Solutol HS 15, Nontoxic Polyoxyethylene Esters of 12-Hydroxystearic Acid, Reverses Multidrug Resistance

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ABSTRACT

A recently developed non-ionic surfactant called Solutol HS 15 (polyoxyethylene esters of 12-hydroxystearic acid), with low toxicity in vitro, was shown to reverse completely the multidrug resistance of KB 8-5 and KB 8-5-11 human epidermoid carcinoma cells in vitro but did not potentiate drug toxicity in drug-sensitive KB 3-1 cells. At a concentration of 10% of its own IC50 (mean concentration of drug that causes 50% inhibition of cell growth compared to controls), Solutol HS 15 produced a 35-, 28-, and 42-fold reduction in the resistance of KB 8-5-11 cells to colchicine, vinblastine, and doxorubicin, respectively. Solutol HS 15 was relatively much more potent than the prototypic reversing agent, verapamil, for reversing colchicine resistance, compared to the ability of each agent to reverse vinblastine resistance. Like verapamil, Solutol HS 15 promoted a 50-fold accumulation of rhodamine 123 in KB 8-5-11 cells, as measured by flow cytometry. Also, Solutol HS 15 and verapamil reduced the efflux of rhodamine 123 from KB 8-5-11 cells previously loaded with rhodamine 123 to a similar low rate. Solutol HS 15 did not affect the transport of alanine or glucose into KB 8-5-11 cells, indicating that its effect upon membrane active transport is not entirely nonspecific. Considering their different structure and different relative potency for reversing colchicine resistance, Solutol HS 15 and verapamil probably reverse multidrug resistance by different mechanisms. Solutol HS 15 merits consideration as a potential therapeutic agent because of its effectiveness for reversing multidrug resistance in vitro and its low toxicity in vivo.

INTRODUCTION

MDR3 due to high expression of a cell membrane drug efflux transporter called Mdr1 protein (also called P-glycoprotein) may be important in the drug resistance of human malignant disease (1). Strategies to reverse MDR are of both great theoretical interest and, potentially, of practical importance, as shown by preliminary studies in human cancer patients treated with RMA in combination with cytotoxic chemotherapy (2). Although many agents have been identified as RMA in vitro, a large proportion have little or no therapeutic potential because of high toxicity in vivo at the doses used to reverse MDR. For example, metabolic poisons, e.g., azide, reverse MDR in vitro but obviously have no usefulness in vivo (3). Most other highly effective RMA, e.g., verapamil, appear to work as competitive antagonists of a drug binding site on the Mdr1 protein (4). Many of these agents also have toxicity which limits their usefulness in vivo (5). Developing alternate pharmacological strategies for reversing MDR may provide better RMA and/or may allow the use of a combination of RMA for additive or even synergistic effect with lower overall toxicity (6). Such an approach should also provide valuable insight into the physiology of MDR and Mdr1 protein. This paper describes a novel, highly effective, relatively nontoxic RMA called Solutol HS 15 which is structurally unlike typical RMA and which probably works by a different mechanism.

MATERIALS AND METHODS

Gel Permeation Chromatography of Solutol HS 15. A solution of Solutol HS 15 (lot 343; BASF, Wyandotte, MI) at 10 mg/ml (approximately 7 mg) was analyzed using a Beckman model 110B liquid chromatograph (Fullerton, CA) equipped with a model 160 selective wavelength detector. Samples were analyzed on a 7.8-min x 30-cm Waters Protein Pak 300 SW gel filtration column (Millipore Corp., Bedford, MA) under isocratic conditions and at a constant flow rate of 1.0 ml/min. Effluent fractions were monitored for absorbance at 254 nm. The buffer used was phosphate-buffered saline, pH 7.4, containing 1 M guanidinium hydrochloride, 2.5% butanol, and 2.5% 3-(cholamidopropyl)dimethylammonio-1-propanesulfonate (Sigma Chemical Co., St. Louis, MO).

Cell Lines and Culture Conditions. The MDR cell lines KB 8-5 and KB 8-5-11 and the parental, drug-sensitive line KB 3-1 were generously provided by Dr. Michael Gottesman's laboratory. The characteristics of these human epidermoid carcinoma cell lines have been described in detail elsewhere (7). Cells were grown as adherent monolayers on 80-cm2 flasks (Nunc, Roskilde, Denmark) in DMEM (pH 7.4) with 2 mm l-glutamine, nonessential amino acids, and vitamins (Gibco, Grand Island, NY) with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin (50 units/ml), and streptomycin (50 µg/ml) (Sigma). To prevent loss of the MDR phenotype, KB 8-5 cells were maintained in 25 µM colchicine and KB 8-5-11 in 250 µM colchicine. All cell cultures were split at 1:5 twice weekly for routine maintenance. After 10 to 12 passages, new aliquots of frozen cells were thawed for further experiments. The presence of Mycoplasma was excluded by the Gen-Probe Rapid Detection System-Mycoplasma TCCII (Gen-Probe, San Diego, CA).

