In Vitro and in Vivo Enhancement of Ricin-A Chain Immunotoxin Activity by Novel Indolizine Calcium Channel Blockers: Delayed Intracellular Degradation Linked to Lipidosis Induction

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ABSTRACT

With regard to increasing the clinical potential of ricin A-chain immunotoxins (RTA-ITs), a novel class of calcium channel blockers, indolizines SR33557 [2-isopropyl-1-((4-(3-N-methyl-N-(3,4-dimethoxy-3-phenethyl)amino)propoxy)benzenesulfonyl)indolizine] and SR33287 [isopropyl-2-((1-butylamino-3-propyl)oxy-4-benzoyl)-3-indolizine], were evaluated for their ability to enhance RTA-IT activity in vitro and in vivo. Five μM SR33287 and 5 μM SR33557 were potent enhancers of both anti-Thy 1.2 AT15E RTA-IT (84- and 64-fold, respectively) on T2 cells and anti-CD5 T101 (622- and 538-fold) and T101 F(ab’2); RTA-IT (34- and 28-fold) on CEM III cells. This was superior to the effect achieved by both 10 μM verapamil and 10 mM NH4Cl, albeit slightly inferior to that of 50 μM monensin and 5 μM perhexiline. Murine T2 lymphoma cells bearing the Thy 1.2 antigen were injected i.v. in Thy 1.2 (−) BL. 1.1 mice (median survival time, 17.7 days). Intravenous treatment with 10 μg of AT15E RTA-IT prolonged the survival of mice (median survival time, 26.8 days). When 400 μg of SR33287 were conjected i.v. with 10 μg of AT15E RTA-IT, mouse survival was further increased, with 5 of 6 mice surviving, disease-free, over 42 days.

SR33287 had a significant impact on the intracellular routing of 125I-AT15E RTA-IT, which induced a greater than 2-fold increase in intracellular intact AT15E RTA-IT at 90 min. This effect on RTA-IT half-life was distinctly different from that observed with either NH4Cl or monensin and may be linked to the inhibition of acid lysosomal sphingomyelinase by SR33287, leading to cellular lipidosis.

In conclusion, indolizines appear to be promising agents not only for immunotoxin enhancement but also for increasing the activity of any number of targeted therapeutic agents where modifying either the intracellular routing or increasing the activity of the ligand would be beneficial to its cytotoxic activity.

INTRODUCTION

RTA-ITs are the most potent immunotoxins being evaluated for their potential use as anticancer agents (1). One of the most active protein inhibitors at the acellular level, ricin A-chain is comparable to other toxins of bacterial (diphtheria toxin and Pseudomonas exotoxin) and higher plant (pokeweed antiviral protein and gelonin) origin (2). Abundant, easily purified, and less immunogenic than bacterial toxins, the toxic protein of Ricinus communis seeds proves to be the best-suited starting material for pharmacological development (1).

The general procedure for the synthesis of RTA-IT conjugates relies on heterobifunctional reagents, such as N-succinimidyl-3-(2-pyridyl-dithiopropionate), which permit cross-linking between antibody and ricin A chain (3). The absence of the ricin B chain, which plays a role in facilitating the translocation of the A chain from the endosome to the cytosol, greatly decreases the in vitro activity of RTA-ITs (4). Therefore, although these conjugates show stringent specific cytotoxicity, their clinical potential depends not only on their construction (5–7) but equally on ways of increasing their cytotoxic activity.

Among several strategies engaged to enhance RTA-IT cytotoxicity, such as the development of more potent conjugates [e.g., using monoclonal antibody fragments (6) or chimeric fusion proteins (8)], potentiation of RTA-IT cytotoxicity has been achieved in vitro with the use of agents such as carboxylic ionophores (9, 10), lysosomotropic amines (9, 11), and calcium antagonists (12–15). Some of these enhancers have clinical uses, such as bone marrow purging in humans (16), but they are ill suited to in vivo use, where the major obstacles are either their toxicity or short half-life after i.v. administration (17, 18).

