(51) International Patent Classification: G01N33/5 74 (2006.01)

(21) International Application Number: PCT/US201 1/047846

(22) International Filing Date: 16 August 2011 (16.08.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/374,106 16 August 2010 (16.08.2010) US
61/379,226 1 September 2010 (01.09.2010) US


(72) Inventors; and


(54) Title: CAMKK-BETA AS A TARGET FOR TREATING CANCER

(57) Abstract: Provided herein are compounds, compositions, including pharmaceutical compositions, having anti-cancer activity. Also provided are methods for diagnosing, detecting, and treating cancer in a subject, as well as a method for evaluating cancer stage in a subject, wherein the methods include determining the amount of a Ca^2+/calmodulin dependent kinase kinase (CaMKK) in a sample. Further provided are methods of screening and identifying a compound that inhibits CaMKK.

[Continued on next page]

Published:

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))
CaMKK-β AS A TARGET FOR TREATING CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is related to and claims the benefit of priority to U.S. Provisional Patent application serial number 61/374,106, filed August 16, 2010, and U.S. Provisional Patent application serial number 61/379,226, filed September 1, 2010. The content of both applications are incorporated herein by reference.

FIELD

[0002] The disclosure relates to cancer, including diagnostic markers of cancer, methods for the diagnosis of cancer, methods and compounds for the treatment of cancer, methods for identifying cancer stage in a subject, methods for identifying a cancer that is responsive to particular therapies, and methods for evaluating efficacy of cancer therapy.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0003] This work was supported in part by grants R01 CA139818 (U.S. National Institutes of Health, National Cancer Institute); 1K01 DK084205 (U.S. National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases), and R01 GM033976 (U.S. National Institutes of Health, National Institute of General Medical Sciences). The United States government has certain rights in this invention.

SEQUENCE LISTING

[0004] An electronic version of the sequence listing ("028193_9098_SeqList.txt") which is 233,472 bytes in size and created on August 16, 2011, is submitted herewith and is herein incorporated by reference.

BACKGROUND

[0005] Prostate cancer is the most common malignancy in men and is second only to lung cancer in terms of cancer mortalities [Cancer Facts and Figures: American Cancer Society; 2007]. Early diagnosis of prostate cancer usually allows for successful surgical treatment of localized tumors and thus, good patient outcomes. However, as with many cancers, the
treatment of the advanced disease state requires a systemic approach to inhibit the growth and spread of secondary metastases. Prostate cancers express the androgen receptor (AR) and rely on androgens for growth and survival [Isaacs JT, Isaacs WB., Nat Med 2004; 10:26-7.]. Consequently, androgen ablation therapies are the standard of care for late-stage disease. While 80% of patients with prostate cancer respond favorably to initial androgen ablation therapy, most patients experience a relapse of the disease within 1-2 years [Isaacs JT, Isaacs WB., Nat Med 2004; 10:26-7.]. Despite the unresponsiveness of the hormone-refractory disease to androgen-deprivation therapy, AR-regulated signaling pathways remain active and are necessary for cancer progression [Chen CD., et al, Nature Med 2004; 10:33-9.].

[0006] Several approaches are currently used to target the AR signaling axis in prostate cancer. Existing therapies focus on decreasing the levels of circulating androgens and/or competitively blocking the AR transcriptional complex. Specifically, gonadotropin-releasing hormone (GnRH) agonists are used to suppress the testicular production of testosterone whereas antiandrogens, such as bicalutamide, function by competitively inhibiting the interaction of androgens with AR. The initial response to either form of androgen deprivation is very high. Nevertheless, the rapid onset of resistance to these interventions highlights the need for other strategies that target the hormone-independent activities of AR.

[0007] Most of the studies on the role of androgens in prostate cancer have focused on defining the mechanisms underlying the mitotic actions of this class of hormone [Balk S.P., Nucl Recept Signal 2008; 6:e001]. However, there is a growing body of evidence that AR signaling also influences tumor cell migration and invasion. For example, different clinical trials of goserelin (a GnRH analog) in prostate cancer patients demonstrate reduced incidences of distant metastases [Lawton C.A., et al. Int J Radiation Oncology Biol Phys 2001; 49:937-46; Bolla M., et al. The Lancet 2002; 360:103-8.]. Furthermore, it has recently been reported that MDV3100, a second generation AR-antagonist, decreases the number of circulating tumor cells in approximately half of the treated patients having a castration-resistant type cancer [Scher H.I., et al. The Lancet; 375:1437-46].

