Abstract: The present invention provides novel T type calcium channel inhibitors of formula (I), the use thereof in the treatment of a disease or condition in a mammal associated with influx of extracellular calcium via T type calcium channels, wherein R₁ is C₁-C₄ alkyl, hydroxy, or C₁-C₄ alkoxy; Z is NH, NCH₃, O, or CH₂; Y is NH, O, or CH₂ with the proviso that Y and Z are not the same; R₂ is H, halo, NH₂, C₁-C₄ alkyl, hydroxy, or C₁-C₄ alkoxy; and m and n are independently selected from integers ranging from 1-5 with the proviso that m + n = an integer ranging from 2-9; and R₃ is H, halo, NH₂, C₁-C₄ alkyl, hydroxy, or C₁-C₄ alkoxy.
Declarations under Rule 4.17:


— as to the applicant’s entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the “Guidance Notes on Codes and Abbreviations” appearing at the beginning of each regular issue of the PCT Gazette.
T TYPE CALCIUM CHANNEL INHIBITORS

BACKGROUND OF THE INVENTION

Influx of extracellular calcium is critical for a number of vital cellular processes. Calcium influx is generally mediated by calcium channels, which are grouped into several families one of which is the T type calcium channel family. Pharmacological modulation of the T type calcium channel’s function is tremendously important in the practice of medicine; for example, T type calcium channel inhibitors are in widespread use in the treatment of neurological diseases (e.g. epilepsy, petit mal seizure, absence seizure, neuropathic pain, and etc.) and cardiovascular diseases (e.g. hypertension, unstable angina, and etc.). For example, mibebradil, a T type calcium inhibitor, was clinically efficacious in treating hypertension and cardiac arrhythmia. Studies also suggest that T-type calcium channels may play an important role in age related macular degeneration. Recently, we have showed that the α1H and δ25 isoforms of T type calcium channels are present in cancer cell lines and that novel chemical agents could be synthesized to block calcium entry via this channel thus inhibiting cancer cell proliferation.

Ca2+ entry is critical for cellular proliferation. Because unusually rapid proliferation is a hallmark of cancer, elucidation of the mechanism of Ca2+ entry is of scientific importance with potential clinical significance. However, the mechanism or mechanisms of Ca2+ entry in electrically non-excitable cells, which constitute most types of cancer, remain elusive. The majority of cancers arise from cell types considered "electrically non-excitable." This is to distinguish the means of regulation of Ca2+ entry in these cells from those having action potentials, that is "electrically excitable" cells. In electrically excitable cells, an action potential opens voltage gated Ca2+ channels that admit the Ca2+ required for initiating events such as neurosecretion. In electrically non-excitable cells, the regulatory mechanism activating Ca2+ entry is unclear in part because of the uncertainty about the molecular mechanism by which Ca2+ entry occurs.

The two types of ion channels or transporters most commonly implicated in capacitative Ca+ entry are ICRAC and members of the TRP. These two
channel types do not share many properties other than relative selectivity for Ca$^{2+}$, albeit sometimes very weak, and lack of voltage dependent gating. It has been difficult, however, to tie these proteins' function to capacitative Ca$^{2+}$ entry. This may reflect a particular complexity of Ca$^{2+}$ signaling in electrically non-excitatory cells arising from participation of numerous channels and transporters in this process. It is also possible that the primary means of Ca$^{2+}$ entry in electrically non-excitatory cells has yet to be fully elucidated.

We have taken an alternative approach to dissecting the Ca$^{2+}$ entry pathway in electrically non-excitatory cells. We first took advantage of Ca$^{2+}$ entry blockade by Ni$^{2+}$, as measured by fluorescence techniques (Merritt, J.E. and Rink, T.J. 1987. Regulation of cytosolic free calcium in fura-2-loaded rat parotid acinar cells. *J.Biol.Chem.* 262:17362-17369; Merritt, J.E., Jacob, R., and Hallam, T.J. 1989. Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J.Biol.Chem.* 264:1522-1527; Skryma, R., Mariot, P., Bourhis, X.L., Coppenolle, F.V., Shuba, Y., Abeele, F.V., Legrand, G., Humez, S., Boilly, B., and Prevarskaya, N. 2000. Store depletion and store-operated Ca$^{2+}$ current in human prostate cancer LNCaP cells: involvement in apoptosis. *J.Physiol.(Lond.*) 527 Pt 1:71-83), to identify compounds in the published literature with a similar ability. The structure/activity relationship of these known compounds was used to guide the synthesis of novel compounds with enhanced potency to block Ca$^{2+}$ entry into and proliferation of several cancer cell lines. Administration of one of these compounds, TH-1177, significantly extended the lifespan of nude mice inoculated with human PC3 prostate cancer cells (Haverstick, D.M., Heady, T.N., Macdonald, T.L., and Gray, L.S. 2000. Inhibition of human prostate cancer proliferation *in vitro* and in a mouse model by a compound synthesized to block Ca$^{2+}$ entry. *Cancer Res* 60:1002-1008). We show here that two representative compounds block the Ca$^{2+}$ current through the heterologously expressed α1H isoform of T type Ca$^{2+}$ channels and inhibit proliferation of HEK293 cells stably transfected with this isoform. These two compounds blocked the Ca$^{2+}$ current and capacitative Ca$^{2+}$ entry with similar potencies and identical stereoselectivity. Importantly, cell lines sensitive
to our novel compounds express message for α1H, its 825 splice variant, or both while a cell line resistant to our compounds does not detectably express either message.