We have previously reported that the calcium antagonist perhexiline was a potent RTA-IT enhancer in vitro, comparable to both NH4Cl and monensin (15). We also suggested that the increase in RTA-IT cytotoxicity in the presence of perhexiline was due to alterations in intracellular routing and degradation of RTA-ITs, which could be linked to perhexiline-induced lipidosis via the inhibition of acid lysosomal sphingomyelinase.

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Although the concentrations of perhexiline needed for RTA-IT enhancement are relevant to the pharmacokinetics of this agent, a number of considerations suggest that the prospect of using perhexiline in a clinical setting may be hindered by both its previously documented hepatotoxicity and neurotoxicity in humans (see Ref. 19) as well as its unfavorable dose effect in RTA-IT enhancement. However, our previous study suggested that it would be possible to discover other potent RTA-IT enhancers among sphingomyelinase inhibitors.

We report here that indolizines SR33287 and SR33557, which represent a novel class of calcium channel blockers (20) and which inhibit acid sphingomyelinase activity, are equally potent RTA-IT enhancers in vitro. Furthermore, we show that SR33287 significantly potentiates RTA-IT therapy in an in vivo model. Finally, we observed that the SR33287 impact on intracellular RTA-IT degradation was distinct from that of NH4Cl and monensin and may be linked to lipidosis induction.

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MATERIALS AND METHODS

Chemicals. Novel calcium channel blockers SR33287 and SR33557, as well as analogues SR33510 and SR45813, were synthesized in our Chemical Department (21) (see Fig. 1). Perhexiline maleate, NADCl, and verapamil-HCl were purchased from Sigma Chemical Co. (St. Louis, MO). Monensin was purchased from Calbiochem (Behring Diagnostics, La Jolla, CA). Being poorly soluble in water at high concentrations, these drugs were dissolved in dimethyl sulfoxide (0.01% final concentration). All other reagents were purchased from Sigma Chemical Co.

Cells. The human lymphoblastic T-cell line CEM III is permanently maintained in our laboratory by serial passage. CEM III were subcloned from CEM using a FACS IV cell sorter (Becton-Dickinson, Mountain View, CA). These cells express a mean CD5 density of 30,000 molecules/cell (22). The mouse T2 cell line, carrying the Thy 1.2 antigen, is from the Salk Institute for Biological Studies, was a generous gift from Dr. B. J. Bourrié (Sanoft, Montpellier, France). All of these cell lines were cultured in RPMI 1640 (Mérieux, Lyon, France) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, VA), 2 mM glutamine, 20 μM β-mercaptoethanol, and antibiotics (200 units/ml penicillin and 100 μg/ml streptomycin).

Immunotoxins. T101, T101-F(ab')2, AT15E monoclonal antibodies, and ricin A-chain were purified and then conjugated by the cross-linking agent N-succinimidyl-3-(2-pyridyldithio)propionate (Pharamcia, Montigny, France) as described previously (3, 6). Mouse monoclonal IgG2a antibody T101 and its fragment T101-F(ab')2 react with the CD5 surface antigen (23). Rat monoclonal IgG2c antibody AT15E is specific for the mouse Thy 1.2 antigen (24). RTA-ITs, which contained no detectable ricin B-chain, are expressed as molar concentrations of bound A-chain.

Protein Synthesis Inhibition Assay. RTA-IT efficacy was evaluated by the inhibition of [3H]leucine incorporation as determined previously (22). Briefly, 1 x 10⁶ cells/ml in complete medium were treated over 18 h at 37°C with various concentrations of toxin or RTA-IT and, when specified, in the presence of an enhancing agent. [3H]Leucine (specific activity, 120 Ci/ml; 1 μCi/100-μl well) CE, GiF/Yvette, France) was added 6 h before the end of treatment. Washed cells were harvested on filter paper (Titertek, Flow Laboratories, Les Ulis, France), and radioactivity was measured with a Kontron MR 300 automatic liquid scintillation system (Kontron, Vélizy, France). Results of triplicate experiments are expressed as the percentage of control (cells not treated by toxin or RTA-IT).

