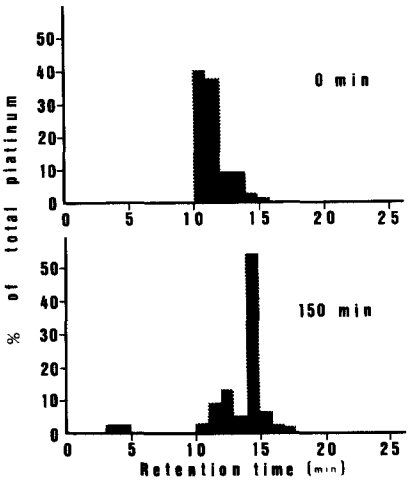

Fig. 6. Platinum contents of HPLC eluate from TNO_6 (43 μg/ml) in NaCl/water solution (9 g/l) after 0 and 150 min

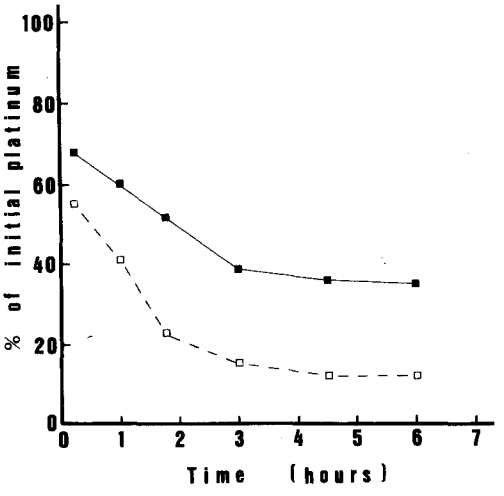

Fig. 7. Loss of platinum in plasma with TNO_6 (10 μg/ml) previously incubated with ultrafiltrate (■) or not previously incubated (□)

The major problem connected with protein binding of platinum in ultrafiltrates, then concerns the ability of the new platinum-containing compounds to react with plasma proteins.

To determine the binding capacity of these we added a TNO_6 (10 μl/ml) ultrafiltrate solution incubated for 2 h 30 min to a sample of total plasma. We compared the loss of platinum in ultrafiltrate with the similar loss of platinum in a solution containing the same plasma and the same ultrafiltrate not previously incubated with TNO_6.

Figure 7 shows that the loss of platinum is slower when TNO_6 has previously been incubated with ultrafiltrate, and that the constant concentration reached after some hours is higher in this case. This result seems to indicate that some of the platinum compound in ultrafiltrate can react with proteins, but at a lower rate than initial TNO_6. Some of the rest of the platinum in ultrafiltrates does not react with proteins within 6 h.

Reaction of TNO_6 with total plasma

We have previously described the evolution of platinum compound monitored by HPLC analysis of an ultrafiltrate

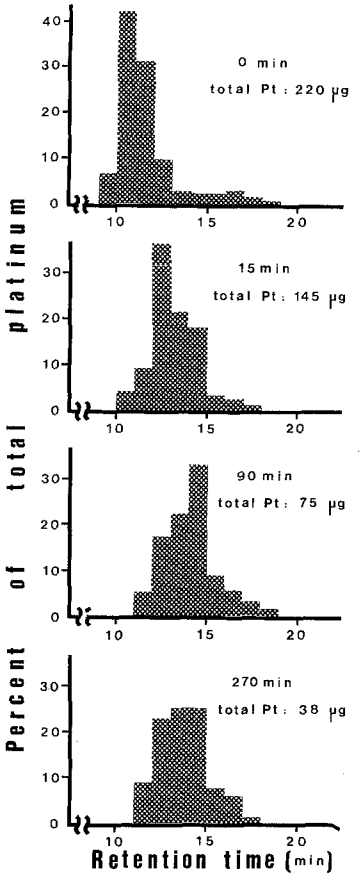

Fig. 8. Platinum contents of HPLC eluate from ultrafiltrates of plasma incubated for several times with TNO_6 (220 µg/ml)

incubated with TNO_6. We then examined the same evolution in ultrafiltrate (molec. wt < 50,000) of total plasma incubated with TNO_6.

