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Short communication

Protein-binding patterns of the antitumor antibiotic cryptophycin 52 as measured with a two-phase partitioning system

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Abstract

Exposure of murine leukemia L1210 cells to the antitumor antibiotic cryptophycin 52 (C52) resulted in a rapid and dose-dependent increase in cell-surface hydrophobicity, as measured with a two-phase partitioning system. This effect was not observed with inactive drug analogs that lacked an epoxy residue. While the C52 has distinctly hydrophobic properties, the drug does not uniformly bind to all proteins. Affinity for human high- and low-density lipoprotein and albumin was demonstrated, but the drug binds only to the albumin fraction of mouse plasma, in spite of the high HDL level in the latter species.

Keywords: Protein-binding patterns; Two-phase partitioning; Cryptophycin 52

1. Introduction

The cryptophycins are potent antitumor antibiotics which are derived from blue–green algae [1,2] and, like the Vinca alkaloids, cause microtubule depolymerization [3,4]. Among the more active in the series is cryptophycin 52 (C52) a macrolide bearing an epoxide residue (Fig. 1). Cryptophycins are highly hydrophobic molecules that show extensive binding to albumin and to a variety of surfaces including glass and plastic [5]. In this study, we used murine leukemia L1210 cells to examine the effects of C52 and two drug analogs lacking the epoxide residue on cell-surface hydrophobicity. Additional studies were carried out to determine binding affinity of C52 toward a model series of biologically-significant proteins: albumin and lipoproteins from human and murine plasma in vitro.

2. Materials and methods

2.1. Reagents

[^{3}H-Methoxyl]-cryptophycin 52 (82 mC/mmol) was obtained from Amersham Life Sciences, Arlington Heights, IL, USA, and provided by Eli Lilly Research Labs., Indianapolis, IN, USA. This was diluted with carrier C52 so that a 10 n*M* concentration=$2\cdot10^6$ dpm. C52 was insoluble in

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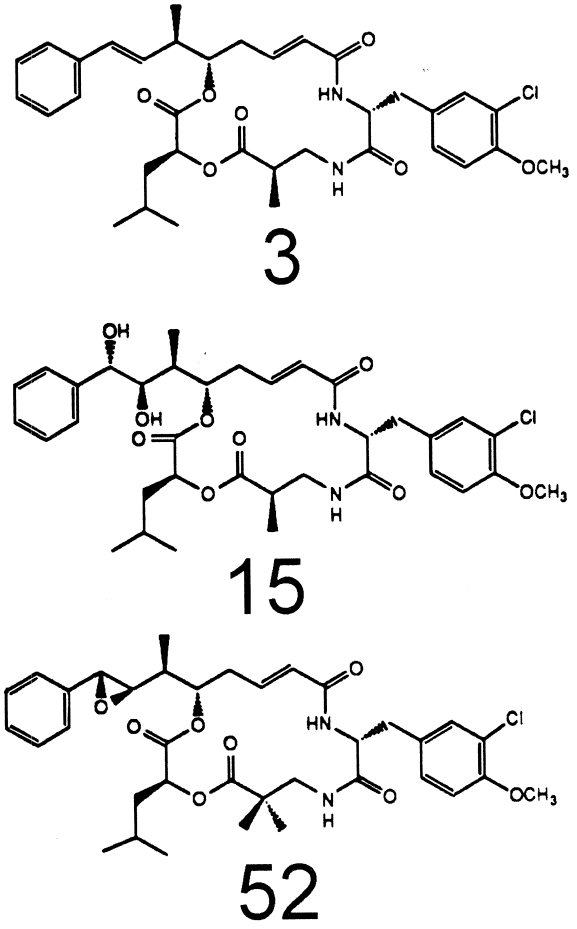

Fig. 1. Structures of C3, C15 and C52.

aqueous solution and was formulated in dimethyl sulfoxide.

2.2. Biological preparations

Pooled human and mouse plasma were brought to 1 m*M* EDTA to retard lipid oxidation, and stored at −80°C until used. Murine leukemia L1210 cells were grown in Fischer's medium (Gibco, Grand Island, NY, USA), supplemented with 10% horse serum, 1 m*M* mercaptoethanol, 1 m*M* glutamine and gentamicin. Cell viability was assessed by MTT assays in quadruplicate, carried out after 96 h of cell growth on 96-well plates. Under conditions employed here, a 1-h exposure of cells to 50 n*M* C52 yielded a 90±2% cell kill.