The library of compounds we have developed has apparently broad activity against cancer cell lines from various tissues. They act cytostatically and are equally potent at inhibiting hormone sensitive and insensitive breast and prostate cancer lines. We demonstrate here the likely target of their anti-proliferative activity. Taken together, these observations raise the possibility of directed chemical synthesis of compounds that inhibit Ca2+ entry into and thereby proliferation of cancer cells.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides a method for the treatment of a disease or condition in a mammal associated with influx of extracellular calcium via T type calcium channels, which comprises administering to the mammal a therapeutically effective amount of a T type calcium channel inhibitor, a prodrug thereof, or a pharmaceutically acceptable salt of said inhibitor or prodrug. Preferably, the T type calcium channel inhibitor has a structure represented by Formula (I):

![Chemical Structure](image)

wherein

- R₁ is C₁-C₄ alkyl, hydroxy, or C₁-C₄ alkoxy;
- Z is NH, NCH₃, O, S, or CH₂;
- Y is NH, O, or CH₂ with the proviso that Y and Z are not the same;
- R₂ is H, halo, NH₂, C₁-C₄ alkyl, hydroxy, or C₁-C₄ alkoxy;
- m and n are independently selected from integers ranging from 1-5 with the proviso that m + n = an integer ranging from 2-9; and
- R₃ is H, halo, NH₂, C₁-C₄ alkyl, hydroxy, or C₁-C₄ alkoxy.

More preferably, R₁ is hydroxy or C₁-C₄ alkoxy; Z is N or O; R₂ is H, halo, NH₂ or
hydroxy; and R₃ is H. The disease or condition is preferably selected from the group consisting of unstable angina, hypertension, epilepsy, neuropathic pain, petit mal seizure, absence seizure, age related macular degeneration, cancer, and pre-cancerous condition.

The present invention also provides a method for reducing proliferation of electrically non-excitatory cells, which comprises administering a T type calcium channel inhibitor of formula (I) as described above.

In another aspect, the present invention provides a compound of Formula (I)

\[
\text{Formula (I)},
\]

wherein

- R₁ is C₁-C₄ alkyl, hydroxy, or C₁-C₄ alkoxy;
- Z is NH, NCH₃, O, S, or CH₂;
- Y is NH, O, or CH₂ with the proviso that Y and Z are not the same;
- R₂ is H, halo, NH₂, C₁-C₄ alkyl, hydroxy, or C₁-C₄ alkoxy;
- m and n are independently selected from integers ranging from 1-5 with the proviso that m + n = an integer ranging from 2-9; and
- R₃ is H, halo, NH₂, C₁-C₄ alkyl, hydroxy, or C₁-C₄ alkoxy;
- or a pharmaceutically acceptable salt thereof.

Preferably, R₁ is hydroxy or C₁-C₄ alkoxy; Z is N or O; R₂ is H, halo, NH₂ or hydroxy; and R₃ is H. More preferably, the compound of formula (I) is selected from the group consisting of the following compounds:
In another aspect, the present invention provides a pharmaceutical composition comprising a therapeutically effective amount of a compound of formula (I) as described above, a prodrug of said compound or a pharmaceutically acceptable salt of said compound or prodrug; and a pharmaceutically acceptable carrier, vehicle or diluent.

In another aspect, the present invention provides a method for the treatment of cancer or pre-cancerous condition in a mammal, which comprises administering to the mammal a therapeutically effective amount of a compound of formula (I) as described above, a prodrug thereof, or a pharmaceutically acceptable salt of said compound or prodrug in combination with one or more anti-tumor agent.

The present application also provides pharmaceutical combination composition comprising a therapeutically effective amount of a combination of a compound of formula (I) as described above, a prodrug thereof, or a pharmaceutically acceptable salt of said compound or prodrug; and one or more anti-tumor agent.
BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Identification of known compounds that block Ca2+ entry and proliferation and design of novel compounds with increased potency. Proliferation and Ca2+ entry were determined in Jurkat cancer cells as described below in the Materials and Methods. Individual concentration-response curves for each activity and compound were constructed and IC50 values were calculated. The calculated least squares regression in shown as a solid line and a line with a slope of one in shown as a dashed line. Panel A: Using Ni2+ sensitivity as a guide, known compounds were identified that block Ca2+ entry and proliferation in Jurkat cells.

Panel B: An SAR was developed from the data depicted in panel A leading to the synthesis of novel compounds that block Ca2+ entry and proliferation Jurkat cells.

Figure 2. The Ca2+ ionophore ionomycin overcomes the inhibition of Ca2+ entry into and proliferation of Jurkat cells produced by TH-1177. Panel A. Ca2+ entry into Jurkat cells was determined as described in Materials and Methods.

Ionomycin at the indicated concentrations was added 30 s and the mitogenic monoclonal antibody OKT3 was added at a concentration of 1 ug/ml at 60 s. Either EGTA at 2.5 mM or TH-1177 at the indicated concentrations was added at 150 s. The percentage inhibition was determined as described in Material and Methods. Panel B. Jurkat cells were grown for 48 hrs in the presence (open symbols) or absence (closed symbols) of 30 nM ionomycin and the indicated concentrations of TH-1177. Percent control growth was determined as described in Materials and Methods.

Figure 3. Amplicons of two sizes were identified in Jurkat and SK-N-SF cancer cell lines using α1H Ca2+ channel specific PCR primers. Messenger RNA was extracted and amplified as described in Materials and Methods using the primers described previously (Mariot, P., Vanoverberghe, K., Lalevée, N., Rossier, M.F., and Prevarskaya, N. 2002. Overexpression of an alpha 1H (Cav3.2) T-type calcium channel during neuroendocrine differentiation of human prostate cancer cells. J Biol Chem 277:10824-10833). The resulting products were isolated by gel electrophoresis and visualized by ethidium bromide staining and visualized by UV illumination.
Figure 4. The sequences of the amplicons shown in Figure 4 are virtually identical to either α1H or its 825 splice variant. The amplicons shown in Figure 4 were sequenced as described in Materials and Methods. The GenBank database was then queried and the alignments shown were obtained.