AT15E RTA-IT Therapy in an in Vivo Model. Target cells used for in vivo experiments were a Thy 1.2-positive murine lymphoma (T2), grown in congenic Thy 1.2-negative BL. 11 mice (17, 22). T2 cells (5 x 10⁶) were injected i.v. on Day 1, followed, on Day 4, by a single i.v. injection of 10 μg of AT15E RTA-IT or other control products (10 μg AT15E monoclonal antibody and free ricin A-chain), with or without 400 μg SR33287 (injected i.v. 2 min after the proteins). Each group had six mice; the animals were observed for the appearance of disease, and mortality was recorded for calculations of survival. Antitumor activity was determined by comparing the MST of the treated group with that of the control group, and the results are expressed as T/C, where:

\[ T/C = \frac{\text{MST of treated group}}{\text{MST of control group}} \times 100 \]

Statistical Analysis. Statistical comparisons between in vivo survival curves were determined by the Mann-Whitney test. The paired Student's t test was performed to compare internalization and degradation rates of 125I-AT15E RTA-IT.

125I Labeling of AT15E RTA-IT. RTA-IT (50 μg) was labeled with 1 mCi of 125I (Amersham, Paris, France) in the presence of chloramine T (25). After 1 min of incubation the reaction was stopped by the addition of sodium metabisulfite followed by potassium iodide. Free 125I was removed by chromatography on a phosphate-buffered PD-10 Sephadex G-25 ml column (Pharmacia). The collected fractions which contained labeled protein were pooled, and the radioactivity was measured on a Packard Auto-Gamma Counter. Specific radioactivity of labeled AT15E RTA-IT was approximately 8 μCi/mg protein. Iodination did not modify the activity of RTA-IT (data not shown).

Kinetics of AT15E RTA-IT Internalization in T2 Cells. AT15E RTA-IT degradation kinetics experiments were performed according to the previously described method (15, 26) with slight modifications. Briefly, T2 cells were incubated for 1 h at 4°C with 125I-labeled AT15E RTA-IT (10-fold the membrane-saturable concentration; 1 x 10⁻⁸ M) in complete medium. After 3 ice-cold washes with saline solution (removing excess 125I-RTA-IT), cells (1 x 10⁶/m) were incubated at 37°C, with or without enhancing agents, for various incubation times. At the end of these times, cells were pelleted by centrifugation and washed twice. The collected supernatants, which represented non-cell-associated 125I-ligand, were treated with a 10% TCA solution, and the radioactivity of the TCA-precipitable and -soluble (degraded ligand) fractions was determined (these fractions were nonexistent before 22 h, after which they represented less than 10% of total recovered radioactivity). Nonendocytosed cell surface-bound 125I-ligand was removed by treatment for 30 min at 4°C with 0.4% (w/v) Streptomyces griseus protease (Sigma Chemical Co.). Protease treatment removed >90% of cell surface-bound radioactivity. Cells were then centrifuged, and the radioactivity in the cell pellet and supernatant was determined. From these determinations, the amount of RTA-IT endocytosed (E) was calculated:

\[ \% E = \frac{P_{t}}{P_{t} + S_{t}} \times 100 \]

where E is endocytosis at time t, Pₜ is cpm in the cell pellet at time t, and Sₜ is cpm in the supernatant (of protease-treated cells) at time t. Results are expressed as the percentage of total recovered radioactivity, which remained constant throughout the experiment (differences between incubation times, <10%). Radioactivity was determined with a gamma counter. P was defined as the addition of the values of degraded and intact intracellular 125I-ligand described below.

Kinetics of AT15E RTA-IT Degradation in T2 Cells. After protease treatment, washed cells were lysed with 50 mM Tris-HCl, 0.5% sodium dodecyl sulfate, pH 8. Intracellular nondegraded and degraded 125I-ligand was assessed by measuring the 10% TCA-precipitable and -soluble radioactivities, respectively. A negative control was performed by incubating the cells at 4°C with cold AT15E monoclonal antibody (6 μg/ml) for 2 h before the addition of 125I-AT15E RTA-IT.

