Elevation of Leukemic Cell Intracellular Calcium by the Ether Lipid SRI 62–834

Charles M. Lazenby, Michael G. Thompson, and John A. Hickman

Cancer Research Campaign Experimental Chemotherapy Research Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET, United Kingdom

ABSTRACT

SRI 62–834 is a novel antineoplastic ether lipid which is currently undergoing a Phase 1 clinical trial in the United Kingdom. Its mechanism of action has not been defined. Incubation of 7.5 × 10⁶/ml HL-60 human myelomonocytic leukemia cells with between 10 and 50 μM SRI 62–834 brought about a concentration-dependent, biphasic rise in intracellular calcium, as measured by the calcium-sensitive fluorescent dye Quin-2 AM. Incubation with 30 μM SRI 62–834 elevated intracellular calcium from 110 to 415 nM after 10 min in a typical experiment; this concentration inhibited cell growth by >90% (50% inhibition of growth was observed at 8 μM). The calcium channel blockers verapamil and prenylamine did not inhibit the SRI 62–834-induced elevation of intracellular calcium. Incubation of SRI 62–834 with K562 erythroleukemia cells also brought about a rise in the intracellular calcium. The growth of K562 cells was less sensitive to SRI 62–834 (dose to produce 50% growth inhibition, 65 μM) compared to HL-60 cells, and significant intracellular calcium rises, which were monophasic, required >40 μM SRI 62–834. At a concentration of SRI 62–834 which inhibited both HL-60 and K562 growth by 90% (30 and 140 μM, respectively), an equivalent rise in intracellular calcium was observed (circa 400 nM). Preincubation of HL-60 or K562 cells with 1 to 100 mM 12-O-tetradecanoylphorbol-13-acetate for 10 min prior to the addition of SRI 62–834 inhibited the rise in intracellular calcium in a concentration-dependent manner. It is suggested that SRI 62–834-induced changes in intracellular calcium may contribute to its cytotoxicity and that the rise is not due to an early and grossly disruptive effect of the agent on membrane structure.

INTRODUCTION

The ether lipids are a novel class of membrane active antitu- mour drugs (1). SRI 62–834 (Fig. 1) is a cyclic ether analogue of the prototypical ether lipid 1-O-octadecyl-2-O-methyl-(±)-glycero-3-phosphocholine and has antineoplastic activity in vivo and in vitro (2). SRI 62–834 also has highly selective toxicity toward leukemic cells compared with bone marrow stem cells (3). It has recently entered a Phase 1 clinical trial in the United Kingdom as part of the Cancer Research Campaign rapid Phase 1 program. The mechanism of action of this class of compounds has not been clearly defined, although SRI 62–834 does not appear to inhibit protein kinase C (2) unlike other members of this class of drug (5). The ether lipids may have mechanical effects on membrane structure, disrupting them to cause blebbing and pitting of the plasma membrane (6). This loss of membrane integrity is likely to be cytotoxic because transmembrane gradients would be predicted to be dissipated. Among the most important of these is the 10,000-fold gradient of calcium ions (<1 μM intracellular, >1 mM extracellular). A collapse of this gradient has been implicated as an important factor in xenobiotoic-induced cell death (7). We wished to establish the effects of SRI 62–834 on the maintenance of this ionic gradient as part of an attempt to explain the selective nature of the cytotoxicity of SRI 62–834. Accordingly, we have measured intracellular calcium concentrations in leukemic cells with differing sensitivities to this ether lipid (8), using the calcium-sensitive dye Quin-2 AM.

MATERIALS AND METHODS

Materials. SRI 62–834 was the gift of Dr. William J. Houlihan, Sandoz Research Institute, East Hanover, New Jersey. Quin-2 AM, verapamil, prenylamine lactate, 4α-phorbol-12,13-didecanoate and TPA were obtained from Sigma (Poole, United Kingdom).

Cell Culture. Human HL-60 myelomonocytic leukemia and K562 erythroleukemia cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, Glasgow, Scotland) at 37°C in an atmosphere of 10% CO₂ in air. Both cell lines had a doubling time of approximately 24 h, and they were maintained in the logarithmic phase of cell growth by serial subculture of between 5 × 10⁶ and 1 × 10⁷ cells/ml. Cell numbers were measured by a Coulter Counter (model ZB). Cell membrane integrity, one measure of viability, was determined after various treatment protocols by light microscope evaluation of the exclusion of a 0.1% solution of trypan blue.

