Inhibition of Human Prostate Cancer Proliferation in Vitro and in a Mouse Model by a Compound Synthesized to Block Ca$^{2+}$ Entry

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ABSTRACT

Accelerated Ca$^{2+}$ entry may be one component of the pathway regulating the proliferative phenotype of some types of cancer. Thus, a pharmacological agent with the ability to retard Ca$^{2+}$ influx in susceptible cancers might inhibit proliferation of them by a cytostatic mechanism rather than by inducing cytotoxicity. We have developed a chemical synthetic scheme that has produced a small library of novel compounds that block Ca$^{2+}$ entry induced by occupancy of the P2 receptor in two prostate cancer cell lines and inhibit proliferation of these cells in vitro. One of the agents, named TH-1177, was used to treat severe combined immunodeficient mice inoculated with the human prostate cancer line PC-3. Although the doses used and treatment schedule were chosen arbitrarily, treatment extended the mean life span of mice bearing tumors by up to 38%. Treatment of mice without cancer at doses 18 times that used in mice with tumors was not associated with any obvious toxicity, either grossly or on histological examination. These results suggest that novel cytostatic agents with efficacy against human prostate cancer cells can be developed by chemical synthesis of agents directed at the Ca$^{2+}$ entry pathway.

INTRODUCTION

Malignant transformation is often associated with acquisition of a phenotype that is consistent with an abnormally high sensitivity to ambient concentrations of growth factors. In prostate cancer, the source of these growth factors can be autocrine, from the cancer cells themselves, or paracrine, from the surrounding stroma (1, 2). The molecular role of growth factors and their corresponding receptors in malignant transformation and prostate cancer progression is not wholly clear but is likely to be complex (2). This complexity derives at least in part from the ability of various growth factors to either promote or inhibit cellular proliferation (3–5). Complexity is compounded by the ability of proteins other than growth factor receptors to participate in and modify the final expression of the malignant phenotype in a particular tumor (6). Despite the intricacy of growth regulation in prostate cancer, the mitogenic properties of growth factors and their receptors make them a tempting target for therapeutic intervention.

Growth factor receptors are often linked to the pathway that regulates Ca$^{2+}$ homeostasis. As a general rule, engagement of a growth factor receptor by an appropriate ligand results in the activation of phospholipase C by tyrosine phosphorylation (7). Activated phospholipase C metabolizes phosphatidylinositol bisphosphate to produce diacylglycerol and inositol 1,4,5-trisphosphate (7, 8). Via engagement of a specific receptor, inositol trisphosphate releases Ca$^{2+}$ from an internal storage depot, and this release of intracellular Ca$^{2+}$ triggers the influx of extracellular Ca$^{2+}$ (8). Thus, the mitogenic interaction of a growth factor with its receptor can activate a pathway that includes enhancement of the entry of extracellular Ca$^{2+}$, which in turn is a component of the proliferative cascade (9).

What role enhanced Ca$^{2+}$ entry might play in the proliferation of prostate cancer cells is not well understood. It has been shown, however, that proliferation of at least some cancer cell lines can be slowed or stopped at specific points in the cell cycle by removal of extracellular Ca$^{2+}$ (9, 10). Consistent with this is the observation that a drug that blocks Ca$^{2+}$ entry can retard the metastasis of human melanoma cells in immune-deficient mice (11). It is unlikely that blockade of Ca$^{2+}$ entry could reverse the malignant phenotype, even if it could ameliorate excessive proliferation. However, inhibition of prostate cancer proliferation in vivo would be a therapeutically appealing goal by, for example, lengthening remission duration or treating early, limited disease. With these ideas in mind, we have taken as our working hypothesis the idea that a pharmacological agent capable of inhibiting receptor-linked Ca$^{2+}$ entry in vitro might also slow prostate cancer proliferation in vivo.