Figure 5. Blockade of the α1H Ca2+ current by the known T type Ca2+ channel mibefradil or TH-1177 inhibits proliferation of stably transfected HEK293 cells. HEK293 cells were stably transfected the α1H gene as described in Materials and Methods. Proliferation and the Ca2+ current were determined as described in Materials and Methods.

Figure 6. TH-1177 and TH-1211 are stereoisomers about one of two chiral centers and have different potencies at inhibiting the proliferation of PC3 prostate cancer cells. Panel A: The structures of TH-1177 and TH-1211 were determined as described in Materials and Methods. The diastereomers are racemic at the benzhydrol center (solid arrows) and enantiomeric at the proline center (open arrows) with TH-1177 having the S configuration and TH-1211 having the R. Panel B: The proliferation of PC3 human prostate cancer cells was determined as described in Materials and Methods. The IC50 for TH-1177 was 14 uM (open boxes) and for TH-1211 was 42 uM (inverted triangles).

Figure 7. TH-1177 and TH-1211 have different potencies at inhibiting the Ca2+ current through transfected α1H channels. The current carried by transfected α1H Ca2+ channels was determined as described in Materials and Methods. The concentration response for TH-1177 and TH-1211 is shown in Fig. 6D. The IC50 for TH-1177 was 0.8 uM and for TH-1211 was 7 uM.

Definitions

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

As used herein, the term "purified" and like terms relate to an enrichment of a molecule or compound relative to other components normally associated with the molecule or compound in a native environment. The term
"purified" does not necessarily indicate that complete purity of the particular molecule has been achieved during the process. A "highly purified" compound as used herein refers to a compound that is greater than 90% pure.

As used herein, the term "treating", "treat" or "treatment" includes administering therapy to prevent, cure, or alleviate/prevent the symptoms associated with, a specific disorder, disease, injury or condition. For example treating cancer includes inhibition or complete growth arrest of a tumor, reduction in the number of tumor cells, reduction in tumor size, inhibition of tumor cell infiltration into peripheral organs/tissues, inhibition of metastasis as well as relief, to some extent, of one or more symptoms associated with the disorder. The treatment of cancer also includes the administration of a therapeutic agent that directly decreases the pathology of tumor cells, or renders the tumor cells more susceptible to treatment by other therapeutic agents, e.g., radiation and/or chemotherapy. As used herein, the term "treating" includes prophylaxis of the specific disorder or condition, or alleviation of the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms.

As used herein, the term "pharmaceutically acceptable carrier, vehicle or diluent" includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The term also encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in animals, including humans.

The term “therapeutically effective amount” means an amount of a compound of the present invention that ameliorates, attenuates or eliminates a particular disease or condition or prevents or delays the onset of a particular disease or condition.

By “mammal” it is meant to refer to all mammals, including, for example, primates such as humans and monkeys. Examples of other mammals included herein are rabbits, dogs, cats, cattle, goats, sheep and horses. Preferably, the mammal is a female or male human.
The phrase "compound(s) of the present invention" or "compound(s) of Formula (I)" or the like, shall at all times be understood to include all active forms of such compounds, including, for example, the free form thereof, e.g., the free acid or base form, and also, all prodrugs, polymorphs, hydrates, solvates, tautomers, and the like, and all pharmaceutically acceptable salts, unless specifically stated otherwise. It will also be appreciated that suitable active metabolites of such compounds are within the scope of the present invention.

The expression "prodrug" refers to compounds that are drug precursors which following administration, release the drug in vivo via some chemical or physiological process (e.g., a prodrug on being brought to the physiological pH or through enzyme action is converted to the desired drug form).

The expression "pre-cancerous condition" refers to a growth that is not malignant but is likely to become so if not treated. A "pre-cancerous condition" is also known as "pre-malignant condition" by one of ordinary skill in the art.

As used herein the term "anti-tumor agent" relates to agents known in the art that have been demonstrated to have utility for treating neoplastic disease. For example, antitumor agents include, but are not limited to, antibodies, toxins, chemotherapeutics, enzymes, cytokines, radionuclides, photodynamic agents, and angiogenesis inhibitors. Toxins include ricin A chain, mutant Pseudomonas exotoxins, diphtheria toxoid, streptonigrin, boamycin, saporin, gelonin, and pokeweed antiviral protein. Chemotherapeutics include 5-fluorouracil (5-FU), daunorubicin, cisplatinum, bleomycin, melphalan, taxol, tamoxifen, mitomycin-C, and methotrexate as well as any of the compounds described in US Patent No. 6,372,719 (the disclosure of which is incorporated herein by reference) as being chemotherapeutic agents.

Radionuclides include radiometals. Photodynamic agents include porphyrins and their derivatives. Angiogenesis inhibitors are known in the art and include natural and synthetic biomolecules such as paclitaxel, O-(chloroacetyl-carbomyl) fumagillo ("TNP-470" or "AGM 1470"), thrombospondin-1, thrombospondin-2, angiotatin, human chondrocyte-derived inhibitor of angiogenesis ("hCHIAMP"), cartilage-derived angiogenic inhibitor, platelet factor-4, gro-beta, human interferon-inducible
protein 10 ("IP10"), interleukin 12, Ro 318220, tricyclodecan-9-yl xanthate ("D609"),
irsogladine, 8,9- dihydroxy-7-methyl-benz[8]quinolizinium bromide ("GPA 1734"),
medroxyprogesterone, a combination of heparin and cortisone, glucosidase inhibitors,
genistein, thalidomide, diamin-antraquinone, herbimycin, ursolic acid, and oleanolic
acid. Anti-tumor therapy includes the administration of an anti-tumor agent or other
therapy, such as radiation treatments, that has been reported as being useful for
treating cancer.

As used herein, the term "halogen" or "halo" includes bromo, chloro, 
fluoro, and iodo.

The term "haloalkyl" as used herein refers to an alkyl radical bearing at
least one halogen substituent, for example, chloromethyl, fluoroethyl or
trifluoromethyl and the like.

