

Discontinuous Drug Binding to Proteins: Binding of an Antineoplastic Benzyl Styryl Sulfone to Albumin and Enzymes In Vitro and in Phase I Clinical Trials

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ABSTRACT:

Sodium (*E*)-[*N*-[2-methoxy-5-(2',4',6'-trimethoxy-styrylsulfonyl)methylenephyl]amino]acetate ($C_{21}H_{24}NNaO_8S$, ON 01910.Na) is a novel, synthetic benzyl styryl sulfone, currently in phase I clinical trials in cancer patients. Our objective was to use electrospray mass spectrometry to determine, in intact complexes, the number of drug molecules bound to albumin and selected enzymes. Native and recombinant albumin incubated with the drug, at various molar ratios, revealed simultaneous and discontinuous progression of drug binding, yielding intact albumin-drug complexes containing up to 22 drug molecules. Comparable complex protein-drug patterns were obtained for several enzymes, e.g., carbonic anhydrase. Intact albumin-ON 01910 complexes were also found in all patient samples. The drug-binding profiles were comparable, but not iden-

tical, for increasing sampling times and different doses (400–1700 mg/m²). We concluded that the techniques developed are capable of detecting the simultaneous formation of intact protein-drug complexes and of determining the number of drug molecules bound to proteins. The results enhance our hypothesis that drug binding may lead to conformational changes in proteins that, in turn, account for the exclusion of specific binding complexes and may influence protein behavior and activity. Application of these techniques reveals new insights about the nature of the antineoplastic drug ON 01910 in patient plasma, and the information obtained may have significance in understanding drug delivery to tumors.

Introduction

ON 01910.Na (473.5 Da), is a novel, synthetic benzyl styryl sulfone, designed to inhibit cell cycle progression (Gumireddy et al., 2005). The chemical composition of ON 01910.Na is sodium (*E*)-[*N*-[2-methoxy-5-(2',4',6'-trimethoxy-styrylsulfonyl)methylenephyl]amino]acetate, $C_{21}H_{24}NNaO_8S$ (Fig. 1A). It is a cell cycle-active drug, and as such it inhibits mitotic progression, induces apoptosis, and has activity against most human cancer cell lines in vitro (>100 tumor types tested, including drug-resistant lines) and against a broad spectrum of human xenografts in mice (Chun et al., 2009; Prasad et al., 2009). Currently, the drug is in several phase I clinical trials in adult patients with a variety of solid tumors (Jimeno et al., 2008) as well as hematologic malignancies. A liquid chromatography-mass spectrometry assay was developed for pharmacokinetic studies (Li et al., 2007). In a preclinical application to pancreatic cancer, an ex vivo predictive assay was developed (Jimeno et al., 2009).

Based on a previous preliminary study of intact albumin-suramin (Roboz et al., 1998) and other intact protein-drug complexes, we hypothesized that drug binding to proteins displays a discontinuous

progression and might lead to conformational changes that could influence protein activity. The objective of the present work was to determine, using electrospray ionization (ESI) mass spectrometry, the molecular masses and thus the number of drug molecules bound in intact complexes of ON 01910.Na with native and recombinant human albumin and selected enzymes. These studies included both in vitro incubations and serum/plasma samples, taken at a variety of time points, from patients in phase I trials.

Materials and Methods

Drugs, Albumin, Enzymes, Chemicals, and Reagents. ON 01910.Na and ON 01911 (451.5 Da), one of its structural analogs (Fig. 1B) that is biologically inactive in systems in which ON 01910.Na is active, were provided by Onconova Therapeutics, Inc. (Newtown, PA). Native and recombinant human albumin (~66 kDa), papain (~21 kDa), elastase (~25 kDa), trypsin (~24 kDa), and carbonic anhydrase (~29 kDa) were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Acetonitrile, formic acid, and water were of high-performance liquid chromatography grade, and all other chemicals and reagents were of the highest available purity (Thermo Fisher Scientific, Waltham, MA).

In Vitro Incubation of Albumin and Enzymes with ON 01910.Na. In one set of experiments with excess albumin, molar ratios of albumin/ON 01910.Na, and also the analog ON 01911, ranged from 1:1 to 50:1, in eight steps. In another set of experiments, with excess drug, the molar ratios were reversed. Incubations were performed at 37°C for periods ranging from 0 min to 24 h.