Previously, we identified a Ca$^{2+}$ current named I$_{Ca}$ in a malignant T lymphocyte cell line that is augmented by a mitogenic stimulus (12). Guided by the general biophysical features of this current, we surveyed the literature for compounds that inhibited Ca$^{2+}$ currents with roughly similar characteristics in other systems. Several known compounds were identified and tested for their ability to inhibit receptor-linked Ca$^{2+}$ entry and proliferation. Although this structurally diverse group of agents was not strikingly effective, they nonetheless suppressed receptor-linked Ca$^{2+}$ influx and proliferation with a high degree of concordance. From the structures of these compounds and their relative efficacy, we constructed a structure-activity series. This series was used to direct the synthesis of novel compounds with the intent of blocking Ca$^{2+}$ entry and proliferation. In vitro testing showed that many of these compounds, including one named TH-1177, were more effective at blocking Ca$^{2+}$ influx and proliferation of prostate cancer cells than those already known. We also examined TH-1177 in a murine model of human prostate cancer. Our results suggest that TH-1177 is cytostatic for prostate cancer cells in vitro and can significantly prolong the life of immune-deficient SCID mice inoculated with the human prostate cancer line PC-3.

MATERIALS AND METHODS

Synthesis of TH-1177. TH-1177 was synthesized in three simple steps (Fig. 1). L-Proline methyl ester was coupled with 4-methoxyphenylacetic acid using benzotriazol-1-yloxytripyrrolidinephosphonium to generate methyl 1-[2-(4-methoxyphenyl)acetyl]pyrrolidine-2-carboxylate, a yellowish oil. The resulting amide was subsequently reduced to the amino alcohol with LiAlH$_4$ and AlCl$_3$ in tetrahydrofuran. The resulting colorless oil was coupled with 4-chlorobenzhydrol under Williamson conditions with catalytic 4-chlorobenzhydrol and alkylating agent 4-chlorobenzhydrol under Williamson conditions, which in turn is a component of the proliferative cascade (9).

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The abbreviations used are: SCID, severe combined immunodeficient; [Ca$^{2+}$], intracellular Ca$^{2+}$.
vitro and in ethanol for use in vivo. Details of the synthesis of TH-1177 and related compounds will be presented elsewhere.4

Cell Lines and Maintenance. Hormone-responsive LNCaP-FRG and hormone-resistant PC-3 prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were maintained in RPMI 1640 and supplemented with glutamine and 5% fetal bovine serum containing SerXtend (Irvine Scientific). The fetal bovine serum used for culture was heat-inactivated by maintaining the serum at 56°C for 1 h.

Measurement of the [Ca2+]i Concentration. Cells were incubated in growth media containing 1 μM of the acetoxy-methyl ester of the Ca2+-sensitive fluorescent dye indo-1 (indo-1/AM; Molecular Probes, Eugene, OR) for 1 h at 37°C. Cells were washed three times in buffer A [10 mM HEPES (pH 7.4), 1 mM MgCl2, 3 mM KCl, 1 mM CaCl2, 140 mM NaCl, 0.1% glucose, and 1% fetal bovine serum] and suspended to a final concentration of 10⁶ cells/ml.

Before stimulation, cells were warmed to 37°C. Changes in [Ca2+]i were monitored in an SLM 8100C spectrofluorometer (SLM/Aminco; Urbana, IL) using previously published methods (13, 14).

Measurement of Cellular Proliferation. LNCaP cells at 2.5 × 10⁴ cells/well or PC-3 cells at 5 × 10⁴ cells/well, both in a final volume of 100 μl, were plated in triplicate in standard flat-bottomed 96-well tissue culture plates in the presence of drug or vehicle (DMSO). Unless otherwise indicated, cells were grown for 48 h at 37°C in a CO2 incubator. Relative cell growth was determined with the CellTiter 96 aqueous cell proliferation assay (Promega, Madison, WI) as described by the manufacturer using an automated plate reader. Results were calculated in a blinded fashion and are the means of triplicate determinations.

Determination of Apoptosis. Cells at 2.5 × 10⁵ cells/ml (LNCaP) or 5 × 10⁵ cells/ml (PC-3) were cultured in growth medium for 24 h in the presence or absence of 100 μM TH-1177 on chambered microscope slides that were coated with 0.01% poly-L-lysine to promote cell adhesion. Apoptotic cells were identified by the terminal deoxynucleotidyl transferase-mediated nick end labeling method using a commercially available kit (Promega, Madison, WI). Staining with propidium iodide identified all cells, both living and apoptotic. Slides were scored in a blinded fashion, and a minimum of 200 cells under each condition were counted.