Electron Microscopy. Approximately 2 x 10⁶ cells treated with or without drugs for various time intervals at 37°C were prepared for electron microscopy as described previously (15). Cells were fixed in complete medium with 1.5% glutaraldehyde (w/v) in 40 mM cacodylate buffer, pH 7.2, and 2 mM MgCl₂. Cells were then centrifuged in BEEM (Beem, WA) conical capsules for 15 min at 4000 x g and stored for 3
h at 20°C. Samples were then rinsed three times with 100 mM sodium cacodylate (pH 7.2), 180 mM saccharose, and 2 mM MgCl₂. These were then left for 3 h at 0°C in 1% osmium tetroxide; 100 mM sodium cacodylate, pH 7.2; 120 mM saccharose; and 2 mM MgCl₂. After a second series of rinses, samples were dehydrated in ethanol followed by epoxy-1,2-propane and embedded in Epon for 48 h at 60°C. Ultrathin sections obtained with a diamond knife (Diatome Co., Bienne, Switzerland) were counterstained with 5% uranyl acetate in a 30% ethanol solution, followed by lead citrate. These sections were observed with a Philips EM 301 electron microscope (Eindhoven, Holland).

Determination of Acid Sphingomyelinase Activity. Enzymatic activity was determined as described previously (27). Briefly, the cells (5 × 10⁶) treated with or without drugs for various time intervals at 37°C were washed with 0.9% NaCl and the cell pellets, resuspended in distilled water, and sonicated. Protein concentrations were determined by the procedure of Lowry et al. (28). A fluorescent pyrenesulfonilamiloundecanoyl sphingomyelin substrate was added to sonicated suspension in the presence of Triton X-100 for 2 h at 37°C. The reaction was stopped by the addition of 1.5 ml heptane and 0.45 ml isopropyl alcohol, followed by 0.25 ml water, and centrifugation at 1500 × g for 10 min. The product of the enzyme reaction, ceramide (which is linked to the fluorescent moiety), was determined fluorimetrically by analyzing the upper heptane-rich phase at 351 and 378 nm (excitation and emission wavelengths, respectively). This product was quantitated by measuring the fluorescence intensity of known quantities of fluorescent ceramide (standard curve). The sphingomyelinase activity in treated cells is expressed as nmol of ceramide/h and nmol of ceramide/mg protein and is compared to that of untreated cells.

**RESULTS**

Toxicity of Indolizines and Analogues. To determine the cytotoxicity of indolizines and analogues (Fig. 1), T2 cells were incubated for 18 h with increasing concentrations of each drug. The IC₅₀ values were comparable between SR33287, SR33557, and SR33510 (8, 10, and 13 JIM, respectively), whereas SR45813 showed significantly less toxicity (IC₅₀ of 20 μM). At 5 μM, which is the highest concentration used in our investigation, all four drugs induced less than 10% inhibition of protein synthesis (see Fig. 2).

Enhancement of RTA-IT Activity by Indolizines on Cultured Cell Lines. To evaluate the ability of indolizines and analogues to enhance RTA-ITs, various concentrations of the drugs and RTA-ITs were added to both murine and human leukemic cell lines. Perhexiline, NH₄Cl, monensin, and verapamil were used as comparative standards. Results were compared using either IC₅₀ or ligand enhancement-specific activity (IC₅₀ of ricin A chain/IC₅₀ of RTA-IT) factors.

T2 cells were sensitive to the cytotoxic activity of native ricin and of purified A chain (IC₅₀ of 9.4 × 10⁻¹³ and 2.0 × 10⁻¹⁰ M, respectively) (see Fig. 3). Of the eight potential RTA-IT enhancing agents tested, only monensin (50 nM) slightly increased the cytotoxicity of uncoupled ricin A chain on T2 cells (IC₅₀ of 2.3 × 10⁻¹⁰). Anti-Thy 1.2 AT15E RTA-IT cytotoxicity on the murine T2 lymphoma cell line was increased over 80-fold by 5 μM SR33287 and by 60-fold by 5 μM SR33557. A ligand enhancement-specific activity factor of 105,000 and 83,000 for AT15E RTA-IT in the presence of 5 μM SR33287 and 5 μM SR33557, respectively, revealed the effectiveness of these as compared to 10 mM NH₄Cl (45,000) and 10 μM verapamil (8,000). Only 50 nM monensin (255,000) and 5 μM perhexiline showed a higher RTA-IT-enhancing effect (241,000). SR33510 and SR45813 had no effect (Table 1). Moreover, dose-effect experiments revealed that SR33287 was a much more potent RTA-IT enhancer at submicromolar concentrations as compared to perhexiline (see Fig. 4). Indeed, for concentrations ranging from 0.1 to 2 μM, SR33287 presented a far superior enhancement of RTA-IT cytotoxicity as compared to perhexiline.