[0008] Compounds of Formula I are known and have been used as dye molecules. See, for example, U.S. Patent 2,820,037 which describes:
wherein \( R_i \) is selected from CN, COOH, or COCl. The dye industry has generated a number of compounds that are structurally related to those of Formula I. *See, e.g.*, U.S. Patents 2,835,674; 2,965,644; 2,949,467; 3,953,452; 3,960,867; 4,239,868; and 4,336,383.

[0009] Japanese Patent Application No. 2003-012516 (Sumitomo Pharmaceutical Co.) identifies compounds as Ca\(^{2+}\)/calmodulin dependent kinase kinase (CaMKK) inhibitors. The compounds are described as Formula II:

\[
\text{Formula II}
\]

wherein \( R_1 \) and \( R_2 \) are independently selected from H, halo, alkyl, or haloalkyl; and \( R_3 \) is H, alkyl, or substituted alkyl, or three \( \text{COOR}_3 \) groups can be substituted at any location on the naphthalene ring.

wherein $R_1, R_2, R_3, R_4, R_5, R_7, R_8, R_9, R_{10},$ and $R_{11}$ are each independently selected from the group consisting of $H$, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, cycloalkylalkenyl, cycloalkylalkynyl, heterocyclo, heterocycloalkyl, heterocycloalkenyl, heterocycloalkynyl, aryl, arylalkyl, arylalkenyl, arylalkynyl, heteroaryl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, alkoxy, halo, mercapto, azido, cyano, formyl, carboxylic acid, hydroxyl, nitro, acyl, aryloxy, alkylthio, amino, alkylamino, arylalkylamino, disubstituted amino, acylamino, acyloxy, ester, amide, sulfoxyl, sulfonyl, sulfonate, sulfonic acid, sulfonamide, urea, alkoxyacylamino, and aminoacyloxy; or a pharmaceutically acceptable salt or prodrug thereof.

None of these documents disclose or suggest that any of the compounds of Formula I-III would be useful in methods relating to cancer, or that CaM KKp represents a therapeutic target in the treatment of cancers.

SUMMARY OF THE INVENTION

In an aspect, the disclosure provides a method of diagnosing prostate cancer in a subject comprising: determining an amount of at least one of CaM KKp, CaM KKp splice variant 2, CaM KKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in a sample from the subject; and comparing the amount to a control sample comprising an amount of CaM KKp, CaM KKp splice variant 2, CaM KKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in a control sample; wherein the subject is diagnosed as having prostate cancer when the amount of at least one of CaM KKp, CaM KKp splice variant 2, CaM KKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject is greater than the amount in the control sample.
In an aspect the disclosure relates to a method for determining disease stage in a subject having prostate cancer, the method comprising: determining an amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in a sample from the subject; and comparing the amount to a control sample comprising an amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit; wherein the disease stage of prostate cancer is determined by the difference in the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject and the amount in the control sample.

Aspects also relate to a method for predicting the likelihood of success of hormone-based therapeutic treatment of a subject having prostate cancer, the method comprising determining an amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in a sample from the subject; and comparing the amount to a control sample comprising an amount of the CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit. Embodiments provide for a likely successful response to hormone-based therapeutic treatment when the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject is greater than the amount in the control sample.

In an aspect the disclosure relates to a method for early detection of prostate cancer in a subject comprising obtaining a sample from the subject; determining an amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject; and comparing the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit from the sample from the subject to an amount of the CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, AMPK, and AMPK subunit.
in a control sample; wherein early detection of prostate cancer is made when the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject is greater than the amount in the control sample.

[0016] In another aspect the disclosure provides a method for identifying a selective inhibitor of CaMKKp where the method includes contacting CaMKKp and a substrate therefor, in the presence and in the absence of the test compound, under conditions such that CaMKKP-dependent phosphorylation of the substrate can be effected, and determining the level of phosphorylation of the substrate resulting from the contacting, and comparing the amount of phosphorylated substrate with a level of phosphorylation of the substrate in the absence of the test compound, wherein an decrease in phosphorylation of the substrate in the presence of the test compound indicates that the test compound is a selective inhibitor of CaMKKp.

[0017] In an aspect the disclosure provides a method of screening a test compound for anti-cancer activity comprising: contacting CaMKKp and a substrate therefor in the presence of the test compound, under conditions that allow for CaMKKP-dependent phosphorylation of the substrate; determining the level of phosphorylation of the substrate resulting from the contacting; and comparing that level with a level of phosphorylation of the substrate obtained in the absence of the test compound, wherein a reduction in the level of phosphorylation of the substrate in the presence of the test compound indicates that the test compound has anti-cancer activity.