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ABBREVIATIONS: ON 01910.Na, sodium (*E*)-[*N*-[2-methoxy-5-(2',4',6'-trimethoxy-styrylsulfonyl)methylenephyl]amino]acetate, $C_{21}H_{24}NNaO_8S$; ESI, electrospray ionization.

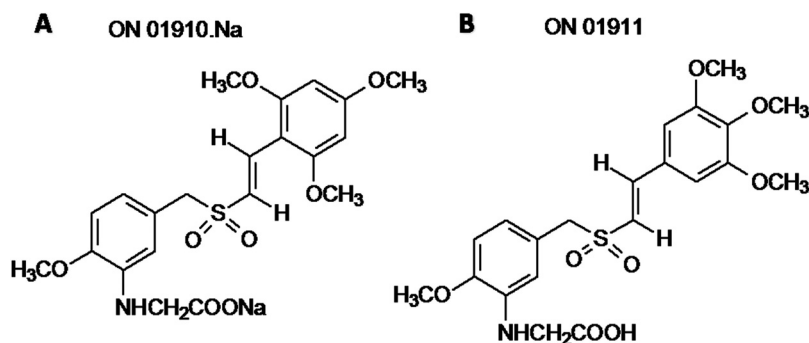


FIG. 1. Chemical structures of ON 01910.Na (A) and its structural isomer ON 01911 (B).

Similar experiments were performed by incubating ON 01910.Na with enzymes (molar ratios in the 1:10 to 10:1 range).

Clinical Protocol in Phase I Trial of ON 01910.Na. We obtained blood samples from patients who were treated in two clinical protocols, designed in accordance with all applicable regulations. Protocol 1 was entitled "A Phase I Dose Escalation of ON 01910.Na by a 3-Day Continuous Infusion in Patients with Advanced Cancer." Protocol 2 was entitled "Phase I Dose Escalation Study of ON 01910.Na with Increasing Duration of an Initial 3-Day Continuous Infusion in Patients with Refractory Leukemia or Myelodysplastic Syndrome (MDS)." These clinical studies are still ongoing and have been supported by Onconova Therapeutics, Inc. The drug studied, ON 01910.Na (Investigational New Drug no. 66,780), was supplied by the same company. These protocols called for blood collection for pharmacokinetic and biochemical studies. Both protocols were approved by the institutional review board of the Mount Sinai School of Medicine. Signed informed consent was obtained before the start of study in all volunteer patients with advanced cancer.

In the first protocol, the starting dose of the drug was a $50 \text{ mg}/(\text{m}^2 \cdot \text{d})$ continuous intravenous infusion for 3 consecutive days, and dosing was repeated every 2 weeks. In the absence of grade 2 or more toxicity, the dose of the drug was escalated according to a modified Fibonacci search scheme in subsequent patients. After observation of grade >2 toxicity, dose escalation was slowed to a half escalation in three or more patients in each cohort. The maximally administered dose was $1700 \text{ mg}/(\text{m}^2 \cdot \text{d})$ for 3 days. Patient samples for this study were obtained randomly from six different patients who represented all dose levels higher than $400 \text{ mg}/(\text{m}^2 \cdot \text{d})$. Blood samples were taken at 0, 1, 3, 6, 24, 48, 72, 72.5, 73.5, 75, 78, 96, 120, and 144 h, in either red-top tubes (for serum) or green-top tubes (for plasma) (BD Biosciences, San Diego, CA), followed by centrifugation at 300g (model 5415D centrifuge; Eppendorf North America, New York, NY) for 10 min. For comparison, one patient was randomly selected from those on protocol 2 (hematologic tumors), receiving a drug dose of $1375 \text{ mg}/\text{m}^2$.