RTA-IT enhancers were further compared using the anti-CD5 T101 whole-Ig and T101-F(ab')₂ RTA-ITs on the human lymphoblastic T-cell line CEM III. Table 1 shows that both SR33287 and SR33557 were potent anti-CD5 RTA-IT enhancers, comparable to the other drugs, albeit much more potent than verapamil.

**Effect of SR33287 on the Kinetics of Protein Synthesis Inhibition by AT15E RTA-IT.** The effect of SR33287 on the kinetics of AT15E RTA-IT cytotoxicity was studied on T2 cells. As shown in Fig. 5, the kinetics of AT15E RTA-IT (1 × 10⁻⁸ M)-mediated inhibition of protein synthesis was rapid, with 90% inhibition of protein synthesis occurring at 13.9 h. In the presence of AT15E RTA-IT and 5 μM SR33287 90% of protein synthesis was inhibited at 6.4 h.

**In Vivo Enhancement of AT15E RTA-IT.** SR33287 was solubilized in dimethyl sulfoxide and then diluted in saline solution. The toxicity of the compound (LD₅₀ = 61 mg/kg) has previously been evaluated in healthy mice.⁵ To evaluate the impact of SR33287 on RTA-IT treatment in vivo, murine T2 lymphoma cells (5 × 10⁸), bearing the Thy 1.2 antigen, were

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⁵ P. Chatelain, unpublished data.
Table 1 Comparative RTA-IT enhancement on cultured cell lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (µM)</th>
<th>AT15E RTA-IT</th>
<th>T101 RTA-IT</th>
<th>T101-F(ab')2 RTA-IT</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>5</td>
<td>1600</td>
<td>28,000</td>
<td>3100</td>
</tr>
<tr>
<td>SR33287</td>
<td>5</td>
<td>19 (84)</td>
<td>45 (622)</td>
<td>92 (34)</td>
</tr>
<tr>
<td>SR33557</td>
<td>5</td>
<td>24 (67)</td>
<td>52 (538)</td>
<td>112 (28)</td>
</tr>
<tr>
<td>SR33510</td>
<td>5</td>
<td>1600 (1)</td>
<td>28,000 (1)</td>
<td>3100 (1)</td>
</tr>
<tr>
<td>SR45813</td>
<td>5</td>
<td>1600 (1)</td>
<td>28,000 (1)</td>
<td>3100 (1)</td>
</tr>
<tr>
<td>Perhexiline</td>
<td>5</td>
<td>8.3 (193)</td>
<td>19 (1474)</td>
<td>86 (36)</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>10,000</td>
<td>44 (36)</td>
<td>21 (1333)</td>
<td>22 (141)</td>
</tr>
<tr>
<td>Monensin</td>
<td>0.05</td>
<td>2.7 (593)</td>
<td>10 (2800)</td>
<td>9 (344)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>10</td>
<td>250 (6)</td>
<td>820 (34)</td>
<td>620 (5)</td>
</tr>
</tbody>
</table>

Mice treated with a control saline solution died by Day 18 (MST, 17.7 days) (Fig. 6). The administration of 10 µg of AT15E RTA-IT alone resulted in an increase in survival with a T/C of 151% (MST, 26.8; P < 0.01) (Fig. 5). The administration of 10 µg of AT15E antibody and noncovalent ricin A chain with or without 400 µg of SR33287 only modestly affected survival, with a T/C of 113% (MST, 20; P < 0.01) and 111% (MST, 20; P < 0.01), respectively, whereas SR33287 had no effect when injected alone (T/C = 101%; MST, 17.8). However, treatment of mice with 10 µg of AT15E RTA-IT along with 400 µg of SR33287 resulted in a significant increase in survival, with only one mouse dying on Day 21 and all of the other five surviving disease free until Day 42, when these were sacrificed (T/C > 243.5%).