Assessment of Toxicity. HL-60 or K562 cells (1 × 10⁶/ml) were incubated continuously with various concentrations of SRI 62–834, added in tissue culture medium, for 72 h and cell numbers were estimated. The percentage of inhibition of cell growth was calculated as the percentage of change in the increase in cell number in the treated cells compared to the increase observed in the untreated controls.

Measurement of Intracellular Calcium. Cells were washed in fresh medium, then resuspended in growth medium at 1.5 × 10⁶ cells/ml containing 20 μM Quin-2 AM, and incubated for 1 h at 37°C. Cells were harvested when required, and 1.5 × 10⁶ cells were resuspended in a buffer which contained 140 mM NaCl, 5 mM KCl, 2.8 mM NaHCO₃, 1.5 mM CaCl₂, 1 mM MgCl₂, 60 mM MgSO₄, 5.6 mM glucose, and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2). They were then transferred to a 1-cm² quartz cuvette and fluorescence monitored at 37°C in a Perkin-Elmer LS-5 spectrometer equipped with a thermally controlled cuvette holder and magnetic stirrer. The excitation and emission wavelengths were 339 and 492 nm, respectively. Intracellular "free" calcium concentrations were calibrated as described previously by Tsien et al. (9). The various drugs and reagents were added in protocols detailed in "Results."

RESULTS

The effect of continuous incubation with SRI 62–834 on the growth of HL-60 and K562 cells in suspension culture is shown in Fig. 2. The 50% inhibitory concentrations were 8 and 65 μM, respectively.

Incubation of HL-60 or K562 cells with the calcium fluorescent dye Quin-2 AM showed, by measurement of fluorescence at 492 nm, that they had low basal concentrations of intracellular calcium of 131 ± 11 (SD) nM (n = 18) and 76 ± 5 nM (n = 12), respectively. Addition of SRI 62–834 to Quin-2 AM-loaded HL-60 cells produced a biphasic rise in intracellular calcium, which was sustained over a 10-min period (Fig. 3A). The rise was drug concentration dependent (Fig. 4) and it appeared that it was a controlled event and not due to membrane disruption since the calcium gradient could later be dissipated by the addition of detergent (Fig. 3A), which was used in the standard way to calibrate the fluorescence signal (9). The detergent triton...
was also added to HL-60 cells to establish whether a similar response as to SRI 62-834 could be obtained: Fig. 3C shows that, as when digitonin was added to these cells, there was a distinctive and immediate sharp rise in calcium, quite different from the pattern seen in response to SRI 62-834. The typically biphasic response of HL-60 cells to SRI 62-834, which was preceded by a short lag period, suggested that two discrete pools of calcium were being mobilized, and in preliminary experiments with the calcium-sensitive dye INDO-2, we have found the second phase of calcium increase to be inhibited by preincubation with an extracellular medium which contained manganese, suggesting that this phase constitutes an influx of calcium from the extracellular milieu. A similar concentration-dependent rise in intracellular calcium was observed when Quin-2 AM-loaded K562 cells were incubated with SRI 62-834 (Figs. 3B and 4), although the rise in these cells was monophasic in comparison to the biphasic rise observed in HL-60 cells.

Preincubation of Quin-2 AM-loaded HL-60 or K562 cells with TPA for 10 min prior to the addition of a calcium-elevating concentration of SRI 62-834 inhibited the rise of intracellular
calcium observed in both cell types in a TPA concentration-dependent manner (Fig. 5). The inactive phorbol ester 4a-phorbol-12,13-didecanoate (100 nM), which does not activate the enzyme protein kinase C, had no effect under these conditions. Preincubation of HL-60 or K562 cells with 100 nM TPA had no effect on their membrane integrity over a 24-h period.

Preincubation of Quin-2 AM-loaded HL-60 cells for 10 min with either of the voltage-dependent calcium channel blockers verapamil (100 µM) or prenylamine (10 µM) failed to inhibit the rises in intracellular calcium induced by SRI 62-834 (data not shown). A higher concentration of prenylamine (100 µM) enhanced the calcium rise induced in HL-60 cells by 30 µM SRI 62-834, and it is presumed that at this high concentration chaotropic effects may be involved.