Characterization of Intact Protein-Drug Complexes. Aliquots of the incubated samples of in vitro models or patient serum/plasma were injected directly into the mass spectrometer. The eluent was acetonitrile-water (1:1,

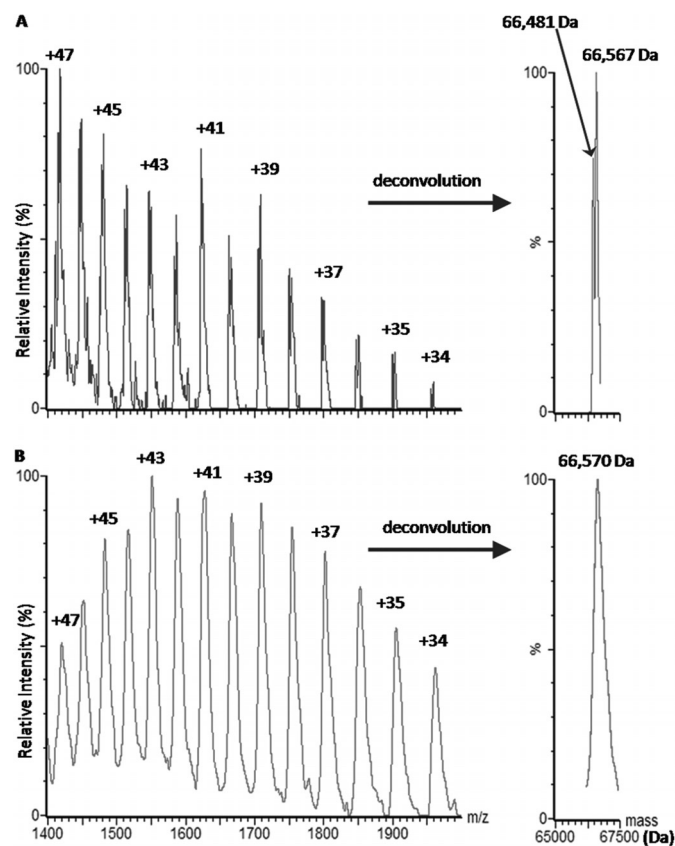


FIG. 2. Mass spectra of human albumin (A) and human recombinant albumin (B) (each $10 \mu\text{M}$). Left side, "raw" mass spectra; the numbers show the number of positive charges in the multiply charged ion profiles, m/z 1000 to 2000 range. Right side, molecular masses (daltons) obtained by deconvolution.

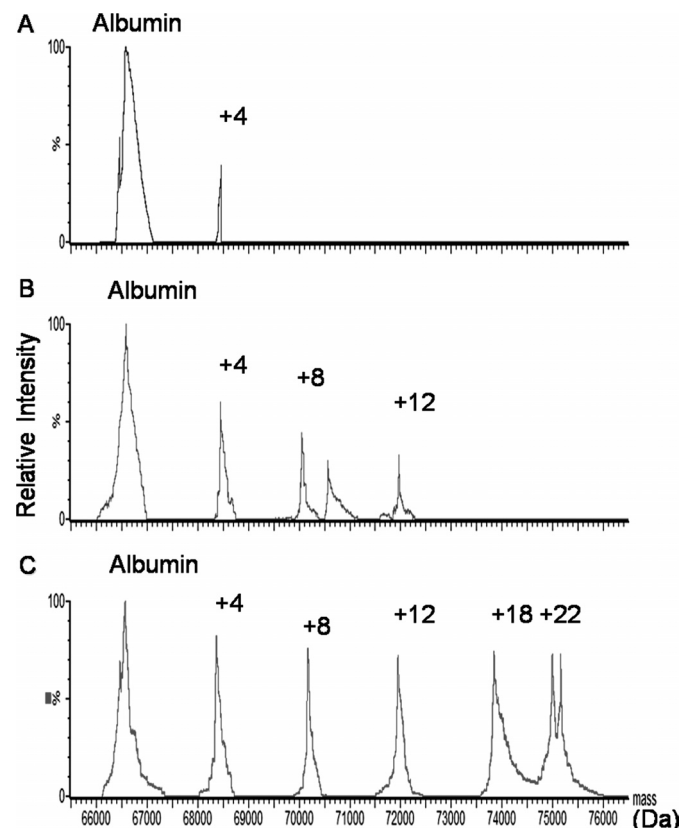


FIG. 3. Comparison of deconvoluted mass spectra (65–75 kDa range) of recombinant human albumin incubated (2 h) with ON 01910.Na. Albumin/drug molar ratio: A, 10:1; B, 1:1; C, 1:10.