Animal Studies. The Animal Care Committee of the University of Virginia approved all protocols. SCID mice were housed in a barrier isolation facility of the University of Virginia Department of Comparative Medicine, and all personnel observed sterile technique when entering the facility and handling animals. TH-1177 was dissolved in ethanol and diluted 10-fold in sterile buffered NaCl solution [PBS; 150 mM NaCl, 20 mM sodium phosphate (pH 7.4)] immediately before each day’s injection. Injection volumes of 0.5 ml/animal were used. Vehicle consisted of PBS-diluted ethanol. PC-3 cells for injection were prepared by washing in sterile PBS three times before suspension to 2 × 10⁶ cells/ml. Each animal received 0.5 ml of cells by i.p. injection on day 0 of the experiment shown in Fig. 10. Each animal received a daily i.p. injection of vehicle or drug beginning on day 1.

Statistical Methods. In vivo survival data were analyzed with Prism 2.01 (GraphPad Software, San Diego, CA). The results of the Kaplan-Meier analysis are presented as one-tailed probabilities because there was no reasonable expectation that drug treatment would cause the mice to succumb more rapidly to the implanted cancer than would control animals.

RESULTS

General Overview of Chemical Synthesis of TH-1177

Design. It is thought that in dihydropyridines, orientation and ortho, meta, para substitution of the aryl ring(s) affect the activity, as do varying degrees of hydrophobicity in benzothiazepines. The efficacy of phenylalkylamines and derivatives varies with the enantiomer as well as with the type of group substituted on the aryl rings (15). Calcium channel blockers used in preliminary experiments designed to test our hypothesis included dihydropyridines, benzothiazepines, and phenylalkylamines. This survey of known blockers provided structural features and functionality thought to be important for activity. These include a basic tertiary nitrogen, varying regions of hydrophobicity, core heterocycles, substituted aryl rings, and ether linkages. Our proline (specifically, TH-1177) and nipecotate targets incorporate these features and are typically N– and O– dialkylated.

Nature of the Interaction with a Potential Target. Dihydropyridines, phenylalkylamines, and benzothiazepines are classified by their binding to specific sites on the four-subunit calcium channel protein. Phenylalkylamines are known to covalently incorporate into the α1 subunit, specifically the 42-residue segment from Glu1349 to Ser1357.

Fig. 1. Chemical synthesis of TH-1177. TH-1177 was synthesized as outlined in “Materials and Methods.” The chemical steps and structures are indicated in the figure. THF, tetrahydrofuran; NMM, 4-methylmorpholine; p-TSOH, p-toluene-sulfonic acid; PyBOP, benzotriazolyl-1-yl-oxytrypyrrolidinophosphonium.

Fig. 2. Effect of TH-1177 on Ca2+ entry stimulated by thapsigargin in LNCaP cells. LNCaP cells were stimulated with 300 nM thapsigargin to initiate Ca2+ entry in a receptor-independent manner. The indicated concentrations of TH-1177 were added before stimulation with thapsigargin (A) or after the influx pathway had been opened (B). Times of addition are indicated on the figure.
Cancer Cells

epines is also located on the lamine and dihydropyridines, the receptor site for the benzothiaz-

because the was added at 180 s, and TH-1177 at 1, 3, and 10 m

internal storage pool. LNCaP cells were incubated with indo-1 to measure changes in

has been called capacitative Ca\(^{2+}\) entry. The influx component of changes in [Ca\(^{2+}\)], TH-1177 also reduced the increase in [Ca\(^{2+}\)], initiated by thapsigargin when added after the stimulus (Fig. 2B). The elevated [Ca\(^{2+}\)], seen at 100 s in Fig. 2B is generally attributable to Ca\(^{2+}\) entry alone (18), suggesting that the effect of TH-1177 is mediated at least in part by inhibition of Ca\(^{2+}\) entry.

To confirm an inhibitory action of TH-1177 on capacitative Ca\(^{2+}\) entry, we performed the experiment outlined in Fig. 3, A and B. The [Ca\(^{2+}\)], of a suspension of LNCaP prostate cancer cells was monitored while the extracellular Ca\(^{2+}\) concentration was reduced by the addi-
tion of EGTA. The intracellular Ca\(^{2+}\) storage pool was depleted by thapsigargin, which is a conventional means of initiating capacitative Ca\(^{2+}\) entry (21). Capacitative Ca\(^{2+}\) entry was then monitored after the readdition of Ca\(^{2+}\) to the extracellular medium. TH-1177 was added immediately before or shortly after readdition of Ca\(^{2+}\). As shown in Fig. 3, TH-1177 caused a concentration-dependent inhibition of Ca\(^{2+}\) entry under both circumstances, suggesting that its effects are on capacitative Ca\(^{2+}\) entry. When added after readdition of Ca\(^{2+}\), the IC\(_{50}\) for TH-1177 was 3.2 \(\mu\)M, which is comparable to the value of 3 \(\mu\)M for the experiment shown in Fig. 2. When the experiment shown in Fig. 3 was performed with PC-3 cells, the IC\(_{50}\) for TH-1177 was 17 \(\mu\)M, whereas it was 16 \(\mu\)M for the experiment shown in Fig. 2 when using the same cell line (data not shown). Taken together, these results strongly suggest that TH-1177 inhibits capacitative Ca\(^{2+}\) entry in these two prostate cancer cell lines.