Determination of IC50. To assess the effect of different concentrations of Solutol HS 15 and verapamil (Abbott Laboratories, North Chicago, IL) on the IC50 of colchicine (Sigma), vinblastine (Sigma), and doxorubicin (Farmitalia Carlo Erba, Milan, Italy), two dimensional titrations in 96 wells (16 mm) in plastic plates (Falcon; Becton-Dickinson, Lincoln Park, NJ) were performed with increasing concentrations of cytoxic drug along one axis and increasing concentrations of a RMA along the other. One-dimensional titrations were performed to determine the IC50 of individual drugs. Two × 104 cells were plated in each well and incubated without drug for 24 h to permit attachment and resumption of growth. The desired concentrations of drugs were then added, and the cells were incubated for an additional 5 days. Wells were washed with phosphate-buffered saline and adherent cells were stained with crystal violet (Sigma) (8). Cell-associated dye was then solubilized with 10% acetic acid and the viable (adherent) cell number was assessed by counting trypsinized cells in a hemacytometer in a duplicate experiment (data not shown).

Measurement of Rhodamine 123 Efflux by Flow Cytometry. The KB 8-5-11 cells for rhodamine 123 studies were harvested in a two stage procedure because of the possibility that routine trypsinization might...
Components. Gel chromatography of Solutol HS 15 revealed cell suspension with 90% viability as determined by propidium iodide. These monolayers were harvested for staining with 0.25% trypsin and 1 mM EDTA (Gibco) and reseeded to approximately 40% confluence. These monolayers were harvested for staining with 10 mM EDTA and agitation of the flask. This procedure yielded a single cell suspension with 90% viability as determined by propidium iodide (9). The suspension of cells (5 × 10^6/ml) was washed and incubated in 0.5 μg/ml rhodamine 123 (Sigma) and 24 μM verapamil in complete medium for 3 h at 37°C. The cells were washed in ice cold DMEM, split into 3 aliquots, and incubated in either complete medium alone or complete medium with 24 μM verapamil or Solutol HS 15 (70 μM) at 37°C. The rhodamine 123 fluorescence of the cells was measured periodically in both the primary and secondary incubations by withdrawing an aliquot of cells, washing in ice cold DMEM, and resuspending in DMEM with propidium iodide, 0.15 mg/ml, for immediate flow cytometric analysis.

Flow cytometric analysis of stained tumor cells was performed using a Coulter Epics V flow cytometer with confocal optics and a 3-decade logarithmic amplifier in the green fluorescence channel (Coulter Electronics, Hialeah, FL). Excitation was provided with an argon ion laser (Coherent, Worcester, MA) operating at 200 mW at 488 nm. Forward angle light scatter was measured from 0.75 to 18 degrees. The fluorescent light was separated from the exciting laser light using a 495 nm absorbance and a 515 nm interference filter. A 560 nm dichroic mirror was used to separate the red and green signals, with a 530 nm short pass filter in front of the green detector and a 610 nm long pass filter in front of the red detector. After setting light scatter and red fluorescence gates to exclude debris and dead cells (dead cells were excluded by uptake of propidium iodide) 1-parameter or 2-parameter correlated histograms were collected for 10,000 viable cells. The instrument was calibrated daily to allow comparison of staining profiles obtained on different days using 10-μm 1/8 bright beads (Coulter Electronics).

Determination of the Effect of Solutol HS 15 on Cell Membrane Transport of Alanine and Glucose. For the alanine uptake assay, 2.5 × 10^5 KB 8–5-11 cells were added to each well of 24-well plates (Falcon) and allowed to attach and grow for 24 h. Plain medium was then replaced with medium containing L-[2-3H]alanine, 60 Ci/mmol, (Amersham, Arlington Heights, IL), 10 μCi/well, and where appropriate, either Solutol HS 15 (70 μM) or ouabain (2 mM) (Sigma). At 30-min intervals, duplicate wells were harvested for the control group, the Solutol HS 15 group, or the ouabain group. Cells were washed and disrupted with 1% Triton X-100 (Sigma). Scintillation grade fluid was added (ICN, Cleveland, OH) and cell-bound radioactivity was counted in a Beckman LS7800 liquid scintillation spectrometer.