Figure 8 shows that initial TNO_6 disappears very quickly from the ultrafiltrates. Most of the TNO_6 lost is bound to the high-molecular-weight proteins, while a little reacts with ultrafiltrate to give new platinum peaks, as observed previously. The parent compounds of TNO_6 in ultrafiltrate (products of degradation or associated forms) do not appear and decline simultaneously. On the contrary, the evolution of the peaks seems to indicate either successive conversion of the platinum compounds or, more probably, differences in the reactivity of these compounds towards proteins.

Discussion

Until the introduction of new platinum-containing compounds, such as TNO_6, into phase I and phase II testing, cisplatin was the only platinum derivative that has been commonly used in cancer treatment [6].

The ability of cisplatin to bind to proteins is an important aspect of its pharmacology [1–5, 7], and as expected, TNO_6 exhibits the same property.

In fact TNO_6 shows a greater affinity to plasma proteins in vitro (molec. wt > 50,000) than cisplatin, but reacts with different kinetics (Figs. 1 and 2). While cisplatin binding is a first-order reaction, TNO_6 binding is very fast in the early

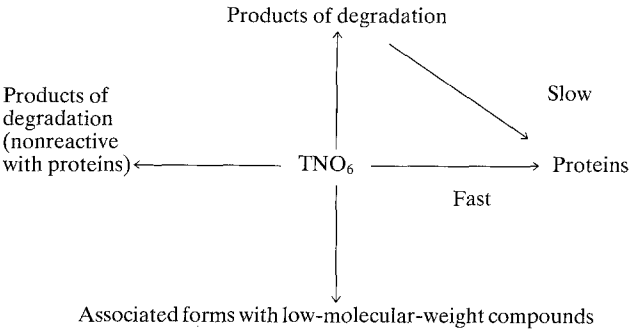

Fig. 9. Schematic showing in vitro protein binding of TNO_6

minutes of the reaction, then decreasès until no fixation of the platinum compounds remaining in the plasma.

Incubation of TNO_6 and cisplatin together in plasma shows that the two compounds do not compete for protein binding (Table 1), at least with concentrations in the range of 2 µg/ml to 50 µg/ml. When incubated with TNO_6 the plasma content of unbound platinum reaches a plateau after 4 or 5 h (Figure 1). The level of the unbound platinum concentration depends on the particular plasma studied and varies from about 5% to 20% of the initial platinum concentration (Fig. 3).

In contrast, binding of TNO_6 to a single protein in water is very fast and complete (Fig. 4). Otherwise TNO_6 reactions depend on the plasma contents.

These results are in agreement with the HPLC chromatography of TNO_6 incubated with plasma ultrafiltrate (without any possible reaction on proteins). The initial chromatographic peak quickly declines, while new platinum-containing peaks appear (Fig. 5). At least some of these new platinum compounds cannot react with the plasma proteins, while another fraction reacts much slowly than TNO_6 (Fig. 7).

In complete plasma there is competition between protein binding and formation of new platinum species with new kinetic properties (Fig. 8).

The nature of the new platinum species is problematic. There may be several explanations for their formation. TNO_6 may react with one or many of the numerous low-molecular-weight molecules present in plasma (peptides for example), or may be transformed to degradation products.

Although we are currently unable to exclude any of these possibilities, there are some arguments for the presence, at least, of degradation products, the main one being that TNO_6 is unstable even at a low concentration in an isotonic solution of NaCl (Fig. 6). Moreover, while TNO_6 reacts quickly with a single protein in water it reacts slowly with the same protein in an isotonic solution (Fig. 4). This fact is in keeping with a difference in kinetic properties between TNO_6 and its degradation products.

In conclusion, in vitro protein binding of TNO_6 is more complex than cisplating binding, and it can be summarized by the schematic of Fig. 9.

The initial velocity of the TNO_6 binding, the sensitivity of the reaction to Cl^- concentration and the possible presence of unreactive platinum species may be important pharmacodynamic properties for the explanation of TNO_6 behavior in vivo.

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