Internalization of 125I-AT15E RTA-IT. Measuring the difference between the proteolytically releasable cell membrane-bound radioactivity and the total cell pellet radioactivity enabled us to estimate the internalization rates of 125I-AT15E RTA-IT. In the absence of enhancers, internalization of 125I-ligand was rapidly cleared in T2 cells (Fig. 7A). Within 2 h 100% of membrane-bound RTA-IT was internalized. This rate of internalization was not affected in the presence of 10 mM NH4Cl or 50 nM monensin (Fig. 8). However, 5 µM SR33287 induced a significant decrease in the internalization of RTA-IT (P < 0.05). At 2 h, 30% of AT15E RTA-IT was still membrane bound (Fig. 7B).
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Fig. 6. Effect of SR33287 on the activity of AT15E RTA-IT in vivo. T2 cells (5 x 10⁶) were injected i.v. in BL.1.1 Thy 1.2 (-) mice. On day 4, mice were administered either saline solution (O), 10 μg of AT15E RTA-IT alone (A), 10 μg of AT15E MoAb and free ricin A-chain in the absence (data omitted for clarity) or in the presence of 400 μg SR33287 (B), 400 μg SR33287 alone (data omitted for clarity), or 10 μg AT15E RTA-IT and 400 μg SR33287 (•).

Fig. 7. Internalization and degradation of ¹²⁵I-AT15E RTA-IT in T2 cells. T2 cells (1 x 10⁶/ml) were incubated with ¹²⁵I-AT15E RTA-IT (1 x 10⁻⁴ M) in the absence (A) and in the presence (B) of 5 μM SR33287 for the indicated times, at the end of which cells were washed. Nonintemalized RTA-IT (C) was measured after proteolytically removing cell-bound ¹²⁵I-ligand. Intracellular degraded RTA-IT (•) was measured by collecting the TCA-soluble fractions of lysed cells, while intracellular intact RTA-IT (O) was determined by collecting the TCA-precipitable fractions. Points, representative of 3 independent experiments with SD no greater than 10%.

Fig. 8. Internalization rate of ¹²⁵I-AT15E RTA-IT in T2 cells. T2 cells were incubated with ¹²⁵I-AT15E RTA-IT (1 x 10⁻⁴ M) in the absence (O) (r = 0.92) and in the presence of 5 μM SR33287 (•) (r = 0.94), 10 mM NH₄Cl (A), and 50 nM monensin (U) for the indicated times, at the end of which cells were washed. Internalized RTA-IT was measured as described in “Materials and Methods.” Points, representative of three independent experiments, with SD no greater than 10%. Linear regressions for NH₄Cl and monensin were essentially identical to those for control and are omitted for clarity.

Fig. 9. Intracellular acid-precipitable ¹²⁵I-AT15E RTA-IT. T2 cells were incubated with ¹²⁵I-AT15E RTA-IT (1 x 10⁻⁴ M) in the absence or in the presence of 5 μM SR33287, 10 mM NH₄Cl, and 50 nM monensin for the indicated times, at the end of which cells were washed. Intact RTA-IT was measured as described in “Materials and Methods.” Columns, representative of 3 independent experiments.

DISCUSSION

In an effort to provide experimental evidence to support the clinical potential of RTA-IT enhancement, we evaluated the ability of a novel class of calcium antagonists, indolizines SR33287 and SR33557 and two analogues, to enhance the in vitro and in vivo activity of RTA-ITs. The effectiveness of these drugs was compared to that of four of the most potent in vitro RTA-IT enhancers, NH₄Cl, monensin, verapamil, and perhexiline (9–15).

