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EFFECTS OF KS-501, KS-502 AND THEIR ENANTIOMERS ON CALMODULIN-SENSITIVE ENZYME ACTIVITY AND CELLULAR PROLIFERATION

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Abstract—Calmodulin plays an important role in cellular proliferation as part of a signal transduction pathway activated by phospholipase C. Drugs that block the ability of calmodulin to bind to and activate its target enzymes inhibit the growth of a wide variety of malignant cells. To identify more potent and selective inhibitors of this potential target for new drug development, we studied two recently synthesized compounds, KS-501 and KS-502, for their activity against calmodulin-sensitive enzymes and for their ability to block the growth of parental and multidrug-resistant leukemic cells. KS-501 and KS-502 inhibited the activation of a calmodulin-sensitive cyclic nucleotide phosphodiesterase. The mechanism of enzyme inhibition was through interfering with calmodulin activation rather than through a direct effect on the enzyme. KS-501 was more potent than KS-502 and was studied in greater detail. This compound inhibited the activation of calmodulin kinase I and II, but had less effect against cyclic adenosine 3′,5′-monophosphate (cyclic AMP)-sensitive kinase. KS-501 was also more effective than KS-502 in inhibiting the growth of sensitive L1210 leukemic lymphocytes. Both compounds were less effective inhibitors of multidrug-resistant L1210 leukemia than of the parental line. These studies identify a new class of calmodulin inhibitor, with selectivity for calmodulin-dependent kinases over cyclic AMP-dependent protein kinase. Since the total synthesis of the KS-compounds has been accomplished, it should now be possible to develop derivatives with greater activity and selectivity.

Key words: calmodulin inhibitors; kinase; phosphodiesterase; multidrug resistance

For many years it has been appreciated that calmodulin plays an important role in cellular proliferation [1–3], and yet the details of how this limb of the calcium messenger system is activated in response to mitogens are uncertain. Recent evidence links the stimulation of receptor-activated phospholipase C by certain growth factors to the activation of calmodulin-sensitive enzymes. Phospholipase C activity generates two second messengers, diacylglycerol and IP_3† [4]. IP_3 interacts with specific receptors on the endoplasmic reticulum to release calcium from intracellular stores, thereby achieving cytosolic concentrations of calcium able to saturate the four binding sites on calmodulin. Once saturated, calmodulin interacts with its target proteins, thereby initiating calmodulin-dependent processes.

Receptor-linked increases in calcium mobilization can activate a variety of calmodulin-dependent processes important in mitogenesis. Perhaps the most carefully characterized has been calmodulin kinase II, whose activity regulates the phosphorylation of microtubular associated proteins, depolymerization of tubulin, and induction of DNA synthesis [5]. In addition, Palfrey *et al.* [6] reported that calmodulin kinase III is activated in response to mitogenic growth factors such as bradykinin, vasopressin and epidermal growth factor, leading to the phosphorylation of the unique substrate for this enzyme, elongation factor 2. We have demonstrated the selective activation of calmodulin kinase III and the phosphorylation of elongation factor 2 in rapidly proliferating malignant cells [7, 8]. Unlike calmodulin kinase II, the precise role of this kinase in cell division is unknown.

Receptor-linked activation of calmodulin kinases may help to clarify the mechanism of the antiproliferative and cytotoxic effects of calmodulin antagonists such as phenothiazines, naphthalene sulfonamides, and CGS 9343B. For example, Lee and Hait [9] have shown that proliferating L1210 leukemic cells are significantly more sensitive to the cytotoxic effects of phenothiazines compared with that of non-proliferating cellular populations. Bagaglio *et al.* [7, 8] found that anticalmodulin drugs and peptides can block the activation of calmodulin kinase III and the phosphorylation of elongation factor 2 in proliferating cellular populations.