The term "C_{1-n} alkyl" wherein n is an integer, as used herein,
represents a branched or linear alkyl group having from one to the specified number of
carbon atoms. Typically C_{1-6} alkyl groups include, but are not limited to, methyl,
ethyl, n-propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, hexyl and the
like.

The term "C_{2-n} alkenyl" wherein n is an integer, as used herein,
represents an olefinically unsaturated branched or linear group having from 2 to the
specified number of carbon atoms and at least one double bond. Examples of such
groups include, but are not limited to, 1-propenyl, 2-propenyl, 1,3-butadienyl, 1-
butenyl, hexenyl, pentenyl, and the like.

The term "C_{2-C_n} alkynyl" wherein n is an integer refers to an
unsaturated branched or linear group having from 2 to the specified number of carbon
atoms and at least one triple bond. Examples of such groups include, but are not
limited to, 1-propynyl, 2-propynyl, 1-butylnyl, 2-butylnyl, 1-pentynyl, and the like.

The term "C_3-C_n cycloalkyl" wherein n = 4-8, represents cyclopropyl,
cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl.

As used herein, the term "optionally substituted" refers to from zero to
four substituents, wherein the substituents are each independently selected. Each of
the independently selected substituents may be the same or different than other substituents.

As used herein the term "aryl" refers to a mono- or bicyclic carbocyclic ring system having one or two aromatic rings including, but not limited to, phenyl, benzyl, naphthyl, tetrahydronaphthyl, indanyl, indenyl, and the like. Substituted aryl includes aryl compounds having one or two C₁-C₆ alkyl, halo or amino substituents. The term (C₅-C₈ alkyl)aryl refers to any aryl group which is attached to the parent moiety via the alkyl group. The term "heterocyclic group" refers to a C₃-C₈ cycloalkyl group containing from one to three heteroatoms wherein the heteroatoms are selected from the group consisting of oxygen, sulfur, and nitrogen.