v/v) + 0.1% formic acid, at a flow rate of 200 $\mu\text{L}/\text{min}$. ESI mass spectra were obtained with a triple quadrupole mass spectrometer system (Quattro LC; Waters, Milford, MA), operated in the positive ion mode. The source temperature was kept at 80°C; the cone voltage was 42 V. The mass scale was calibrated (up to m/z 2500) using sodium iodide. After injection of 10- μL sample aliquots into the ESI source, total ion currents were obtained in the “continuum” mode (not centroid mode) by monitoring masses in the m/z 1000 to 2000 range (scanned in 4 s). After several steps of handling and smoothing mass spectral data in the relevant portions of the total ion currents, “raw” mass spectra of the number of multiply charged ion patterns formed from the large protein-drug complexes were obtained.

Molecular masses of unreacted albumin and the intact protein-drug complexes were generated from the raw mass spectra using Transformation software, which is part of the MassLynx program (Waters). Several parameters were optimized, including the selection of the number of multiply charged ions (in the range of 15 to 19) that must be present in the m/z 1000 to 2000 range and the corresponding mass range of unreacted albumin and possible albumin-ON 01910 complexes, i.e., 66 to 75 kDa.

The number of ON 01910 molecules bound to albumin was obtained by subtracting the molecular mass of albumin from those calculated for the complexes and dividing the remainder by the molecular mass of ON 01910. Note that the sodium content was omitted because the drug ionizes when it is dissolved in water; thus, sodium does not contribute to the molecular mass of the complexes. The presence of an albumin-drug complex in the observed mixture of complexes was accepted only if the number of bound ON 01910 molecules thus calculated was within ± 0.1 from a nominal (“whole”) number, e.g., an albumin-ON 01910 complex (at 68,369 Da; see Fig. 6B) containing four bound drug molecules. Similar calculations were used to determine the number of ON 01910 molecules bound to several enzymes (see *Results*).

The reproducibility of individual complexes was estimated by analyzing several samples repetitively (10–13 times) and counting the number of times the complex was detected. The cutoff for acceptance was set, arbitrarily, at 50%. The reproducibility of data reported was in the 60% (good) to 100% (excellent) range.

Results

In Vitro Incubation of ON 01910.Na with Albumin. Transformed mass spectra of native albumin revealed the presence of at least two forms of native albumin (Fig. 2A). As expected, these were also observed in all serum/plasma from patients. In contrast, recombinant albumin yielded a significantly “cleaner” spectrum, and deconvolution revealed a single, predominant molecular mass (Fig. 2B). Recombinant albumin was used in all in vitro incubation experiments.

Formation of complexes commenced immediately upon exposure of albumin to ON 01910.Na solutions. A set of experiments, using widely differing albumin-drug molar ratios, revealed that the number of bound molecules of ON 01910 was usually even (Fig. 3). A complex containing 4 drug molecules bound was always observed, regardless of widely differing albumin/drug molar ratios, incubation temperatures, and duration of incubation. Complexes containing more drug molecules, e.g., 8 and 12, up to 22, were often, but not always, detected, depending on experimental conditions and the origin of patient samples. Complexes containing 13 and/or 15 bound drug molecules were occasionally detected in albumin-drug incubates with very large drug excesses, e.g., 1:10 albumin/drug molar ratio. We note that at high drug-albumin molar