Consequently, it would be of value to identify more active and selective classes of drugs capable of inhibiting calmodulin. Recently, Dushin and Danishefsky described the total synthesis of KS-501 and KS-502 [10], compounds initially isolated from the culture broth of *Sporothrix* sp. KAC-1985 by Nakanishi *et al.* and shown by these investigators [11] and by our laboratory [12] to inhibit calmodulin-sensitive cyclic nucleotide phosphodiesterase from

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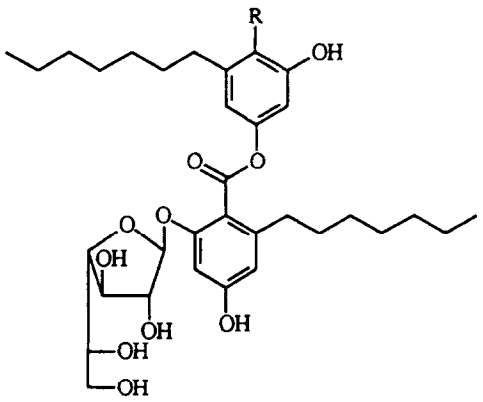
† Abbreviations: IP_3, inositol tris phosphate; and PKA, cyclic adenosine 3′,5′-monophosphate-sensitive kinase.



1: KS-501 R=H
2: KS-502 R=CO2H

Fig. 1. Structures of KS-501 and KS-502.

brain. We now report the results of experiments describing the activity of these new compounds against calmodulin-sensitive enzymes and malignant cellular proliferation.

MATERIALS AND METHODS

KS-501 and KS-502 (Fig. 1), and their enantiomers, were synthesized by the method of Dushin and Danishefsky [10].

Calmodulin-sensitive cyclic nucleotide phosphodiesterase was purified from rat brain by high pressure liquid chromatography [13] and assayed by the luciferin/luciferase method [14].

Calmodulin kinase II was measured by *in vitro* phosphorylation of synapsin I [15] using the 100,000 *g* cytosolic fraction of C6 glioma cells as an enzyme source [7, 8]. Reaction mixtures included 50 mM HEPES, pH 7.6, 10 mM Mg^{2+}, 0.5 μg synapsin I, 10 μg protein, 20 μM/2–4 μCi [γ-^{32}P] ATP in a final volume of 50 μL. Additional assays included 1.5 mM Ca^{2+}, 3 μg calmodulin, a 1 μM concentration of the selective peptide inhibitor of cyclic AMP-sensitive protein kinase (PKI-tide; Santa Cruz Biotechnology, Santa Cruz, CA), or various concentrations of KS-501 and KS-502. After a 1-min preincubation at 30°, reactions were initiated by adding [γ-^{32}P] ATP for 30 sec. The reactions were terminated by adding 3× Laemmli's sample buffer and boiling for 5 min. Phosphoprotein bands were resolved by 10% SDS–PAGE. After autoradiography, the phosphosynapsin I bands were excised and quantitated by Cerenkov counting.

Limited peptide mapping of phosphosynapsin I was performed as previously described [16]. The phosphorylated synapsin I bands (86 and 80 kDa) were excised from the dried gels and digested overnight with 2 μg *Staphylococcus aureus* V8 endoproteinase during electrophoresis in a 15% (5% stacking) acrylamide gel. The dried gel was subjected to autoradiography, bands were excised, and the radioactivity was quantitated by scintillation spectroscopy.

Sensitive L1210 mouse leukemia cells (L1210/S) were infected with an *MDR*1 gene expression vector, pHaMDR1/A, using polycation polybrene [17]. Resistant cells were selected and expanded subsequently in medium containing 60 ng/mL colchicine. The resistant line was designated L1210/VMDRC.06. Both L1210/S and L1210/VMDRC.06 lines were cultured in RPMI 1640 medium supplemented with 10% horse serum, penicillin (50 U/mL) and streptomycin (50 U/mL) at 37° in an atmosphere of 95% air, 5% CO_2. Cells were checked routinely and found to be free of contamination with mycoplasma or fungi.

Effects of drugs on cellular proliferation were determined as previously described [18]. Briefly, cells were grown in supplemented RPMI 1640 medium in the presence of the KS-compounds or their vehicles, and then were incubated for an additional 48 hr. Cells were enumerated using an electronic counter.