In our in vitro models, SR33287 and SR33557 were potent enhancers of both anti-Thy 1.2 and anti-CD5 RTA-ITs. However, neither drug had any effect on native ricin or free ricin A chain. In the perspective of in vivo treatment SR33287 presented a more favorable dose effect than perhexiline, with a 46-fold enhancement of AT15E RTA-IT cytotoxicity at 1 μM (see Table 1). After an 18-h incubation with optimal RTA-IT enhancement concentration of SR33287 and SR33557, acid sphingomyelinase inhibition was over 80% in T2 cells. The kinetics of inhibition was rapid, with about 70% inhibition achieved in only 30 min (data not shown). Neither SR335120, SR45813, NH₄Cl, monensin, nor verapamil had any effect on acid sphingomyelinase activity.
Fig. 10. Electron microscopy of T2 cells treated by SR33287. T2 cells were incubated for 18 h with or without 5 μM SR33287, after which they were fixed for electron microscopy. Compared to controls (A), cells treated with SR33287 (B and C) presented concentric osmiophilic laminated inclusion, whereas both mitochondria and the Golgi apparatus (arrow) remained intact.

Table 2: Inhibition of acid sphingomyelinase activity by RTA-IT enhancers
Percentage of sphingomyelinase activity of T2 cells, treated with drugs, as compared to untreated cells were determined. Values are mean of at least 3 independent experiments (SD < 20%).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (μM)</th>
<th>Sphingomyelinase activity (%)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>SR33287</td>
<td>0.1</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>18</td>
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<tr>
<td></td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.5</td>
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<tr>
<td>SR33557</td>
<td>5</td>
<td>19</td>
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<tr>
<td>SR33510</td>
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<td>SR45813</td>
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<td>99</td>
</tr>
<tr>
<td>Perhexiline</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>10,000</td>
<td>105</td>
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<tr>
<td>Monensin</td>
<td>0.05</td>
<td>110</td>
</tr>
<tr>
<td>Verapamil</td>
<td>10</td>
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</table>

RICIN A-CHAIN IMMUNOTOXIN ENHANCEMENT BY INDOLIZINES

... compared to only 2-fold by 1 μM perhexiline. Both 5 μM SR33287 and 5 μM SR33557 also enhanced the cytotoxicity of anti-CD5 T101 and T101-F(ab')2 RTA-ITs on the human T-lymphoblastoid cell line, CEM III. In the case of the F(ab')2 fragment counterpart of the whole T101 immunoglobulin RTA-IT, SR33287 and SR33557 proved to be as potent as perhexiline. We have previously described such variability by the degree to which different RTA-ITs can be activated (15). And although it appears that the more potent RTA-ITs are less sensitive to enhancement than weaker RTA-ITs, several factors, such as distinct internalization rates, intracellular routing, and degradation processes, may explain this and make it critical to select the appropriate enhancer and RTA-IT (15-29).

As previously shown by Jansen's group the anti-Thy 1.2 RTA-IT is an attractive candidate for therapeutic studies in animal models and represents an example of high potential for clinical use of an almost nontoxic RTA-IT if it can be adequately potentiated in vivo (17, 24). Indeed, although AT15E MoAb alone is cytotoxic in vitro, only AT15E RTA-IT activity is sensitive to enhancement with, as in the case of monensin, an increase in cytotoxicity which is comparable to that of native whole ricin. However, in vitro RTA-IT enhancement cannot necessarily be considered predictive of the in vivo response. Indeed, not only are there many problems linked to the in vivo administration of RTA-ITs (low tumor localization, short serum half-life, rapid liver uptake, and the induction of antibody responses to RTA-ITs), there are equally major obstacles in the in vivo use of RTA-IT enhancers. The high concentrations of NH₄Cl needed for RTA-IT enhancement limit its use to ex vivo therapy such as bone marrow purging (16). The potential of monensin, an equally potent agent, is hampered by its general toxicity and unfavorable pharmacokinetics (17-18). Among the calcium antagonists, the indolizines appeared to represent a potential candidate for in vivo therapy since, as we have shown, they were more potent than verapamil and presented better dose effects than perhexiline.