Trp\(^{1391}\) (16). The dihydropyridine receptor site is thought to be formed by the extracellular ends of transmembrane segments in two separate domains also on the \(\alpha1\) subunit. As with the phenylalkyl-
lamine and dihydropyridines, the receptor site for the benzothiaz-
epines is also located on the \(\alpha1\) subunit. Because the design of TH-1177 incorporates basic structural features of all three classes and because the \(\alpha1\) subunit of \(\alpha1\) type calcium channels is highly conserved across many cell types, it is reasonable to assume that it shares the same receptor or a similar receptor (16).

Criteria Used to Select TH-1177. TH-1177 was chosen because of its low IC\(_{50}\) proliferation values and its ability to halt the influx of calcium in both prostate cancer cell lines.

Synthesis of TH-1177. Fig. 1 outlines the three-step synthesis of TH-1177 that allows for a large number of additional target compounds to be made easily and efficiently. Details of the synthetic strategy for these other compounds will be presented elsewhere (3). All steps provided yields of greater than 75%. Efficient reduction of both the amide and the ester is achieved in one step using LiAlH\(_4\) and AlCl\(_3\) in a 1:3 molar ratio. Three different syntheses of TH-1177 were used for the completion of these studies, with no differences in nuclear magnetic resonance or mass spectroscopy characteristics among the batches. Each batch was assessed for its ability to inhibit PC-3 and LNCaP prostate cancer cell proliferation in vitro (see below), and the IC\(_{50}\) values for each batch were within the variance of the assay.

TH-1177 Blocks Capacitative Ca\(^{2+}\) Entry in Human Prostate Cancer Cells

In electrically nonexcitable cells, Ca\(^{2+}\) influx is triggered by re-

entry (17–19). Capacitative entry can be initiated by treatment of cells with thapsigargin. Thapsigargin inhibits the Ca\(^{2+}\)-ATPase of the endoplasmic reticulum, allowing uncompensated leak of Ca\(^{2+}\) from this compartment into the cytosol, thereby causing Ca\(^{2+}\) entry in the absence of engagement of a specific receptor (20, 21). As shown in Fig. 2A, addition of TH-1177 to a suspension of LNCaP human prostate cancer cells before thapsigargin resulted in a dose-dependent inhibition of the increase in [Ca\(^{2+}\)]. TH-1177 also reduced the increase in [Ca\(^{2+}\)], initiated by thapsigargin when added after the stimulus (Fig. 2B). The elevated [Ca\(^{2+}\)], seen at 100 s in Fig. 2B is generally attributable to Ca\(^{2+}\) entry alone (18), suggesting that the effect of TH-1177 is mediated at least in part by inhibition of Ca\(^{2+}\) entry.

Fig. 3. Effect of TH-1177 on capacitative Ca\(^{2+}\) entry. The effect of TH-1177 on the capacitative influx of Ca\(^{2+}\) was examined by inducing Ca\(^{2+}\) entry with thapsigargin in the absence of extracellular Ca\(^{2+}\). Readdition of Ca\(^{2+}\) to the extracellular medium revealed the influx component of changes in [Ca\(^{2+}\)], dissociated from the release of Ca\(^{2+}\) from the internal storage pool. LNCaP cells were incubated with indo-1 to measure changes in [Ca\(^{2+}\)], as described in “Materials and Methods.” EGTA (5 mM) was added at 30 s, as indicated by the arrow, to remove extracellular Ca\(^{2+}\). Thapsigargin (1 \(\mu\)M) was added at 60 s, as indicated by the arrow, to release Ca\(^{2+}\) from the internal storage pool. A, TH-1177 at 1, 3, and 10 \(\mu\)M was added at 180 s. Ca\(^{2+}\) (5 mM) was added at 260 s. B, Ca\(^{2+}\) (5 mM) was added at 180 s, and TH-1177 at 1, 3, and 10 \(\mu\)M was added at 250 s.