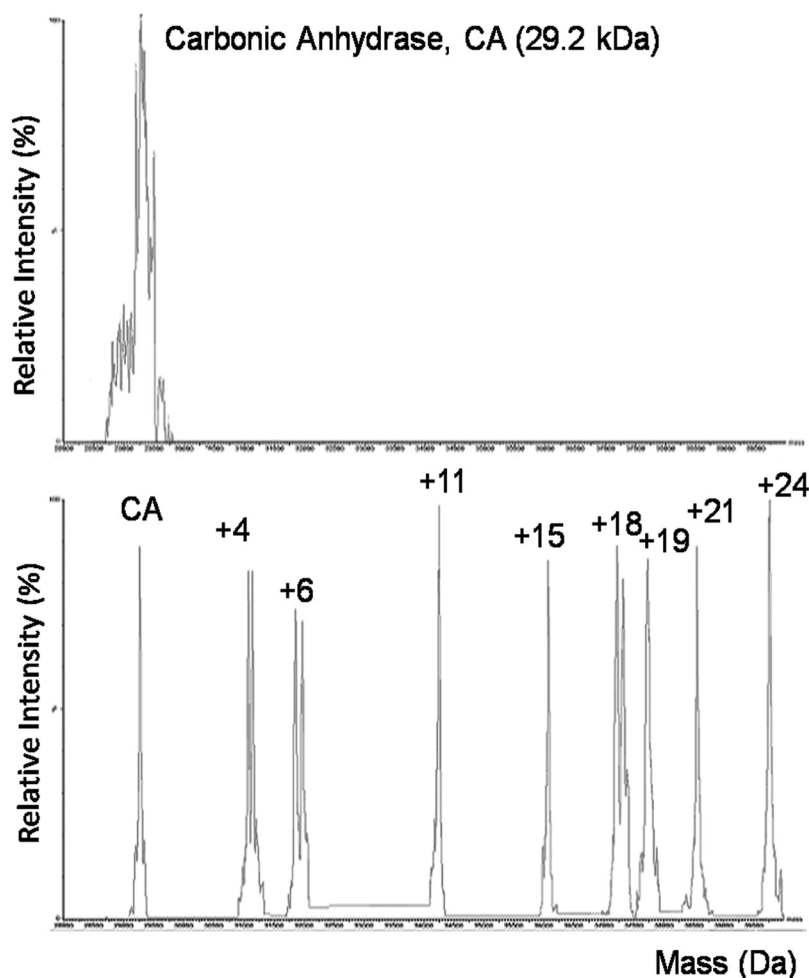


FIG. 4. Top, unreacted carbonic anhydrase (10 μM). Bottom, number of ON 01910 molecules attached to carbonic anhydrase (intact enzyme-drug complex) after incubation at 37°C for 20 min. Enzyme/drug molar ratio = 1:1.

TABLE 1

Number of ON 01910 molecules simultaneously present in intact complexes with carbonic anhydrase, papain, trypsin, and elastase

✓ indicates present.

| Protein | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|-----------------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Carbonic anhydrase (29 kDa) | | | | ✓ | | ✓ | | | | | ✓ | | | | ✓ | | | ✓ | ✓ | | ✓ | | | ✓ |
| Papain (21 kDa) | | ✓ | ✓ | | | ✓ | | ✓ | | | | | | | | | | | | | | | | |
| Trypsin (24 kDa) | | | | | | ✓ | | | ✓ | | ✓ | | | | | | | | | | | | | |
| Elastase (25 kDa) | ✓ | ✓ | ✓ | | | | | | | | | | | | | | | | | | | | | |

ratios a decrease in the quantity of unreacted albumin was also observed.

Comparable albumin-drug patterns were obtained in experiments performed at different incubation periods. A comparison of the binding patterns of ON 01910 and its structural isomer, ON 01911 (Fig. 1), exhibited both qualitative similarities and some minor quantitative differences (data not shown).

In Vitro Incubation of ON 01910.Na with Enzymes. Simultaneous formation of complexes between several enzymes and ON 01910, containing multiple numbers of drug molecules, was detected in in vitro incubations (molar ratios in the 1:10 to 10:1 range), e.g., carbonic anhydrase (Fig. 4). A comparison of the number of ON 01910 molecules simultaneously present in intact complexes of the drug with several enzymes is shown in Table 1.

Confirmation of Measured Masses. In separate experiments, we have used a linear trap quadrupole type of mass spectrometer (model LTQ; Thermo Fisher Scientific) and another type of deconvolution software (Promass Deconvolution 2.0; Thermo Fisher Scientific) to analyze a few randomly selected incubation samples. The results of these experiments were comparable, within expected experimental errors, to those obtained with the instrumentation and techniques described above.