Glucose uptake was estimated by measuring lactic acid production by KB 8–5–11 cells in the presence of Solutol HS 15 (7 μM) versus controls without Solutol HS 15 (10). Cells (20,000/well) were plated in 24-well Falcon plates and incubated for 24 h. Solutol HS 15 was then added to appropriate wells and the plate was incubated for 7 days. Aliquots of medium were analyzed for lactic acid after 24 h and periodically thereafter by a standard method in which lactate and NAD+ (Sigma) were converted stoichiometrically to pyruvate and NADH by lactic dehydrogenase (Sigma) (11). NADH concentration was measured spectrophotometrically at 340 nm with a Gilford spectrophotometer 260. System performance was verified and calibrated with a series of lactic acid controls.

RESULTS

Separation and Analysis of Solutol HS 15 Surfactant and PEG Components. Gel chromatography of Solutol HS 15 revealed one major peak (approximately 70% of total) which eluted at 7 min and a smaller peak which eluted at 12 min (Fig. 1). The smaller peak was presumptively identified as the 30% unesterified PEG component of Solutol HS 15 described by the manufacturer by comparison of elution times with commercial PEG samples (Sigma; M, 600–6,000) all of which eluted at 12–14 min. From the elution pattern, which indicated a molecular weight for the major peak of more than 200,000, it appeared that the surfactant was in the form of micelles under these conditions. One-ml fractions of the eluate were collected and assayed for ability to reverse resistance to colchicine in KB 8–5–11 cells as described below. RMA activity was largely confined to the high molecular weight (micelle) peak (Fig. 1). Commercial PEG samples (Sigma; M, 600–6,000) also showed no activity for reversing colchicine resistance. For convenience, unfraccionated Solutol HS 15 was used for all other experiments. To facilitate comparison with other RMA and chemotherapeutic agents, Solutol HS 15 concentrations are expressed in molar units based on an approximate molecular weight of 960 and the fact that Solutol HS 15 is 70% surfactant by weight with 30% inactive component (12).

Reversal of Resistance to Colchicine in MDR Cell Lines by Solutol HS 15. Parental (KB 3–1) and MDR (KB 8–5 and KB 8–5–11) cells were plated in medium alone or increasing concentrations of colchicine, Solutol HS 15, or both in 96-well plates, until the well with cells in medium alone was confluent (6 days). The IC_{50} of colchicine versus the concentration of Solutol HS 15 in the medium. MDR cells (KB 8–5, KB 8–5–11) or drug-sensitive cells (KB 3–1) were grown for 6 days in incremental concentrations of colchicine and Solutol HS 15. Viable cell number was assessed by staining the adherent cells with crystal violet, solubilizing the dye with acetic acid, and spectrophotometrically measuring dye concentration. Each point represents the mean of 3 experiments. Bars, range of values.
of the concentration of Solutol HS 15 in the medium. Experimental design corresponds to Fig. 2. Each point represents the mean of 3 experiments. Bars, range of values.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Colchicine</th>
<th>Vinblastine</th>
<th>Doxorubicin</th>
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<tbody>
<tr>
<td>Solutol HS 15</td>
<td>8.4</td>
<td>14</td>
<td>4.2</td>
</tr>
<tr>
<td>Verapamil</td>
<td>30</td>
<td>1</td>
<td>6</td>
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Solutol HS 15 reversed resistance completely to both vinblastine and doxorubicin. The same concentration range of Solutol HS 15 versus different concentrations of Solutol HS 15 and verapamil were quite comparable for reversing vinblastine and doxorubicin resistance, but Solutol HS 15 had a much higher index than verapamil for reversing colchicine resistance.

Effect of Solutol HS 15 on Rhodamine 123 Accumulation and Efflux in the MDR Cell Line, KB 8–5–11. Rhodamine 123 is a fluorescent drug which is rapidly effluxed by Mdr1 protein in MDR cells (14). Cellular rhodamine 123 content (green fluorescence) was measured by staining the adherent cells with crystal violet, solubilizing the dye with acetic acid, and spectrophotometrically measuring dye concentration. Each point represents the mean of 3 experiments. Bars, range of values.