RESULTS

To determine the ability of KS-501 and KS-502 to inhibit the activity of a calmodulin-sensitive enzyme, we measured their effects on calmodulin-sensitive cyclic nucleotide phosphodiesterase purified from rat cerebrum. Fig. 2A demonstrates that both KS-501 and its enantiomer, inhibited enzymic activity in a concentration-dependent fashion, with an IC_{50} of 0.8 ± 0.05 μM. Figure 2B demonstrates that KS-502 and its enantiomer were approximately 10-fold less potent than KS-501, having IC_{50} values of 10.3 ± 0.1 μM and 8.8 ± 1.0 μM, respectively. (IC_{50} is defined as the concentration of drug required to inhibit the activation of phosphodiesterase by 50%). These compounds had no effect on the activity of the enzyme in the absence of calmodulin (data not shown).

To determine the mechanism of action of these new compounds, we investigated the effects of increasing concentrations of calmodulin on the ability of KS-501 to inhibit cyclic nucleotide phosphodiesterase. Figure 3 demonstrates that increasing concentrations of calmodulin increased enzymic activity, and that this effect was attenuated at higher concentrations of the drug.

We next compared the effects of KS-501 on calmodulin- and cyclic AMP-dependent protein kinases. Figure 4 demonstrates that KS-501 inhibited the calmodulin-dependent phosphorylation of synapsin I at a concentration of 40 μM in a manner similar to that of trifluoperazine. The compounds did not inhibit basal enzymic activity, i.e. in the absence of calmodulin. In fact, at 10 μM the basal activity was slightly stimulated.

Since synapsin I is a substrate for several other kinases, we subjected the phosphorylated protein to limited proteolysis with *S. aureus* V8 protease and determined which phosphorylated fragment was affected by the compounds. Figure 5 demonstrates that KS-501 and its enantiomer inhibited the phosphorylation of both site 1 and site 2,3, peptide

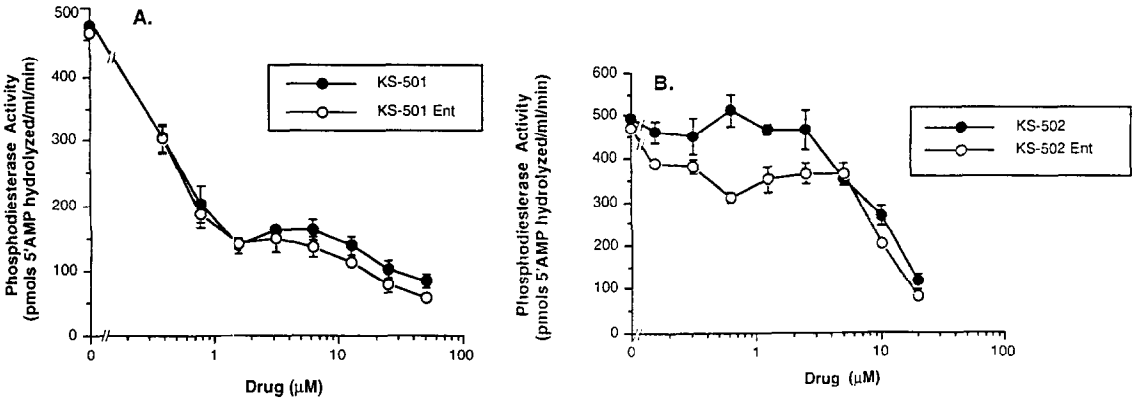


Fig. 2. Effects of KS-501 (A) and KS-502 (B) and their respective enantiomers on calmodulin-sensitive cyclic nucleotide phosphodiesterase activity. Phosphodiesterase was purified from rat cerebrum, and its activity was determined in the presence and absence of calmodulin and various concentrations of drug. Each point is the mean ± SEM of three determinations from one of three similar experiments.