TH-1177 was chosen because of its low IC\(_{50}\) proliferation values and its ability to halt the influx of calcium in both prostate cancer cell lines.

Fig. 4. Effect of TH-1177 on Ca\(^{2+}\) entry stimulated by ATP in LNCaP cells. Ten \(\mu\)M TH-1177 was added to a suspension of LNCaP cells either before stimulation with 1 mM ATP (A) or after the influx pathway had been opened by the addition of ATP (B). Times of addition are indicated on the figure.
TH-1177 Inhibits Receptor-linked Ca\(^{2+}\) Entry

The P2 purinergic receptor is linked to activation of the Ca\(^{2+}\) entry pathway in many types of cells including prostate cancer cells (22). The ability of TH-1177 to block capacitative Ca\(^{2+}\) entry induced by thapsigargin suggested that this compound might also block Ca\(^{2+}\) entry triggered by the engagement of a specific receptor. The P2 receptor binds extracellular ATP, inducing multiple biochemical events including Ca\(^{2+}\) entry (22). Addition of ATP to LNCaP prostate cancer cells resulted in a rapid rise in [Ca\(^{2+}\)], that was inhibited by the prior addition of TH-1177 (Fig. 4A). As shown in Fig. 4B, TH-1177 added after ATP also caused a reduction in the [Ca\(^{2+}\)], that had been augmented by P2 receptor engagement.

PC-3 prostate cancer cells also demonstrated an increase in [Ca\(^{2+}\)], when stimulated by ATP (Fig. 5; Ref. 22). In the experiment depicted in Fig. 5A, TH-1177 was added before the cells were stimulated with ATP. TH-1177 caused a concentration-dependent inhibition of the increase in [Ca\(^{2+}\)], that was otherwise induced by engagement of the purinergic receptor. By 70 s after ATP addition, release of Ca\(^{2+}\) from the internal storage pool is largely over (for example, see Fig. 6), and the maintenance of elevations of [Ca\(^{2+}\)], over baseline is dependent on Ca\(^{2+}\) entry from the extracellular compartment. As shown in Fig. 5B, addition of TH-1177 to cells treated previously with ATP caused a reduction in [Ca\(^{2+}\)]. This suggests that TH-1177 is interacting with the Ca\(^{2+}\) influx pathway initiated by P2 receptor stimulation.

The efficiency of TH-1177 in blocking Ca\(^{2+}\) entry was assessed by comparison to the effect of chelation of extracellular Ca\(^{2+}\) with EGTA, which was considered 100% inhibition of Ca\(^{2+}\) entry. For LNCaP cells, the IC\(_{50}\) for TH-1177 was 3 \(\mu\)M, and for PC-3 cells, the IC\(_{50}\) was 16 \(\mu\)M under these conditions.

The data presented above suggest that TH-1177 was inhibiting stimulated increases in [Ca\(^{2+}\)], by blockade of Ca\(^{2+}\) entry. In Fig. 6, extracellular Ca\(^{2+}\) was markedly reduced by the addition of EGTA to the extracellular medium. Under these conditions, addition of ATP to either PC-3 (Fig. 6A) or LNCaP (Fig. 6B) cells resulted in a rise in [Ca\(^{2+}\)], that is more transient and of smaller magnitude than in the presence of extracellular Ca\(^{2+}\). This increase represents release of Ca\(^{2+}\) from the internal storage pool. Addition of TH-1177 had no effect on the size of this change in [Ca\(^{2+}\)]. Similar results were obtained when Ca\(^{2+}\) release was induced by treatment with thapsigargin (data not shown). This indicates that TH-1177 does not interfere with Ca\(^{2+}\) release and suggests that TH-1177 has no influence on the biochemical events upstream from release of Ca\(^{2+}\) from the internal pool.