In our study, the in vivo anitumor effect of AT15E RTA-IT was significantly increased when SR33287 was coadministered. This effect was specific, since SR33287 had no effect when administered with uncoupled AT15E MoAb and ricin A chain. To our knowledge, although successful treatment of tumors by i.v. RTA-IT immunotherapy has previously been reported (5, 10, 17), this is the first example of successful potentiation of RTA-IT-induced cytotoxicity in vivo by i.v. administration of a potential clinically useful agent.

The mechanism of RTA-IT enhancement by calcium antagonists remains to be determined. In order to study the intracellular degradation kinetics of AT15E RTA-IT in the presence of indolizines, we performed radiolabeled RTA-IT studies with SR33287, NH₄Cl, and monensin. Among these, only SR33287 induced a dramatic change in the kinetics of RTA-IT internalization and intracellular degradation. Indeed, endocytosis of AT15E RTA-IT was completed after 2 h, which is analogous to that described for the AT15E MoAb on the mouse lymphoma cell line EL-4 (26). In the presence of 5 μM SR33287, the rate of endocytosis was significantly reduced, with only about 75% internalization achieved at 2 h. Such an impact on RTA-IT internalization, which has also been seen for another calcium antagonist, perhexiline (15), would appear to be inconsistent with the RTA-IT enhancement activity of SR33287. However, internalization rates have been described as not representing the rate-limiting step in immunotoxin cytotoxicity (30-32).

Although the cytotoxic activity of an RTA-IT requires it to be internalized, it must also be directed toward appropriate compartments (33, 34). The demonstration that NH₄Cl significantly enhances the cytotoxicity of AT15E RTA-IT suggests that at least part of the internalized RTA-IT population is directly routed to lysosomal vacuoles. Our experimental methodology enabled us to follow the intracellular acid-soluble material (degradation of AT15E RTA-IT). In the absence of enhancers, the rate of intracellular degradation of RTA-IT was rapid and paralleled that of internalization. Treatment with 5 μM SR33287 inhibited AT15E RTA-IT intracellular degradation after a 10-min time interval, resulting in an increase in acid-precipitable AT15E RTA-IT, even though its internalization...
tion rate was reduced. This inhibition lasted for over 1 h before degradation returned to a rate similar to that of controls. It is possible that the impact of SR33287 on both RTA-IT internalization and degradation, leading to the observed 2-fold increase in acid-precipitable RTA-IT, may be illustrating a slowing down of intracellular routing processes. Such modifications in intracellular trafficking, limiting the access to the lysosomal route (leading to toxin inactivation), have been described as favoring the release of toxin molecules and their translocation into the cytosol (33, 35). Therefore even a slight modification can lead to profound changes in cytotoxicity. Moreover, the reduction in intralysosomal RTA-IT induced by perhexiline has been previously linked to an observed decrease in RTA-IT degradation (15). These observations suggested changes in intracellular routing which were linked to the inhibition of acid sphingomyelinase activity by perhexiline and to the subsequent induction of lysosomal phospholipidosis in treated cells (36). Interestingly, we found that the impact of SR33287 on T2 cells appeared to be morphologically similar to the effect of perhexiline (e.g., concentric laminated inclusions evoking the induction of liposis). Furthermore, SR33287 and SR33557 (but not inactive analogues) inhibited acid sphingomyelinase in a dose-dependent manner which correlated with RTA-IT enhancement. It would therefore appear that SR33287 and SR33557 (both highly hydrophobic) may act, much like perhexiline, by disturbing membrane lipid composition through their inhibitory action on lysosomal phospholipid hydrolyses, such as acid sphingomyelinase, leading to modifications in intracellular routing and to subsequent degradation of RTA-ITs. Furthermore, these results add strength to the suggestion, in view of the apparent relationship between sphingomyelinase inhibition and RTA-IT enhancement by perhexiline, that other sphingomyelinase inhibitors may be potent RTA-IT enhancers (15).

Finally, although further studies are required, indolizines appear to be promising agents not only for immunotoxin enhancement but also for increasing the activity of any number of targeted therapeutic agents where either modifying the intracellular routing or increasing the intracellular half-life of the ligand would be beneficial to its cytotoxic activity. Present studies on structure/activity relationships may lead us to the development of new molecules with equally potent enhancement activities but with the calcium-antagonistic property of RTA-IT.

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