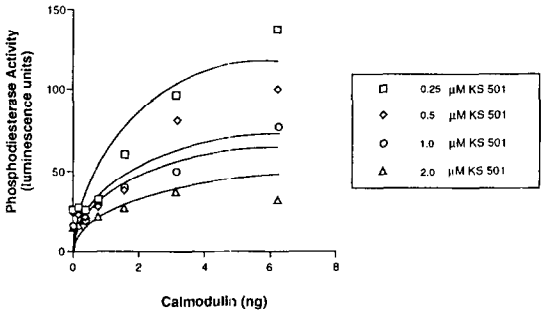


Fig. 3. Effects of increasing concentrations of calmodulin on the inhibition of cyclic nucleotide phosphodiesterase by KS-501. Phosphodiesterase was assayed as described in Fig. 2 in the presence of 0 to 6.25 ng of calmodulin and 0 to 2.0 μM KS-501. Each point is the mean of triplicate determinations from one of three experiments.

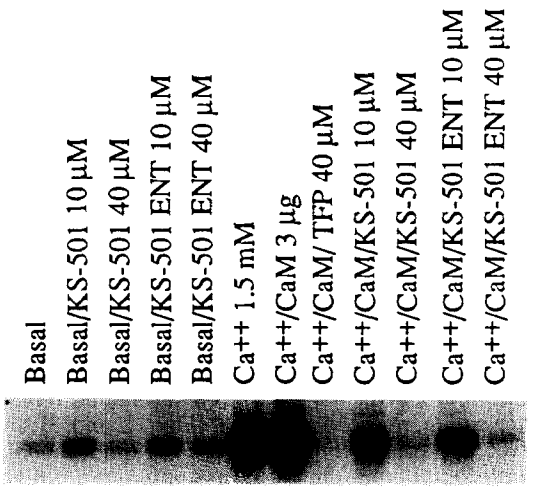


Fig. 4. Effects of KS-501 and its enantiomer on the calmodulin-sensitive phosphorylation of synapsin I. Synapsin I was phosphorylated using the 100,000 *g* cytosolic fraction of C6 glioma cells as an enzyme source at 30° for 30 sec as described in Materials and Methods. Basal assay conditions included 50 mM HEPES, pH 7.6, 10 mM Mg^{2+}, 1 mM EGTA, 1 μM PKI-tide, 0.5 μg synapsin I, 20 μM/ 4 μCi [γ-^{32}P]ATP, and 10 μg of protein in the presence or absence of 1.5 mM calcium, 3 μg calmodulin (CaM), 10 or 40 μM KS-501 or 40 μM trifluoperazine (TFP).

fragments containing the substrates for calmodulin kinase I and II.

KS-501 and its enantiomer were far less potent inhibitors of cyclic AMP-sensitive protein kinase. As shown in Fig. 6, cyclic AMP stimulated the phosphorylation of site 1 on synapsin. This stimulation was nearly abolished by PKI-tide. At 40 μM KS-501, a concentration that inhibited calmodulin-sensitive phosphorylation of synapsin I, KS-501 only partially inhibited the activity of cyclic AMP-sensitive protein kinase. The KS-501 enantiomer was somewhat more effective than the parent compound.

To study the effects of KS-501 and KS-502 on cell growth, we exposed L1210 leukemic lymphocytes to 0–100 μM concentrations of the drugs for 48 hr. Figure 7 shows that KS-501 and its enantiomer (IC_{50} = 80 and 95 μM, respectively) were more effective inhibitors than the KS-502 compounds (IC_{50} > 100 μM).

We next compared the activity of these compounds against the multidrug-resistant cell line, L1210/ VMDRC.06. Figure 8 demonstrates that the compounds were less potent inhibitors of the resistant line and that the KS-501 enantiomer was somewhat more potent than the other compounds.