Albumin-ON 01910 Complexes in Patient Samples. Formation of simultaneous multiple complexes between drug and albumin were found in all patient samples analyzed, even at the lowest dose level, 400 mg/m² of patient body surface area (Fig. 5B). Increasing dose levels to a maximum of 1700 mg/m² (solid tumor) or 1375 mg/m² (hematologic tumor), yielded patterns similar to those at lower dose levels (Fig. 6). Although the general appearance of the patterns was comparable in most cases and at all drug doses, there were both qualitative and quantitative differences between individual patients. The complexes with +4 and +8 molecules appeared in virtually all samples, although often in different relative intensities. Other complexes with even numbers of bound ON 01910, such as 8, 10, 12, and 18 (up to 22) appeared frequently. Odd numbers of bound drug molecules, such as 13 and 17 also appeared, but less frequently, at low drug doses.

The numbers of bound ON 01910 molecules for several drug doses are shown in Table 2. Two time points were selected, 6 h to represent the period during infusion, and 96 h to represent the period after the cessation of infusion. The + signs in the table provide an indication of the relative intensities of the particular complexes with respect to the intensity of the unreacted albumin

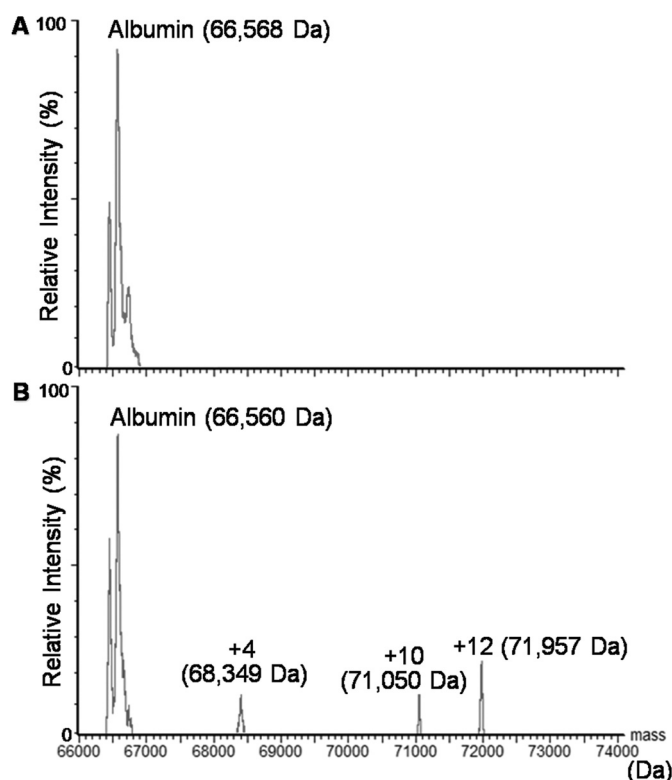


FIG. 5. Comparison of the number (and molecular mass) of ON 01910 molecules bound to albumin in samples from a patient with a solid tumor treated with a drug dose of 400 mg/m². A, before drug infusion; B, 3 h after the start of drug infusion. Mass range of the deconvoluted spectra 66 to 74 kDa.