The term "bicyclic" represents either an unsaturated or saturated stable 7- to 12-membered bridged or fused bicyclic carbon ring. The bicyclic ring may be attached at any carbon atom which affords a stable structure. The term includes, but is not limited to, naphthyl, dicyclohexyl, dicyclohexenyl, and the like. Any ring structure drawn with one or more bonds extending from the center of the ring is intended to designate a series of compounds that have a bond(s) extending from one of the carbon atoms of the ring to another atom. For example, the structure:

```
    R7
   / \  
  S   
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designates a series of compounds including 2-thienyl or 3-thienyl groups that contain an R₇ substituent at one of the remaining ring carbon atoms.

The compounds of the present invention contain one or more asymmetric centers in the molecule. In accordance with the present invention a structure that does not designate the stereochimetry is to be understood as embracing all the various optical isomers, as well as racemic mixtures thereof.

The term "pharmaceutically acceptable salt" refers to salts which retain the biological effectiveness and properties of the compounds of the present invention and which are not biologically or otherwise undesirable. In many cases, the compounds of the present invention are capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto.
It is understood to one skilled in the art that “T type calcium channel inhibitors” are also known as “T type calcium channel inhibitors”.

DETAILED DESCRIPTION OF THE INVENTION

Changes in the cytosolic concentration of calcium are a critical component of numerous cellular processes. The calcium necessary for these changes comes from the extracellular milieu via influx through calcium channels. Calcium channels are grouped into several families based upon sequence analysis, biophysical characteristics and pharmacological sensitivity. Among these is the T type calcium channel family. These calcium channels have been implicated in regulation of blood pressure, cardiac rhythm, and cellular proliferation. However there has been only one pharmacological agent, mibebradil, that has been proven to be clinically effective because of inhibition of T channel function. Unfortunately, mibebradil was withdrawn from market due to adverse interactions with other drugs leaving this therapeutic approach unavailable.

One embodiment of the present invention is directed to a series of novel synthetic compounds that inhibit calcium entry via T type calcium channels while other members of this series activate calcium entry. Inhibitors of calcium entry will be useful for treating hypertension, cardiac arrhythmia and clinically deleterious cellular proliferation while activators of calcium entry will be useful as cardiac stimulants and inducers of programmed cell death with the intent of reducing the burden of clinically unwanted cells such as cancer cells. Examples of these compounds are as follows:
Presence of Oxygen Atom

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NE = no effect at highest concentration of drug tested, all values in micromolar

In all Figures, "prolif" indicates the IC50 for inhibition of proliferation of the indicated cell lines while "Ca2+" is the IC50 for inhibition of calcium entry. As can be seen in this figure, the presence of an ether linkage has a profound effect of biological activity but only when the amine is proximal to it.
Presence of Nitrogen

Series 1

Series 2

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NE = no effect at the highest concentration of drug tested, all values in micromolar

This Figure demonstrates that nitrogen in the hydrocarbon “tether” is required for biological activity.
Basicity of the Nitrogen

Table 5.3 prolif Ca2+ prolif Ca2+ prolif Ca2+ prolif Ca2+ prolif Ca2+ prolif Ca2+

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NE = no effect at the highest concentration of drug tested, all values in micromolar

This Figure demonstrates that the basicity of the nitrogen contributes to the biological activity.
Steric Bulk Around the Nitrogen

From this Figure, it can be seen that steric bulk around the nitrogen decreases biological activity.
Location of the Nitrogen

![Chemical Structures](image)

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NE = no effect at the highest concentration of drug tested, all values in micromolar.

This Figure shows that biological activity is affected by the position of the nitrogen along the hydrocarbon tether.
In accordance with one embodiment a novel compound that inhibit Ca2+ entry, and thereby proliferation of cancer cells is provided. The compounds have the general structure of Formula (I):

![Chemical structure](image)

wherein

- R1 is C1-C4 alkyl, hydroxy, or C1-C4 alkoxy;
- Z is NH, NCH3, O, S, or CH2;
- Y is NH, O, or CH2 with the proviso that Y and Z are not the same;
- R2 is H, halo, NH2, C1-C4 alkyl, hydroxy, or C1-C4 alkoxy;
- m and n are independently selected from integers ranging from 1-5 with the proviso that m + n = an integer ranging from 2-9; and
- R3 is H, halo, NH2, C1-C4 alkyl, hydroxy, or C1-C4 alkoxy; or a pharmaceutically acceptable salt thereof.

Preferably, R1 is hydroxy or C1-C4 alkoxy; Z is N or O; R2 is H, halo, NH2 or hydroxy; and R3 is H.

The novel compounds of the present invention can be combined with standard pharmaceutically acceptable carriers or other known anti-tumor and chemotherapeutic agents.

**Materials and Methods**

Synthesis of TH-1177:

TH-1177 was synthesized in three simple steps as described (Haverstick, D.M., Heady, T.N., Macdonald, T.L., and Gray, L.S. 2000. Inhibition of human prostate cancer proliferation *in vitro* and in a mouse model by a compound synthesized to block Ca2+ entry. *Cancer Res* 60:1002-1008). L-Proline methyl ester
was coupled with 4-methoxyphenylacetic acid using benzotriazol-1-yl-oxytripyrrolidinophosphonium 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 LiAlH₄ and AlCl₃ in tetrahydrofuran. The resulting colorless oil was coupled with 4-chlorobenzhydryl under Williamson conditions with catalytic p-toluenesulfonic acid in refluxing toluene. The final brownish oil was isolated by column chromatography, and its structure was confirmed by nuclear magnetic resonance and mass spectrometry. TH-1177 was dissolved in DMSO for use.

Cell Lines and Maintenance:
Cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were maintained in RPMI1640 supplemented with glutamine and 5% fetal bovine serum containing SerXtend (Irvine Scientific) when appropriate insulin, as directed by the ATCC. The fetal bovine serum used for culture was heat-inactivated by maintaining the serum at 56°C for 1 h. Cell lines used were a T cell leukemia (Jurkat), prostate cancer (PC3, DU145, LNCaP), breast cancer (MDA468, MDA361, MCF7), pancreatic cancer (MIA PaCa2), liver cancer (Hep G2), lung cancer (A549, NCI H460), colon cancer (HT29, HCT 116), and ovarian cancer (SK-OV-3).