DISCUSSION

These studies demonstrate the activity of the

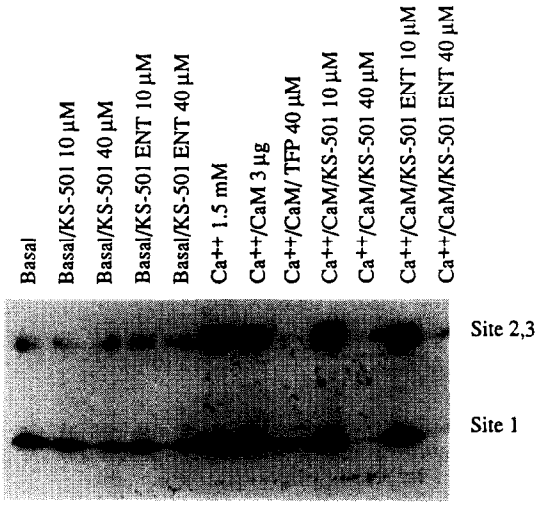


Fig. 5. Effects of KS-501 and its enantiomer on site-specific phosphorylation of synapsin I. Synapsin I was phosphorylated using the 100,000 *g* cytosolic fraction of C6 glioma cells as an enzyme source under conditions described in the legend of Fig. 4. Phosphorylated synapsin I was excised and digested with *S. aureus* V8 protease. SDS–PAGE (15%) resolved a 30 kDa fragment (contains site 2,3) and a 10 kDa fragment (contains site 1). Site 2,3 is the preferred substrate for calmodulin (CaM) kinase II and site 1 is the preferred substrate for CaM kinase I and PKA. The activity of PKA was fully inhibited with the Walsh PKI peptide.

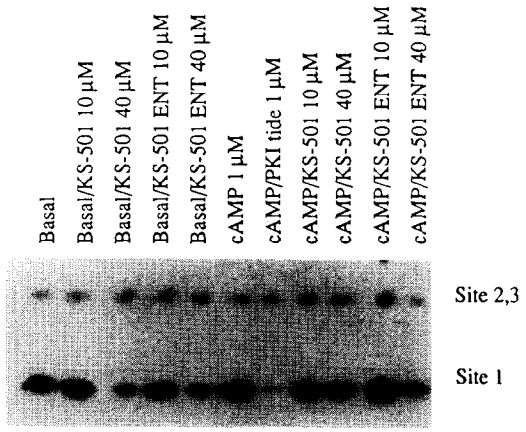


Fig. 6. Effects of KS-501 and its enantiomer on the phosphorylation of cyclic AMP-sensitive phosphorylation of synapsin I. Synapsin I was phosphorylated using the 100,000 *g* cytosolic fraction of C6 glioma cells as an enzyme source under conditions described in the legend of Fig. 4. Basal conditions included 50 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 1 μM EGTA, 1 μM cyclic AMP, 10 μg C_6 cytosol and 20 μM [γ-^{32}P] ATP in the presence or absence of KS-501 or its enantiomer. The reaction was carried out as described in Materials and Methods. The phosphorylated synapsin band was excised from the 10% SDS–PAGE, digested with *S. aureus* V8 protease, and resolved as described in the legend of Fig. 5.

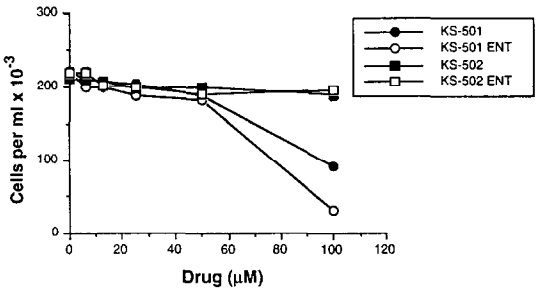


Fig. 7. Effects of KS-501 and KS-502 on the growth of sensitive L1210 leukemic lymphocytes. L1210 cells were grown in supplemented RPMI 1640 medium for 48 hr in the presence or absence of KS-501, KS-502 and their enantiomers and enumerated using an electronic counter. Each point is the mean ± SEM of triplicate samples from one of at least two similar experiments.

newly synthesized compounds KS-501 and KS-502 (Fig. 1) against calmodulin-sensitive enzymes and cellular proliferation. The compounds inhibited calmodulin-activated enzymes including cyclic nucleotide phosphodiesterase (Figs. 2 and 3) and protein kinases (Figs. 4 and 5). KS-501 and its enantiomer were more potent than the KS-502 derivatives. The activities of the parent compounds were similar to those previously reported [11]. However, this is the first report of the activities of the enantiomers against phosphodiesterase, and of the parental compounds against other calmodulin-sensitive enzymes.