Table 2. Resistance modification index at 10% of IC50 of RMA (RMI 0.1)

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<td>Verapamil</td>
<td>4.4 ± 0.5</td>
<td>36.0 ± 2.4</td>
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The RMI 0.1 may be considered as an "in vitro therapeutic index" for RMA, since it assesses the efficacy of different RMA with reference to their own toxicity (13). IC50 values for colchicine, vinblastine, doxorubicin, Solutol HS 15, and verapamil were determined with a one-dimensional microtiter cell growth assay (8). Data used to determine the IC50 values for Solutol HS 15 are shown in Fig. 4. The RMI 0.1 was then determined for both RMAs against all three drugs. The results are summarized in Table 2. By this assay, Solutol HS 15 and verapamil were quite comparable for reversing vinblastine and doxorubicin resistance, but Solutol HS 15 had a much higher index than verapamil for reversing colchicine resistance.

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The experiment was repeated twice with relative fluorescence values within 5% of cytometry. Each point represents the mean fluorescence of 10,000 cells. The of rhodamine 123 from KB 8-5-11 cells. Cells were loaded by incubating them those shown.

Inhibition of drug efflux by both verapamil (as anticipated) and Solutol HS 15 was demonstrated by the greatly reduced rhodamine 123 efflux compared to that of rhodamine 123-loaded cells incubated in medium alone. The parallel efflux curves indicate that the two RMA reduced the efflux to a similar low rate.

Effect of Solutol HS 15 on Other Membrane Transport Functions and Membrane Permeability. In an attempt to define the mechanism by which Solutol HS 15 is able to block the efflux of MDR-related drugs, we tested the hypothesis that Solutol HS 15 nonspecifically inhibits all membrane active transport phenomena. Solutol HS 15 had no effect on uptake of tritiated alanine in KB 8-5-11 cells, whereas ouabain inhibited uptake significantly in the same assay (Fig. 6). Alanine transport is, in part, mediated by a symport mechanism coupled to Na⁺-K⁺-active transport (16). Since ouabain inhibits Na⁺-K⁺ transport, it also significantly inhibits alanine transport. Also, Solutol HS 15 had no effect on glucose uptake as judged by lactic acid production in these cells (10) over a 7-day period (Fig. 7). Solutol HS 15 (7 µM) did not nonspecifically increase cell membrane permeability as judged by measuring uptake of propidium iodide in KB 8-5-11 cells by flow cytometry (data not shown).

DISCUSSION

Solutol HS 15 is significantly different in structure from classical cationic competitive RMA, e.g., verapamil, other calcium channel blockers, reserpine, or quinidine (17). Solutol HS 15 contains soluble non-ionic surfactants (70%) and PEG (30%) formed by the reaction of 12-hydroxystearic acid with ethylene oxide at alkaline pH (12). The principal reaction products are PEG and polyoxyethylene esters of 12-hydroxystearic acid (Fig. 8). A minor component of diesters (12-hydroxystearic acid esterified to itself via the 12-hydroxy group) is also produced (12). It is a colorless odorless paste, highly soluble in aqueous media (12). This newly developed commercial product is intended as an emulsifier of water-insoluble parenteral drugs. This report shows that the toxic concentration (IC₅₀) of Solutol HS 15 in vitro is approximately 10-fold greater than the concentration required to reverse MDR. Toxicity studies in several nonhuman mammalian species in vivo indicate that 200-750 mg/kg body weight can be given repeatedly, i.e., for eight weeks
without ill effects (12). The i.v. 50% lethal dose in beagles was found to be 3.1 g/kg body weight (12). The doses used in vitro to achieve complete reversal of drug resistance in highly MDR cells appear to be tolerable in vivo. However, the local concentration at Solutol HS 15 achievable in solid tumors in vivo has not been investigated.

The data in this report indicate that Solutol HS 15 is a highly effective and potent RMA for MDR cells in vitro. The observation that Solutol HS 15 potentiates the toxicity of colchicine in MDR cell lines, but not a drug-sensitive cell line, and the high RMI 0.1 indicate that the effects observed were not due to the additive toxicity of Solutol HS 15 itself. The effect of Solutol HS 15 thus fits the narrow definition of a RMA, unlike compounds such as cyclosporins, which sensitize both MDR and drug-sensitive cells to anthracyclines (18). The rhodamine 123 studies showed that Solutol HS 15 promotes drug accumulation in MDR cells, in common with most other RMA (3). The rhodamine 123 efflux study showed that the increased drug accumulation is at least partly due to a pronounced decrease in the rate of drug efflux. Whether Solutol HS 15 affects the rate of influx of certain hydrophobic drugs, as has been reported for certain other RMA, including surfactants, will require further study (5). Solutol HS 15 does not appear to increase cell membrane permeability nonspecifically at concentrations used to reverse MDR. This is consistent with the observation that it did not inhibit cell proliferation or potentiate drug toxicity in parental cells. The data also show that the Solutol HS 15 effect is not confined to a single MDR cell line or to a single MDR drug or to MDR drugs of similar structure or cytotoxic mechanism. Solutol HS 15 compares favorably with verapamil as a RMA for vinblastine and doxorubicin in vitro using the in vitro therapeutic index provided by the RMI 0.1. However, Solutol HS 15 has much lower toxicity than verapamil in vivo; the maximum tolerable in vivo level of verapamil is approximately 2 μM (5).