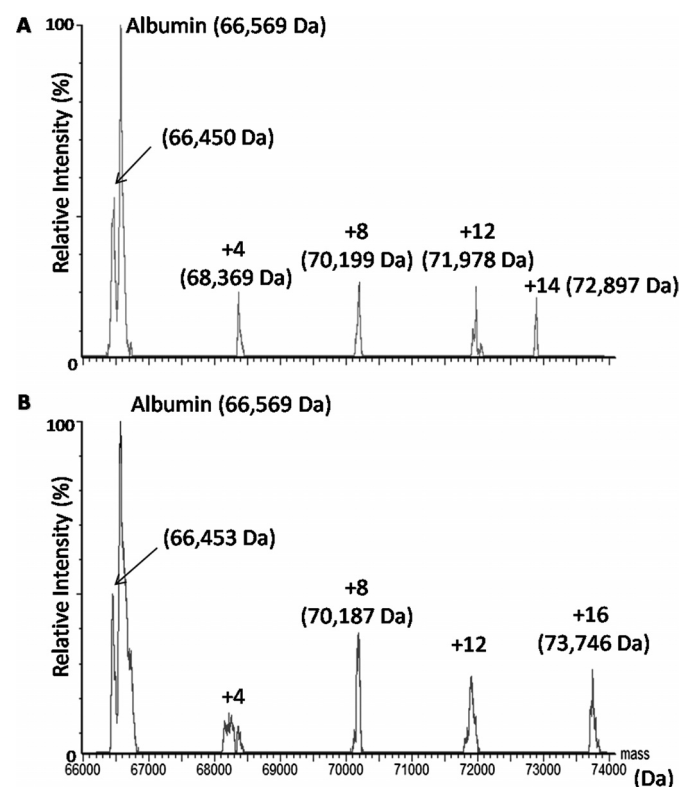


FIG. 6. Comparison of the number (and molecular mass) of ON 01910 molecules bound to albumin in samples from a patient with a solid tumor, drug dose 1700 mg/m² (A) and a patient with a hematologic tumor, drug dose 1375 mg/m² (B). Samples were taken 6 h after the start of drug infusion.

TABLE 2
Numbers of ON 01910 molecules bound to albumin in patient plasma

| Drug Dose | H after Infusion | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 |
|-----------|------------------|-----|----|----|----|----|----|----|----|
| 400 | 6 | + | | | + | + | | | |
| | 96 | + | | | | | + | | + |
| 650 | 6 | + | | | + | | + | | + |
| | 96 | +++ | | | ++ | | + | | |
| 850 | 6 | ++ | + | | + | | | + | + |
| | 96 | +++ | ++ | | | ++ | + | + | + |
| 1050 | 6 | ++ | + | + | + | | | | |
| | 96 | +++ | | + | | | | + | |
| 1375 | 6 | +++ | ++ | + | + | | + | + | ++ |
| | 96 | ++ | + | + | | ++ | + | | + |
| 1700 | 6 | + | | + | | + | + | | |
| | 96 | +++ | | ++ | | ++ | + | + | + |

+, 10% intensity of albumin peak; ++, 30% intensity of albumin peak; +++, 50% intensity of albumin peak.

peak; e.g., +++ shows that the +4 complex was the largest intensity of almost all samples with higher drug doses.

Discussion

It is well known that human serum albumin plays a major role in the transport of drugs, metabolites and endogenous ligands. The physiologically active concentration of a drug, in its free form, is significantly affected by the nature, as well as the degree, of binding to albumin. The albumin-drug complex is likely to provide a reservoir for dissociation to provide free unbound drug, thus prolonging drug action. Every aspect of the processes of absorption, distribution, metabolism, and excretion of a drug may ultimately be influenced significantly by the nature and degree of binding to albumin.

Concerning methodology, we have confirmed that the protein-drug complexes were present regardless of the presence of acetonitrile in the eluent. Note also that proteins were not isolated in patient samples; aliquots of plasma were injected directly. The relevant unreacted albumin and the intact albumin-drug complexes were identified based on their corresponding molecular masses, obtained from multiply charged ion patterns using deconvolution as described under *Materials and Methods*.

In Vitro Incubation with Recombinant Albumin. As noted under *Results*, the mass spectra of native albumin, both commercial (Fig. 2) and from patient samples (Fig. 6), revealed the presence of two (previously observed) "forms" of albumin, differing by approximately 80 to 90 mass units. The relative quantities of these two forms of albumin varied (40–80%), depending on the origin of the sample. Complexes with ON 01910 were formed with both forms of albumin, but in this study we have considered only those complexes formed with the albumin of higher molecular mass. The consequence of the obvious differences between the mass spectral patterns of native and recombinant albumin (Fig. 2) was that the interpretation of the mass spectra of the complexes with recombinant albumin was significantly easier than those of native albumin because of the pattern complexities from the multiple protein forms in native albumin.

In the in vitro experiments, complex formation was "immediate," i.e., the complexes were detected when albumin and drug solutions were mixed, and aliquots were injected into the mass spectrometer (estimated to be 30–45 s). Mixtures of intact complexes formed simultaneously, containing varying numbers of bound drug molecules, e.g., up to 22 drug molecules per albumin molecule (Fig. 3). The spectra reveal the variability and apparently discontinuous progression of drug binding to albumin, e.g., Fig. 3B shows 4, 8, and 12 molecules of ON 01910 bound to recombinant albumin. Figure 3 also illustrates variability due to different molar ratios.