Measurement of the [Ca²⁺]ᵢ Concentration:
Cells were incubated in growth media containing 1 mM 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.

**Measurement of Cellular Proliferation:**

LNCaP cells at 2.5 x 10⁴ cells/well or PC-3 cells at 5 x 10⁴ cells/well, both in a final volume of 100 IJL, 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.

**Results**

**Construction of a novel chemical library:**

Extracellular Ni²⁺ blocks the Ca²⁺ entry pathway in electrically non-excitable cells (Merritt, J.E., Jacob, R., and Hallam, T.J. 1989. Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J. Biol. Chem.* 264:1522-1527; Jones, G.R.N. 1985. Cancer therapy: Phenothiazines in an unexpected role. *Tumori* 71:563-569) as well as the current through T type Ca²⁺ channels (Lee, J.-H., Gomora, J.C., Cribbs, L.L., and Perez-Reyes, E. 2000. Nickel block of three cloned T-type Ca channels: low concentrations selectively block α₁H. *Biophys. J.* 77:3042). We made use of these facts and conducted a search of the Medline database for compounds that block Ca²⁺ entry in any system that was also sensitive to inhibition of Ca²⁺ entry by Ni²⁺. The identified compounds were then used as the basis for a reiterated search. This strategy was continued until the only citations returned were those that had been retrieved.
already indicating that the database had been saturated. These agents, some of which are listed in Table 1, were tested for the ability to block proliferation of and Ca2+ entry into the Jurkat human cancer cell line. These compounds were tested in various cancer cell lines (Materials and Methods) with results similar to those obtained with the Jurkat cell line (data not shown). The correlation between these two inhibitory activities in the Jurkat cell line, expressed as IC50's, is shown in Figure 1, panel A. The resulting structure-activity relationship (SAR) was used as a guide to synthesize novel chemical agents. These novel compounds exhibited enhanced inhibition of Ca2+ into and proliferation (Table 2). The slope of the regression line between the ability of the novel compounds to inhibit proliferation and block Ca2+ entry was 0.97 or very close to unity with an r^2 value of 0.93 (Figure 1, panel B) compared to a slope of 0.73 (r^2 = 0.79) for the known agents (Figure 1, panel A). Because Ca2+ entry is required for proliferation (Berridge, M.J., Lipp, P., and Bootman, M.D. 2000. The versatility and universality of calcium signalling. Nat.Rev.Mol Cell Biol. 1:11-21), the slope of 0.97 should most appropriately be interpreted in a Bayesian fashion. This Bayesian analysis suggests that all of the effect of these compounds on proliferation is mediated through inhibition of Ca2+ entry.

The Ca2+ ionophore ionomycin partially overcomes the effects of

TH-1177. We have used one of our compounds, TH-1177, as the prototype for the others (Haverstick, D.M., Heady, T.N., Macdonald, T.L., and Gray, L.S. 2000. Inhibition of human prostate cancer proliferation in vitro and in a mouse model by a compound synthesized to block Ca2+ entry. Cancer Res 60:1002-1008). If TH-1177 is acting via inhibition of Ca2+ entry, its effects should be at least partially reversed by direct elevation of [Ca2+]i using a Ca2+ ionophore. As shown in Figure 2, panel A, ionomycin overcame inhibition of Ca2+ entry by TH-1177 in a concentration dependent manner although there was no effect on proliferation of 30 nM ionomycin alone. Ionomycin also reduced the ability of TH-1177 to inhibit proliferation (Figure 2, panel B) increasing the IC50 of TH-1177 from 4.6 uM in the presence of 30 uM ionomycin to 17.8 uM in its absence. This suggests that TH-1177 is acting to inhibit
proliferation by inhibition of Ca2+ entry and is in accord with the relationship between Ca2+ entry and proliferation shown in Figure 1.

Cancer cell lines sensitive to our agents express message for the α1H Ca2+ channel or its 825 splice variant.

We have presented data previously suggesting that a member or members of the T type Ca2+ channel family have a role in mediating Ca2+ entry in electrically excitable cells (Densmore, J.J., Haverstick, D.M., Szabo, G., and Gray, L.S. 1996. A voltage operable current is involved in activation-induced Ca2+ entry in human lymphocytes whereas Icrac has no apparent role. Am. J. Physiol. 271:C1494-C1503; Densmore, J.J., Szabo, G., and Gray, L.S. 1992. A voltage-gated calcium channel is linked to the antigen receptor in Jurkat T lymphocytes. FEBS Lett. 312:161-164; Haverstick, D. M. and Gray, L. S. Increased intracellular Ca2+ induces Ca2+ influx in human T lymphocytes. Molecular Biology of the Cell 4, 173-184. 1993; Haverstick, D.M., Densmore, J.J., and Gray, L.S. 1998. Calmodulin regulation of Ca2+ entry in Jurkat T cells. Cell Calcium 23:361-368). It has been shown recently that a prostate cancer line expresses the α1H isoform of T type Ca2+ channels at levels that vary with differentiation status (Mariot, P., Vanoverberghe, K., Lalvee, N., Rossier, M.F., and Prevarskaya, N. 2002. Overexpression of an alpha 1H (Cav3.2) T-type calcium channel during neuroendocrine differentiation of human prostate cancer cells. J Biol Chem 277:10824-10833). Using the same primers, reported in that study, the value for TH-1211 is 24 uM. Thus, when measured by Ca2+ selective fluorescent dyes, Ca2+ entry was similarly sensitive to TH-1177 and TH-1211 as was the Ca2+ current mediated by α1H. As importantly, each of these measures of Ca2+ influx showed the same relative difference in sensitivity to the stereoisomers. This shows the pharmacological correspondence between capacitative Ca2+ entry when measured by fluorescence and Ca2+ entry mediated by α1H when measured by electrophysiological methods.
**Discussion**

Recently, several channels have been suggested as candidates for mediating Ca\(^{2+}\) entry in electrically non-excitatable cells. A common feature of these proposed Ca\(^{2+}\) entry channels is that they are not voltage gated. This reflects the prevalent assumption that the Ca\(^{2+}\) entry pathways in electrically excitable and non-excitatable cells are categorically distinct. It is extremely difficult, however, to understand why such an assumption is necessary particularly in view of the inherently non-categorical nature of biological systems. In addition, these voltage insensitive Ca\(^{2+}\) channels do not fulfill completely the biophysical criteria for the capacitative Ca\(^{2+}\) entry pathway in electrically non-excitatable cells (Haverstick, D. M. and Gray, L. S. Increased intracellular Ca\(^{2+}\) induces Ca\(^{2+}\) influx in human T lymphocytes. Molecular Biology of the Cell 4, 173-184. 1993).