2.3. Cell-surface hydrophobicity

Partitioning studies were carried out in a mixture containing 5% (w/v) Dextran T-500 (Pharmacia, Piscataway, NJ, USA), 4% (w/v), 4% (w/w) poly-(ethyleneglycol) (PEG), mol. mass 6000 (Pierce, Rockford, IL, USA) and 0.0006% PEG palmitate (60% esterified) in 140 m*M* NaCl+10 m*M* sodium phosphate, pH 7. This mixture spontaneously separates into a glycol-rich upper phase and a Dextran-rich lower phase. The phases are separated and stored at room temperature. For each determination, a 2.5-ml portion of each phase was placed in a 10-ml screw-cap tube.

L1210 cells were incubated with C3, C15 or C52 for 60 min at 37°C. An aliquot containing 10^6 cells was collected by centrifugation, resuspended in isotonic NaCl (0.2 ml) and mixed with a 5 ml of the partitioning preparation. The tubes were placed in a horizontal position and the phases allowed to separate for 5 min, then slowly brought to an upright position. After an additional 5 min, 1 ml from the middle of the upper phase was removed and the number of cells determined with a Coulter counter. Partitioning data are reported in terms of % total cells which partition into the upper phase [6,7].

2.4. Drug transport

L1210 cells (7 mg/ml) in growth medium were incubated for 3–60 min at 37°C with a 10 n*M* concentration of [^{3}H]-C52, to determine the time required for a steady-state equilibrium to be achieved. At intervals, samples of the suspension were removed, the cells collected by centrifugation (9500 *g*, 30 s) and washed once with cold isotonic NaCl. After 60 min, the remaining cells were resuspended in growth medium at 37°C and samples removed after 3, 10 and 20 min. Cells were mixed with 10 ml of SafetySolve (Fisher Scientific, Pittsburgh, PA, USA) for the determination of the drug distribution ratio (drug concentration in cells vs. medium) by liquid scintillation counting.

2.5. Plasma binding studies

Human and mouse plasma samples (250 μl) were incubated with radioactive C52 (final concentration=

10 n*M*) at 37°C for 30 min, then brought to a volume of 750 μl and a density of 1.21 g/ml with solid KBr and isotonic saline. The protein and lipoproteins were then separated by density-gradient ultracentrifugation using a modification of a previously published procedure [8]. The plasma samples were layered over 750 μl of KBr (density=1.27 g/ml) in polyallomer centrifuge tubes (Beckman Instruments, Palo Alto, CA, USA). The tubes were then filled with isotonic saline (total volume=3.9 ml). Protein/lipoprotein separation was carried out by centrifugation at 24°C using the Beckman TLV-1 table top ultracentrifuge and a TLN rotor (9E from vertical orientation) for 60 min at 100 000 rpm (r_{av}=254 000 *g*). Tubes were fractionated from the top; a total of 25–27 for determination of radioactivity by liquid scintillation counting.

To confirm the presence of both high density lipoprotein (HDL) and albumin in the murine plasma samples, these were incubated with both C52 and mesoporphyrin. After ultracentrifugation, binding of the porphyrin to low density lipoprotein (LDL), HDL and albumin fractions was assessed by fluorescence (excitation=400 nm, emission=620 nm) after dilution of each fraction to a final volume of 3 ml with Triton X-100 [8].

3. Results and discussion

3.1. Partitioning results

The partitioning procedure is based on observations by Albertsson [6] who initially described the use of two-phase partitioning systems for assessing changes in cell-surface properties. The procedure used here can be used to probe cell-surface hydrophobicity [7]. A cell population will distribute between the upper phase and the interface of a mixture of Dextran and PEG. If the mixture is made isotonic with NaCl and only 10 m*M* phosphate is present, there is no potential difference, and cells migrate to the interface. When PEG palmitate is added, this product is sequestered in the upper (PEG-rich) phase and attracts into that phase cells with a sufficiently hydrophobic surface. The number of cells which are attracted into the upper phase provides an index of relative surface hydrophobicity.

Data shown in Fig. 2 indicate that C52, but not C3 or C15, caused a rapid increase in cell-surface hydrophobicity. In C15, the epoxide residue is hydrolyzed, while in C3, this residue is lacking. These data (mean±SD of three determinations) indicate that an LD_{90} dose of C52 resulted in a readily-detectable increase in the hydrophobicity of the L1210 cell surface. At no concentration of the other drugs could we detect alterations in cell-surface hydrophobicity. These results are consistent with the hypothesis that interactions with the epoxide, likely involving alkylation, results in cell-surface modification. The effect with C52 occurred promptly after addition of drug to the cell culture, several hours before any detectable changes in nuclear morphology were observed.