The compounds had only a small effect on the activity of the enzymes in the absence of exogenous calmodulin (Figs. 4 and 5). In addition, the inhibition of phosphodiesterase could be abrogated by increasing concentrations of calmodulin (Fig. 3). These data suggest that the major site of action of these compounds is through an interaction with calmodulin or through a calmodulin binding site on the target enzymes. This is consistent with the mechanism of many classes of calmodulin antagonists including the phenothiazines [19], naphthalene sulfonamides [20], and bisquinaldiniums [21]. However, proof of the precise site of action will require synthesis of the radiolabeled drugs, which has not been accomplished as of yet.

The KS-compounds are selective for calmodulin-sensitive kinases compared with cyclic AMP-sensitive protein kinase. For example, at concentrations that nearly abolished calmodulin kinase activity (Figs 4 and 5), there was far less inhibition of cyclic AMP-sensitive phosphorylation of synapsin I (Fig. 6).

Like other calmodulin inhibitors, the KS-compounds decreased the viability of malignant cells, and this activity was related to the potency of the drug against calmodulin-activated enzymes (Figs.

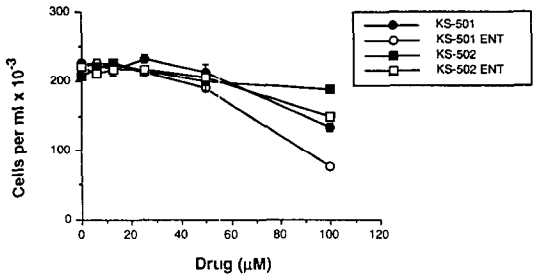


Fig. 8. Effects of KS-501 and KS-502 on the growth of multidrug-resistant L1210 leukemic lymphocytes. L1210/VMDRC.06 cells were grown in supplemented RPMI 1640 medium for 48 hr in the presence or absence of KS-501, KS-502 and their enantiomers and enumerated using an electronic counter. Each point is the mean ± SEM of triplicate samples from one of at least two similar experiments.

7 and 8). For example, KS-501 and its enantiomer were more active anticalmodulin drugs than KS-502 (Fig. 2), and were also more active against the L1210 cell line (Fig. 7). This is consistent with other studies that have demonstrated that the antiproliferative and cytotoxic effects of calmodulin inhibitors are proportional to their effects on calmodulin [22]. Furthermore, Hait *et al.* [23] have shown that irreversible antagonists of calmodulin, such as fluphenazine mustard, inhibit the biological activity of calmodulin *in situ*. The drugs were more potent inhibitors of enzyme activity than of cell proliferation. This was not unexpected based on the size and charge of the drugs which may impede entry into intact cells.

Both compounds and their enantiomers were somewhat less active against the resistant L1210/VMDRC.06 cells than they were against the parental line (Fig. 8). These data suggest that these drugs may be recognized by P-glycoprotein, the *MDR1* gene product [24]. It will require radiolabeled compounds to test this assumption accurately.

The complete synthesis of the KS-compounds provides an opportunity to synthesize additional derivatives to determine structure–activity relationships. This approach may lead to the identification of more potent and selective inhibitors of calmodulin kinases and cellular proliferation. Features such as hydrophobicity and positive charge on amino moieties have been shown previously to influence all three parameters investigated above, including anticalmodulin activity, effects on cell proliferation and viability, and effects on multidrug resistance [24]. In fact, the carboxylic acid substitution on KS-502 may eventually explain its diminished pharmacological properties compared with those of KS-501.

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