This uncertainty about the nature of Ca\(^{2+}\) entry in electrically non-excitatable cells may be the result of a number of technical factors in addition to the constraints imposed by unnecessary assumption. While several of the proposed candidates for mediation of Ca\(^{2+}\) entry in electrically non-excitatable cells have been characterized at the molecular level (Putney, J.W., Jr. and McKay, R.R. 1999. Capacitative calcium entry channels. *Bioessays* 21:38-46), a commonly accepted Candidate, I\(_{\text{CRAC}}\) (Clapham, D.E. 2002. Sorting out MIC, TRP, and CRAC Ion Channels. *J. Gen. Physiol* 120:217-220; Cahalan, M.D., Wulff, H., and Chandy, K.G. 2001. Molecular properties and physiological roles of ion channels in the immune system. *J Clin Immunol* 21:235-252), has not. It is also difficult to tie the function of these channels to inhibition of proliferation of cancer cell lines because of the paucity of specific Ca\(^{2+}\) entry inhibitors for electrically non-excitatable cells. There is as well the unavoidable disjunction between Ca\(^{2+}\) entry as measured by Ca\(^{2+}\) selective fluorescent dyes and electrophysiological methods: The patch-ctamp technique is extraordinarily powerful for examining the biophysical details of the function of an ion channel (Neher, E. and Sakmann, B. 1992. The patch clamp technique. *Scientific American* March:44-51), however the level of membrane control it both achieves and requires makes it less suited to identifying a channel's physiological role. Fluorescence
techniques are very limited in obtaining biophysical detail but better able to study physiological roles. This disconnection makes it difficult to determine if the effects of physiologically relevant stimuli, as determined by fluorescence measurements, are reproduced at the electrophysiological level.

We have suggested that the mechanism of Ca\textsuperscript{2+} entry in electrically non-excitable cells involves a Ca\textsuperscript{2+} channel sharing characteristics with the T type family of voltage gated Ca\textsuperscript{2+} channels (Densmore, J.J., Haverstick, D.M., Szabo, G., and Gray, L.S. 1996. A voltage operable current is involved in activation-induced Ca\textsuperscript{2+} entry in human lymphocytes whereas I\textsubscript{CRAC} has no apparent role. Am. J. Physiol. 271:C1494-C1503; Densmore, J.J., Szabo, G., and Gray, L.S. 1992. A voltage-gated calcium channel is linked to the antigen receptor in Jurkat T lymphocytes. FEBS Lett. 312:161-164; Haverstick, D. M. and Gray, L. S. Increased intracellular Ca\textsuperscript{2+} induces Ca\textsuperscript{2+} influx in human T lymphocytes. Molecular Biology of the Cell 4, 173-184. 1993; Haverstick, D.M., Densmore, J.J., and Gray, L.S. 1998. Calmodulin regulation of Ca\textsuperscript{2+} entry in Jurkat T cells. Cell Calcium 23:361-368). It could be argued that it is difficult to envision a physiologic role for voltage gated Ca\textsuperscript{2+} channels in cells that do not have action potentials. This argument is, however, based upon the assumption that a voltage gated Ca\textsuperscript{2+} channel can be only be activated by an action potential. Such an assumption is false a priori because the means by which a protein can be regulated by imposed experimental conditions is not necessarily identical with, or even similar to, the mechanism by which it is controlled physiologically. Although secondary to regulation by membrane potential, the known biochemical regulation of voltage gated Ca\textsuperscript{2+} channels in a variety of systems also suggests that the categorical distinction between electrical and biochemical regulation of Ca\textsuperscript{2+} channels may be simplistic.

The data presented here strongly suggest the possibility that the \(\alpha_1\)H isoform of nominally voltage gated T type Ca\textsuperscript{2+} channels or its 825 splice variant has a role in Ca\textsuperscript{2+} entry into and proliferation of electrically non-excitable cancer cells. Our data show that novel compounds can be created based upon an structure-activity relationship generated from compounds that are known to inhibit Ca\textsuperscript{2+} entry in systems that are also sensitive to inhibition by Ni\textsuperscript{2+}. These novel compounds also
inhibit proliferation of several cancer cell lines via blockade of Ca2+ entry. The cell lines that are sensitive to our agents express message for α1H Ca2+ channels, its δ25 splice variant, or both. These compounds also inhibit the Ca2+ current mediated by α1H Ca2+ channels and slow the proliferation of HEK293 cells stably transfected with the α1H Ca2+ channel isoform. TH-1177 and TH-1211 stereoselectively inhibit Ca2+ entry into and proliferation of cancer cell lines and show the same-stereoselectivity and potency in blocking canonical α1H. These data strongly suggest that the α1H Ca2+ channel and its δ25 splice variant participate in Ca2+ entry in the cancer cell lines tested in these studies.

Linking biophysical analysis of Ca2+ channel function to a physiological function such as proliferation can pose challenges. We have demonstrated that our compounds block a heterologously expressed Ca2+ channel and that only those cancer cell lines with message for that channel, or its splice variant, are sensitive to inhibition by the same agents. Furthermore, TH-1177 is more potent at inhibiting Ca2+ entry via expressed α1H as measured by biophysical techniques than the stereoisomer of it, TH-1211. TH-1177 and TH-1211 also show the same rank order of potency at inhibiting proliferation and Ca2+ entry in cancer cell lines when these are assayed by more commonly used biochemical methods. The absolute potencies of the agents as measured by IC50 values are strikingly similar whether measured by biophysical or biochemical methods. Thus, the results from a combination of experimental approaches were synthesized into a picture of the likely mechanism of Ca+ entry in some cancer cells.

Expression of the α1H Ca2+ channel has been demonstrated in LNCaP cells and the expression level correlates with differentiation state (Mariot,P., Vanoverberghe,K., Lalevey,N., Rossier,M.F., and Prevarskaya,N. 2002. Overexpression of an alpha 1H (Cav3.2) T-type calcium channel during neuroendocrine differentiation of human prostate cancer cells. J Biol Chem 277:10824-10833). Although the sequence of the δ25 splice variant has been deposited in GenBank (accession number AF223563), its function has not been described to our knowledge. However, both are members of the T type Ca2+ channel