Although the majority of albumin-ON 01910 complexes contained an even number of bound drug molecules, complexes with odd numbers (e.g., 13 and 17) of bound molecules were found occasionally and usually with low abundance, in both in vitro experiments and in samples from patients (see later). Without knowing the exact mechanism of drug binding, there is no rational reason that an odd number of bound molecules should not appear; indeed, we have often found odd numbers in protein-drug complexes involving other proteins and several other drugs (see next paragraph). We have found no direct relationship between the presence of predominantly even and/or a relatively small quantity of odd numbers of drug molecules bound to albumin in either in vitro experiments or in patients.

Similar (but not identical) drug-binding profiles were obtained for compound ON 01911 (Fig. 1B), a structural isomer of ON 01910, but one without antineoplastic activity. We observed a similar pattern of simultaneous formation of multiple complexes between albumin and the antineoplastic drug suramin (Roboz et al., 1998) as well as a number of drugs of widely different types, molecular weights, and traditionally known both highly and lowly bound drugs; although each drug had its own characteristic binding patterns, the discontinuous progression was similar to that presented in the present work (S. Y. Cho and J. Roboz, unpublished observations). These observations suggest that discontinuous progression of small molecule binding to proteins may be a general phenomenon.

Although they have vastly different catalytic functions, all enzymes are proteins; thus, they too would be expected to form complexes with small molecules outside the active enzymatic site. The multiple complex formation and binding pattern with albumin observed for ON 01910 also appeared in incubation experiments with several important enzymes, e.g., carbonic anhydrase (Fig. 4). As seen in Table 1, there are obvious differences in the number of ON 01910 molecules binding to various enzymes, but the patterns add credence to the suggestion that the simultaneous and discontinuous progression of drug binding to proteins is a general phenomenon.

Albumin-Drug Complexes in Patient Samples. Intact albumin-ON 01910 complexes were found in all patient samples analyzed. Even at the low dose of 400 mg/m², the sample taken 3 h after the start of the infusion, the complexes with the highest abundance contained, concurrently, +4 (68,349 Da), +10 (71,050 Da), and +12 (71,957 Da) drug molecules bound per albumin molecule (Fig. 5B). The drug-binding profiles were similar, but not identical, for increasing sampling times and different doses. Complexes containing +4 bound drug molecules appeared in every clinical sample, regardless of dose and sampling time. Up to 22 bound molecules per albumin molecule were detected. At low drug doses, the binding of ON 01910 to albumin also followed a discontinuous progression with +4 and +8

always appearing predominantly, and +13 and +17 drug molecules per albumin appearing occasionally. Occasional differences were also seen between patients with solid tumors versus patients with hematologic malignancies (Fig. 6).

The clinical protocol used in these studies specified that after a "zero time" sample (i.e., before the start of infusion), the first samples should be taken 1 h after the start of the infusion. By that time, a number of complexes were already present, several with considerable intensity, suggesting that complex formation takes place rapidly after exposure to the drug. This is in agreement with the in vitro studies, which suggest that the complex formation might occur in seconds (or microseconds?) after exposure.

As expected, total drug concentrations (determined after precipitation of proteins with acetonitrile) fell precipitously at the end of infusion; however, low levels persisted in many patients, suggesting a possible role of the albumin-drug complex as a reservoir. There was no evidence of a direct relationship between the changes in ON 01910.Na drug dose (from 400 to 1700 mg/m²) during treatment and the presence of the albumin-drug complexes.

We conclude that the techniques developed are capable of detecting intact albumin-drug complexes and of determining the number of drug molecules bound to albumin. Drug binding does not behave as a continuous variable, but rather as discontinuous binding, specific to each protein studied. The results enhance our hypothesis that drug (small molecule) binding may lead to conformational changes in proteins that account for the exclusion of specific binding complexes and may influence protein behavior and enzymatic activity. Applica-

tion of these techniques revealed new insights about the nature of the antineoplastic drug ON 01910 in both in vitro incubation experiments and in patient plasma in phase I clinical trials; the information obtained may have significance in understanding drug delivery to tumors.

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