Certain other non-ionic soluble surfactants, most notably Tween 80, (polyoxyethylene sorbitan monoooleate) were reported several years ago to modify MDR in vitro (19, 20). However, none, to our knowledge, has been documented to be as effective as Solutol HS 15, especially at clearly nontoxic concentrations, as reflected by the high RMI 0.1 of Solutol HS 15. Also, Tween 80 potentiates drug toxicity in both parental and MDR cells, unlike Solutol HS 15 (19). The RMA activity of Tween 80 was ascribed to increased cell membrane permeability leading to enhanced drug accumulation (19). The effect of Tween 80 or other surfactants on drug efflux has apparently not been studied. Much more recently, another non-ionic surfactant, called Cremophor EL (mainly polyoxyethylene glycerol triricinoleate), has been reported to reverse MDR in vitro (21). Like Solutol HS 15 it has been used as a drug emulsifier in vivo. However, Solutol HS 15 administration has a much lower prevalence of adverse reactions than administration of Cremophor EL, which often provokes histamine release (12). Cremophor EL also increases drug accumulation in MDR cells, but whether this effect is mediated by decreased drug efflux has not been reported.

The mechanism whereby Solutol HS 15 reverses MDR and inhibits drug efflux in MDR cells is of interest. However, definitive studies of the mechanism of action of RMA may not be possible until the mechanism whereby Mdr1 protein expression confers MDR is better understood. Decreased drug accumulation is undoubtedly important in some settings, but altered intracellular drug distribution and binding, among other possibilities, may also play a role (22). The best known mechanism for reversing MDR is competition for a drug-binding site on Mdr1 protein which is involved in drug efflux (23). This appears to at least partially explain the activity of many RMA, of which verapamil is the prototype (23). However, the mechanism of reversing doxorubicin resistance by verapamil seems more related to altered intracellular distribution of doxorubicin than decreased accumulation for some cells (22). Also, alternative mechanisms apparently exist for blocking drug efflux. Cepharanthene, an amphiphatic bisbenzylisoquinoline alkaloid, has been reported to inhibit drug efflux by perturbing the plasma membrane by binding phosphatidylserine (17). Phenothiazine has also been postulated to act by an independent mechanism, since it does not inhibit photoaffinity labeling of the binding site by photoactive vinblastine analogues (23). In mutant and wild type MDR transfectants, in which the mutants have selective resistance to colchicine over vinblastine, the binding affinity of the mutant Mdr1 protein was higher than the wild type for vinblastine but lower for colchicine (24). Thus, high affinity binding of drugs to Mdr1 does not appear to be sufficient for optimal efflux, suggesting the existence of additional, rate-limiting steps which may be susceptible to pharmacological intervention. The lack of any similarity in structure between Solutol HS 15 and verapamil or other typical RMA suggests that Solutol HS 15 may inhibit efflux by a different mechanism.

The markedly greater potency of Solutol HS 15 than verapamil for reversing colchicine resistance relative to the ability of each to reverse vinblastine or doxorubicin resistance supports this hypothesis. Considering their dissimilar structures, Solutol HS 15 appears to be an unlikely candidate for blocking colchicine efflux by competition for a second binding site selective for colchicine. Colchicine is known not to interact strongly with the established MDR drug-binding site, since colchicine does not compete for vinblastine binding or inhibit photoaffinity labeling with a photoactive vinblastine analogue (23). The fact that MDR cells are nevertheless highly resistant to colchicine indicates that colchicine efflux is less dependent upon interaction with this drug-binding site than is vinblastine. Since Solutol HS 15 is a highly potent RMA for both colchicine and vinblastine, one possibility is that it inhibits a second event which is necessary for both colchicine and vinblastine efflux, e.g., actual transport through the cell membrane. Whatever the mechanism of blocking efflux of MDR-related drugs, Solutol HS 15 is not entirely nonspecific in its effects on membrane transport, since it did not affect the transport of glucose or alanine at concentrations which completely reversed MDR. Nevertheless the specificity of the effect on membrane transport phenomena compared to verapamil requires clarification.

Solutol HS 15 and related compounds appear to deserve further study, both as possible therapeutic agents for reversing MDR and as probes for critical steps in the drug efflux mechanism.

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