TH-1177 Inhibits Prostate Cancer Cell Proliferation in vitro by a Cytostatic Mechanism

Inhibition of Ca\(^{2+}\) entry has been shown to limit proliferation of cancer cells in vitro (11). The ability of TH-1177 to block Ca\(^{2+}\) entry induced by release of Ca\(^{2+}\) from internal stores such as that stimulated by engagement of the P2 purinergic receptor suggested the possibility that this agent could inhibit proliferation of prostate cancer cells in vitro. As shown in Fig. 7, TH-1177 caused a concentration-dependent inhibition of the proliferation of both LNCaP and PC-3 cells. The IC\(_{50}\) for inhibition of LNCaP proliferation was 4 \(\mu\)M (Fig. 7A), whereas the value for PC-3 prostate cancer cells was 14 \(\mu\)M (Fig. 7B). When compared to the IC\(_{50}\) values for inhibition of Ca\(^{2+}\) entry of 3 and 16 \(\mu\)M for LNCaP and PC-3 cells, respectively, it is clear that TH-1177 inhibits proliferation at a concentration similar to that which blocks Ca\(^{2+}\) entry in these two cell types.

Most conventional cancer chemotherapeutic drugs are cytotoxic and exert their therapeutic benefit by killing cancer cells. On the other hand, agents that act by inhibition of Ca\(^{2+}\) entry would likely arrest
cell proliferation rather than induce cell death (9). To address this possibility, LNCaP prostate cancer cells were allowed to grow unimpeded or exposed to TH-1177 for 2 or 3 days before the agent was washed away (Fig. 8, A and B). As predicted, both cell lines grew in the absence of TH-1177, whereas growth was stopped when the compound was present. Removal of TH-1177 after exposure for 2 or 3 days was associated with resumption of a rate of growth that was similar to that seen in cells never exposed to TH-1177 (Fig. 8). Similar results were seen with PC-3 prostate cancer cells (data not shown). This effect was not due to the induction of apoptosis by TH-1177. In the presence of 100 μM TH-1177 for 24 h, 8.4 ± 0.4% of LNCaP cells and 9.8 ± 0.4% of PC-3 cells were apoptotic, as compared to 10.1 ± 0.4% of cells in control cultures. Taken together, these observations suggest that prostate cancer cells were quiescent in the presence of TH-1177 relative to cells that were cultured in the absence of this agent. We do not know whether TH-1177 blocks proliferation at a specific point in the cell cycle. However, Ca^{2+} influx is required at several specific transition points (23, 24), and it is possible that TH-1177 acts at each of these.

The experiment depicted in Fig. 8 is consistent with the idea that TH-1177 does not induce any long-lasting alteration in the proliferative phenotype of prostate cancer cells. The experiment depicted in Fig. 9 was performed to further evaluate this possibility. LNCaP cells were grown in the continuous presence of TH-1177 at one of three concentrations or in the absence of the compound for 48 h. The cell culture medium was then replaced with medium free of TH-1177 and maintained for an additional 48 h. TH-1177 was then added to all cell cultures at the concentrations indicated in Fig. 9, such that previously treated cell cultures received an identical second treatment. Prior treatment with TH-1177 had no effect on the response to the second exposure to this compound (Fig. 9). This observation is clearly consistent with the idea that TH-1177 must be present to inhibit proliferation and that TH-1177 does not alter the drug-sensitive phenotype of human prostate cancer cell lines under these conditions.

TH-1177 Slows Prostate Cancer Progression in Vivo

To begin an investigation of the possibility that TH-1177 possesses in vivo activity against prostate cancer, SCID mice were inoculated with PC-3 cells by i.p. injection. One day later, daily i.p. injections of TH-1177 or vehicle alone were begun. TH-1177 was administered at doses of either 3 or 10 mg/kg. These doses were selected arbitrarily, based on the general range of doses for Ca^{2+} channel blockers given p.o. to patients for the treatment of hypertension. Daily dosing was started.
also selected arbitrarily, based on the general desirability of a once-a-day treatment regimen. As shown in Fig. 10, there was a dose-dependent increase in longevity associated with TH-1177 administration. Life span was increased by 34% (P = 0.047) by TH-1177 at a dose of 3 mg/kg/day and by 38% (P = 0.0044) at a dose of 10 mg/kg/day.

Although the experiment shown in Fig. 10 suggested a lack of toxicity associated with TH-1177 administration, the drug was further examined specifically for possible toxicity. Four SCID mice without tumors were given TH-1177 at a dose of 180 mg/kg/day by daily i.p. injection for 22 days. Treated mice were all well-groomed and active during treatment, and no gross abnormalities were noted at necropsy (data not shown). Samples of kidney, adrenal gland, heart, and liver were within normal limits on histological examination including active hepatic hematopoiesis.  