In other studies, we found that a subsequent washing of cells for 20 min at 37°C did not affect the partitioning behavior induced by incubation with C52. This result suggests that the pool of drug readily lost during the wash procedure does not contribute to the enhanced cell-surface hydrophobicity.

In related experiments, we established that LD_{90} levels of two other drugs known to interfere with microtubule formation and degradation [9,10]: taxol (60 n*M*) and vincristine (100 n*M*), had no effects on the cell-surface hydrophobicity of L1210 cells.

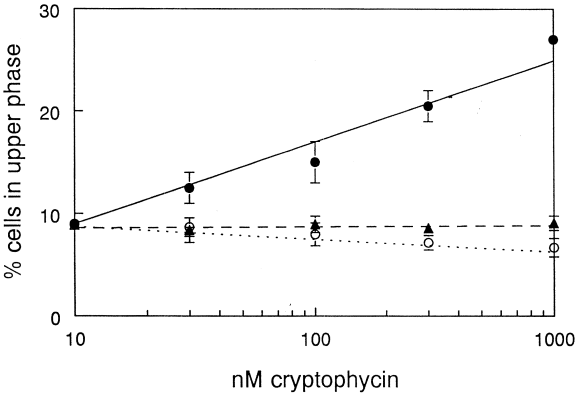

Fig. 2. Cell-surface hydrophobicity, as indicated by two-phase partitioning studies. Cells were incubated with graded levels of the different drugs for 30 min at 37°C, then partitioned as described in the text. C52 (●, solid line); C3 (○, dotted line), C15 (▲, dashed line).

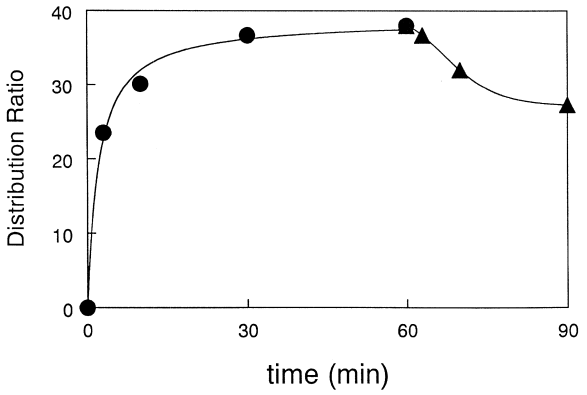

Fig. 3. Uptake and exodus of [^{3}H]-C52 in L1210 cells.

3.2. Transport and binding

Accumulation of C52 by L1210 cells was rapid, with a steady-state reached within 30 min (Fig. 3). With a 10 n*M* extracellular concentration (LD_{90} conditions for a 60 min incubation), the intracellular concentration was approx. 350 n*M*. This is somewhat less than the concentrative uptake of C52 reported for the human lymphoblastic leukemia CCRF-CEM cell line [5]. During a subsequent 20-min wash in growth medium at 37°C, 25% of the intracellular drug pool was lost (Fig. 3), and a new steady-state was reached. These results indicate that a significant fraction of the accumulated drug was not tightly bound. A different result was obtained with two human-derived cell lines, where the interaction between C52 and its biological targets was not reversible [11].

3.3. Affinity for plasma proteins

While it might be expected that an epoxy residue could be capable of interacting with a variety of cell-surface proteins, an additional series of experiments were carried out to assess the affinity of C52 for a group of model proteins, the albumin and lipoprotein species of mouse and human plasma. It was expected that these data might also provide useful information relating to C52 biodistribution.

Ultracentrifugation studies indicated that C52 was bound to albumin>HDL=LDL fractions of human plasma in vitro (Fig. 4, upper). Since albumin greatly predominates in plasma, substantial affinity of this drug for lipoproteins is indicated. In contrast, mouse plasma binding was observed only in the albumin fraction (Fig. 4, lower), although mouse plasma contains high levels of HDL [12]. In a previous study, we had demonstrated binding of mesoporphyrin, a fluorescent dye dicarboxylic dye, to albumin and all lipoprotein species in human and mouse plasma [8]. We therefore included this dye in replicate samples for the mouse binding analysis, to insure that our procedure could separate HDL from albumin.