Our novel synthetic compounds may have clinical utility because treatment of mice bearing xenografted human PC3 prostate cancer cells with TH-1177 significantly extended the lifespan of them (Haverstick,D.M., Heady,T.N., Macdonald,T.L., and Gray,L.S. 2000. Inhibition of human prostate cancer proliferation in vitro and in a mouse model by a compound synthesized to block Ca2+ entry. Cancer Res 60:1002-1008). Thus, it is possible that Ca2+ channel inhibitors
will ultimately provide clinicians with an addition to their armamentarium for the treatment of cancer. The observations presented here, as well as those presented previously (Haverstick, D.M., Heady, T.N., Macdonald, T.L., and Gray, L.S. 2000. Inhibition of human prostate cancer proliferation in vitro and in a mouse model by a compound synthesized to block Ca$^{2+}$ entry. Cancer Res 60:1002-1008), lay the groundwork for further developments in this area.
WHAT IS CLAIMED IS:

1. A method for treating a disease or condition in a mammal associated with influx of extracellular calcium via T type calcium channels, which comprises administering to the mammal a therapeutically effective amount of a T type calcium channel inhibitor, a prodrug thereof, or a pharmaceutically acceptable salt of said inhibitor or prodrug.

2. The method according to claim 1, wherein the T type calcium channel inhibitor has a structure represented by Formula (I):

![Formula (I)](image)

wherein

- $R_1$ is C$_1$-C$_4$ alkyl, hydroxy, or C$_1$-C$_4$ alkoxy;
- $Z$ is NH, NCH$_3$, O, S, or CH$_2$;
- $Y$ is NH, O, or CH$_2$ with the proviso that Y and Z are not the same;
- $R_2$ is H, halo, NH$_2$, C$_1$-C$_4$ alkyl, hydroxy, or C$_1$-C$_4$ alkoxy;
- $m$ and $n$ are independently selected from integers ranging from 1-5 with the proviso that $m + n = $ an integer ranging from 2-9; and
- $R_3$ is H, halo, NH$_2$, C$_1$-C$_4$ alkyl, hydroxy, or C$_1$-C$_4$ alkoxy.

3. The method according to claim 2, wherein $R_1$ is hydroxy or C$_1$-C$_4$ alkoxy; $Z$ is N or O; $R_2$ is H, halo, NH$_2$ or hydroxy; and $R_3$ is H.
4. The method according to claim 1, wherein the disease or condition is selected from the group consisting of unstable angina, hypertension, epilepsy, neuropathic pain, petit mal seizure, absence seizure, age related macular degeneration, cancer, and pre-cancerous condition.

5. A method for reducing proliferation of electrically non-excitable cells, which comprises administering a T type calcium channel inhibitor as described in claim 2.

6. A compound of Formula (I)

\[
\begin{align*}
&\text{R}_1, \text{Z}, \text{R}_2, \text{Y}, \text{R}_3 \\
&\text{R}_1 \text{ is C}_1-\text{C}_4 \text{ alkyl, hydroxy, or C}_1-\text{C}_4 \text{ alkoxy;} \\
&\text{Z} \text{ is NH, NCH}_3, \text{ O, S, or CH}_2; \\
&\text{Y} \text{ is NH, O, or CH}_2 \text{ with the proviso that Y and Z are not the same;} \\
&\text{R}_2 \text{ is H, halo, NH}_2, \text{ C}_1-\text{C}_4 \text{ alkyl, hydroxy, or C}_1-\text{C}_4 \text{ alkoxy;} \\
&m \text{ and } n \text{ are independently selected from integers ranging from 1-5 with the proviso that } m + n = \text{ an integer ranging from 2-9; and} \\
&\text{R}_3 \text{ is H, halo, NH}_2, \text{ C}_1-\text{C}_4 \text{ alkyl, hydroxy, or C}_1-\text{C}_4 \text{ alkoxy;} \\
&\text{or a pharmaceutically acceptable salt thereof.}
\end{align*}
\]

7. The compound according to claim 6, wherein \(\text{R}_1\) is hydroxy or \(\text{C}_1-\text{C}_4\) alkoxy; \(Z\) is N or O; \(\text{R}_2\) is H, halo, NH\(_2\) or hydroxy; and \(\text{R}_3\) is H.
8. The compound according to claim 6, is selected from the group consisting of the following compounds:

9. A pharmaceutical composition comprising a therapeutically effective amount of a compound of formula (I) as described in claim 6, a prodrug of said compound or a pharmaceutically acceptable salt of said compound or prodrug; and a pharmaceutically acceptable carrier, vehicle or diluent.

10. A method for the treatment of cancer or pre-cancerous condition in a mammal, which comprises administering to the mammal a therapeutically effective amount of a compound of formula (I) as described in claim 6 a prodrug thereof, or a
pharmaceutically acceptable salt of said compound or prodrug in combination with one or more anti-tumor agent.

11. A pharmaceutical combination composition comprising a therapeutically effective amount of a combination of a compound of formula (I) as described in claim 6, a prodrug thereof, or a pharmaceutically acceptable salt of said compound or prodrug; and one or more anti-tumor agent.
FIGURE 1

A

Slope = 0.732 ± 0.07

$\text{IC}_{50} \text{ Ca}^{2+} \text{ entry}$

$\text{IC}_{50} \text{ proliferation}$

$r^2 = 0.791$

B

Slope = 0.968 ± 0.03

$\text{IC}_{50} \text{ Ca}^{2+} \text{ entry}$

$\text{IC}_{50} \text{ proliferation}$

$r^2 = 0.934$
FIGURE 2

A

Effect of TH-1177 on Ca²⁺ influx in the presence of ionomycin

- Percent inhibition of Ca²⁺ influx
- [TH-1177] (µM): 100 µM, 30 µM, 10 µM, EGTA

B

Proliferation in the presence of TH-1177 and ionomycin

- Percent Control Growth
- [TH-1177] (µM): 1, 10, 100

Legend:
- No ionomycin
- 1 nM ionomycin
- 3 nM ionomycin
FIGURE 4

Jurkat forward alignment
1    ctcctgggggtaggcattggcgctgcagctgtgcacattgatgggaggggacagcccgtg  60
3348  ctcctgggggtaggcattggcgctgcagctgtgcacattgatgggaggggacagcccgtg  3289

61    ctccttcccctcaggtgcocggtggggtcgccagggctcttcagtctgt  120
3288  ctccttcccctcaggtgcocggtggggtcgccagggctcttcagtctgt  3230

121    ggtttgaggctgtctctgagctgtgtgaggtgctctctgcagag  160
3229  ggtttgaggctgtctctgagctgtgtgaggtgctctctgcagag  3190

SK-N-SH forward alignment
21    caggtgcgtgtggctccctgggtgctccctgcccggtgctgttctgggtgccctgcggc  80
3507  caggtgcgtgtggctccctgggtgctccctgcccggtgctgttctgggtgccctgcggc  3566

81    gggcccgcccccataaggccatcctgggtctttggttggatgtgctgctgtggagccttgcc  140
3567  gggcccgcccccataaggccatcctgggtctttggttggatgtgctgctggtgagccttgcc  3626

141    agctccagggcccttcctcctgtcctggcgccaggtacggtgtcttcctgggcctgctg  200
3627  agctccagggcccttcctcctgtcctggcgccaggtacggtgtcttcctgggcctgctg  3686

201    nocccccaaaaaggccctcctgggttgaggccagggcaacgctgtcctcctccctccatntaacctgac  260
3687  nocccccaaaaaggccctcctgggttgaggccagggcaacgctgtcctcctccctccatntaacctgac  3746

261    agctggccacgccctgcntaccacccaaanaagtcocnnntntccttgg  304
3747  agctggccacgccctgcntaccacccaaanaagtcocnnntntccttgg  3790
FIGURE 5

Does inhibition of proliferation correlate with block of canonical $\alpha 1H$?
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<th>IC₅₀ for inhibition of Ca²⁺ entry</th>
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* Nordihydroguaiaretic acid
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