DISCUSSION

Antimetabolic, cytotoxic therapies for cancer have achieved much success in extending the lives of people with this disease. The goal of this approach to cancer treatment, at its limit, is the complete eradication of cancer cells. Elimination of all residual cancer cells results in cure, although emergence of drug resistance or of disease that is more aggressive not infrequently hampers this outcome. The goal of cytostatic cancer therapies is to retard cellular proliferation rather than eliminate all cancer cells. This should not diminish interest in such an approach because controlling the growth of cancer would, at the limit, render the disease effectively impotent. Failure to achieve this limit could be nonetheless valuable clinically if, for example, cytostatic therapy significantly extended the duration of remissions induced by cytotoxic agents.

A role for Ca\(^{2+}\) entry in controlling the overall rate of cellular proliferation has been speculated about for some time (9, 23, 25). However, the function of Ca\(^{2+}\) entry is paradoxical; although it is critical for cell division (9, 23), it is equally necessary for apoptotic cell death (25). It has, for example, been shown that the normally occurring rate of apoptosis in prostate cancer cell lines is reduced by transfection of the cytosolic Ca\(^{2+}\)-binding protein calbindin (26). On the other hand, a drug that blocks Ca\(^{2+}\) entry into human melanoma cells was shown to retard the metastasis of these cells in immune-deficient mice (11). Using malignant T lymphocytes, we have shown that a previously known and relatively simple organic compound blocks receptor-linked Ca\(^{2+}\) entry and that this blockade is accom-panied by inhibition of proliferation (14). Based on these observations, we undertook the directed synthesis of chemical agents with the intent of developing compounds that, in vitro, block growth factor receptor-linked entry of Ca\(^{2+}\) and, in association, growth factor-driven cellular proliferation. The synthetic scheme was designed based on a structure-activity analysis of known compounds that we have shown to be effective inhibitors of both Ca\(^{2+}\) entry and tumor cell proliferation (data not shown). The resulting synthetic strategy yielded a small library of agents that were effective for the purpose intended. We have no information yet on the identity of the molecular target of TH-1177. However, conventional Ca\(^{2+}\) channel blockers, upon which TH-1177 is roughly based, bind to the \(\alpha 1\) subunit of the target Ca\(^{2+}\) channel (16), and it is possible that TH-1177 acts at a structurally similar site.

The ubiquity of Ca\(^{2+}\) as a second messenger could lead to the speculation that systemic administration of a Ca\(^{2+}\) entry antagonist would have numerous, unwanted consequences. However, this was not observed in mice treated with a dose of TH-1177 that was 18-fold higher than that which showed efficacy in slowing prostate cancer progression. It is possible that polymorphisms among Ca\(^{2+}\) entry pathways may allow relatively specific targeting of drugs to one pathway or another, similar to the strategy used in the treatment of hypertension. The success of therapy for the prevention of cardiovascular disease, as well as our own observations, suggests that certain Ca\(^{2+}\) entry antagonists can be administered without poorly tolerated systemic toxicity.

Whereas a possible role of Ca\(^{2+}\) entry in cancer cell proliferation has been known for some time, the use of directed Ca\(^{2+}\) entry antagonists for the potential treatment of this disease has not yet been developed. Based on our characterization of the Ca\(^{2+}\) entry pathway in one malignant cell type (14), we developed a strategy for the identification of pharmacological agents that could act as Ca\(^{2+}\) entry antagonists in cancer cells more generally. From this assortment of known compounds, we constructed a hypothetical chemical structure to pharmacologically inhibit Ca\(^{2+}\) entry and hence proliferation in the types of cancer cells included in our test panel, namely hormone-sensitive and -insensitive breast and prostate cancer. A library of candidate chemical agents was created based on this hypothetical structure. From this library of compounds, TH-1177, which showed good correlation between inhibition of Ca\(^{2+}\) entry and proliferation of prostate cancer cells in vitro, was chosen for further testing in a murine model of human prostate cancer. In this initial study, administration of TH-1177 to mice significantly retarded the progression of lethal human prostate cancer and did so without apparent toxicity. This observation raises the possibility that directed synthesis of novel Ca\(^{2+}\) channel blockers could be useful in the therapy of cancer by minimizing effects on unintended organs and cells and by maximizing the cytostatic efficacy for the malignant tissue of clinical concern.

REFERENCES


\(5 \text{ Dr. Bruce Williams, personal communication.} \)


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