A comparison between human vs. rodent HDL indicates that the latter is protein-poor and cholesteryl ester rich, and contains higher levels of the phospholipid lysolethicin than human HDL [12]. Whether these differences can account for the poor binding of C52 to murine HDL remains to be established. The apparent affinity of C52 for human but not mouse HDL has two implications: C52 does not appear to bind indiscriminately to any protein, but exhibits a degree of specificity. Moreover, the difference in drug binding patterns in the two different species may play a role in drug biodistribution in vivo. Earlier investigations [13] suggested that lipoprotein binding can be an important factor in drug biodistribution.

4. Discussion

The affinity of C52 for different macromolecules is quite selective, as indicated by the preference of the drug for human vs. mouse HDL. These results suggest variations in drug pharmacology in mouse vs. man, if lipoprotein binding is a factor in biodistribution. An interaction with cell-surface components that appears to involve the epoxide residue, resulted in a rapid increase in cell-surface hydrophobicity in the leukemia cell line, an effect not shared by less active drug analogs. Initial interactions between C52 and neoplastic cells, associated with a cytotoxic outcome, may involve changes in the conformation of membrane structures.

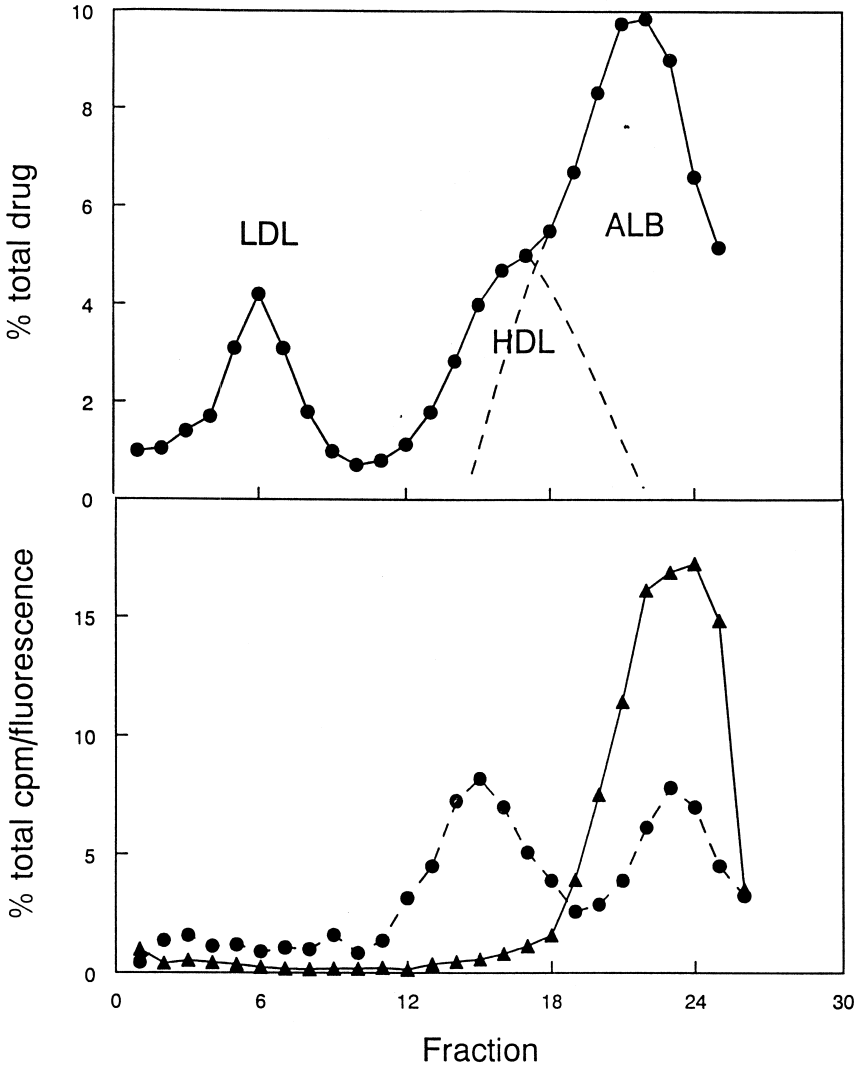

Fig. 4. Binding of radioactive C52 to the lipoproteins and proteins of human (upper) and mouse (lower) plasma. The distribution of the dye mesoporphyrin among the mouse plasma fractions is indicated in the lower curve (dashed line).

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