**DISCUSSION**

It is clear from the structure of a molecule like SRI 62-834 (Fig. 1) that it has the potential to physically interact with the plasma membrane so as to bring about physical disruption. The data presented here suggest that at moderately cytotoxic concentrations mechanical disruption is not a primary event. Rather, SRI 62-834 was found to elevate intracellular calcium concentrations of both HL-60 and K562 cells in a controlled manner (Figs. 3 and 4). Addition of the detergent triton to HL-60 cells also brought about an elevation of intracellular calcium, but the pattern of this rise was dissimilar to that seen after the addition of the ether lipid.

Additional evidence which suggested that the rise in calcium was not due to immediate mechanical rupture of the cell membrane also came from the results of the experiments in which cells were pretreated with TPA (Fig. 5). Inhibition of the SRI 62-834-induced rise in calcium by TPA suggested that it is modulatable by activation of protein kinase C. This result resembles the inhibition by TPA of the calcium rise observed in differentiated neutrophil-like HL-60 cells which had been stimulated by the chemotactic peptide f-Met-Leu-Phe (10). In that case, the elevation of intracellular calcium levels resulted from the initiation of inositol lipid breakdown. Chemotactic peptide-induced inositol lipid cleavage resulted in a distinctive biphasic increase in intracellular calcium (10) which is similar to that observed after incubation of HL-60 cells with SRI 62-834 (Fig. 3A); it remains to establish the precise mechanism whereby intracellular calcium is elevated by SRI 62-834 and its source. Like the chemotactic peptide-induced changes in calcium, those induced by SRI 62-834 were not inhibitable by voltage-dependent calcium channel blockers (see "Results").

The concentrations required to elevate intracellular calcium in each of the leukemic cell lines, HL-60 and K562, was reflected in the differing sensitivity of the lines to the cytotoxicity of SRI 62-834 (Fig. 2), but only at the level of 90% inhibition of cell growth was there an almost equivalent rise in intracellular calcium of 400 nM. The greater sensitivity of the growth of HL-60 cells to SRI 62-834 compared with K562 cells is similar to that which has been observed previously with the ether lipid 1-O-octadecyl-2-O-methyl-(±)-glycero-3-phos-

![Fig. 4. SRI 62-834 concentration-dependent increase in intracellular calcium above resting levels in HL-60 (*) and K562 (□) cells 10 min after the addition of SRI 62-834.](image1)

![Fig. 5. TPA concentration-dependent inhibition of SRI 62-834-induced increases in intracellular calcium concentrations. Top, 30 µM SRI 62-834 added to HL-60 cells; bottom, 60 µM SRI 62-834 added to K562 cells. Bars, SE.](image2)
phocholine (8). The nature of the relationship between the calcium rise and the cytotoxicity observed in the two cell lines will depend not only upon the size of the rise observed but also the inherent sensitivity of each of the cell lines to sustained elevations of intracellular calcium, determined by such criteria as the activity of calcium efflux pumps and/or intracellular mechanisms of calcium sequestration. Such factors may play important roles in determining the selective toxicity of this drug.

If SRI 62–834 initiates changes in calcium metabolism by a discrete biochemical mechanism, this suggests that the selectivity of the drug may additionally depend upon the differential expression of some type of receptor. The cytotoxicity of SRI 62–834 has recently been shown to be inhibited by a platelet-activating factor antagonist, suggesting that platelet-activating factor receptors may be involved with the toxicity of SRI 62–834 (3). Our present hypothesis is that SRI 62–834 may elevate intracellular calcium concentrations via the activation of some type of membrane-specific event, but whether there is a protein receptor involved or some type of biophysical modulation of membrane structure remains to be established. It is not clear why the SRI 62–834–induced increase in calcium was not regulated by normal homeostatic mechanisms of subsequent sequestration and efflux after activation of the calcium pump. This question can only be answered by a study of calcium flux in these cells. Future experiments seek to clarify the role of calcium in ether lipid-induced loss of membrane integrity and cell death and to identify a putative receptor for this drug, which might explain its very considerable selectivity (3).

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