[0018] In an aspect the disclosure provides a method of treating cancer in a subject, comprising administering to the subject an effective amount of a compound that inhibits activity of a CaMK biological cascade in the subject.

[0019] In an aspect the disclosure provides a method of treating cancer in a subject, comprising administering to the subject an effective amount of a compound that inhibits activity of at least one of CaMKK or AMPK.

[0020] In an aspect the disclosure provides a method of treating cancer in a subject, comprising administering to the subject an effective amount of a compound that inhibits activity of at least one of CaMKKp, CaMKKp splice variant 2, or CaMKKp splice variant 7.

[0021] In a further aspect the disclosure provides a method of treating prostate cancer in a subject, comprising administering to the subject an effective amount of a compound that inhibits activity of a CaMK biological cascade in the subject.
In a further aspect the disclosure provides a method of treating prostate cancer in a subject, comprising administering to the subject an effective amount of a compound that inhibits activity of at least one of CaMKK or AMPK.

In a further aspect the disclosure provides a method of treating prostate cancer in a subject, comprising administering to the subject an effective amount of a compound that inhibits activity of at least one of CaMKKp, CaMKKp splice variant 2, or CaMKKp splice variant 7.

In yet another aspect, the disclosure relates to a method of treating prostate cancer in a subject, the method comprising administering to the subject an effective amount of an inhibitor of phosphorylated AMPK, phosphorylated AMPKal subunit, or phosphorylated AMPKa2 subunit.

In another aspect, the disclosure provides a method of determining the efficacy of therapy in a patient being treated for prostate cancer, the method comprising: determining an amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in a series of samples from the subject, where the samples are taken from the subject at different time points during the therapy; and comparing the determined amount over the course of the time points; wherein when the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 in the series of samples is about the same or increases over the course of the time points, the therapy is not effective.

In an aspect the disclosure provides a method of inhibiting androgen-mediated migration of a prostate cancer cell in a subject comprising administering to the subject an effective amount of a compound that inhibits activity of a CaMK biological cascade in the subject.

In an aspect the disclosure provides a method of inhibiting androgen-mediated migration of a prostate cancer cell in a subject comprising administering to the subject an effective amount of a compound that inhibits activity of at least one of CaMKK or AMPK.

In another aspect the disclosure provides a method of inhibiting androgen-mediated migration of a prostate cancer cell in a subject comprising administering to the subject an effective amount of an inhibitor of at least one of CaMKKp, CaMKKp splice variant 7, or CaMKKKp splice variant 2 or any combination thereof.
In an aspect the disclosure provides a method of inhibiting androgen-mediated invasion of a prostate cancer cell in a subject comprising administering to the subject an effective amount of a compound that inhibits activity of a CaMK biological cascade in the subject.

In an aspect the disclosure provides a method of inhibiting androgen-mediated invasion of a prostate cancer cell in a subject comprising administering to the subject an effective amount of a compound that inhibits activity of at least one of CaMKK or AMPK.

In an aspect the disclosure provides a method of inhibiting androgen-mediated invasion of a prostate cancer cell in a subject comprising administering to the subject an effective amount of an inhibitor of at least one of CaMKKp, CaMKKp splice variant 7, or CaMKKp splice variant 2 or any combination thereof.

In an aspect the disclosure provides a method of inhibiting metastasis of prostate cancer in a subject comprising administering to the subject an effective amount of a compound that inhibits activity of a CaMK biological cascade in the subject.

In an aspect the disclosure provides a method of inhibiting metastasis of prostate cancer in a subject comprising administering to the subject an effective amount of a compound that inhibits activity of at least one of CaMKK or AMPK.

In an aspect the disclosure provides a method of inhibiting metastasis of prostate cancer in a subject comprising administering to the subject an effective amount of an inhibitor of at least one of CaMKKp, CaMKKp splice variant 7, or CaMKKp splice variant 2 or any combination thereof.

Aspects also relate to a nucleic acid molecule comprising a sequence that binds under stringent conditions to a region that is about 2.3 kb upstream (5') relative to a CaMKKp transcriptional start site.

Further aspects relate to an antibody that specifically binds to a C-terminal portion of a CaMKKb.

Aspects also relate to polynucleotides (e.g., siRNA) that comprise a sequence that is complementary to CaMKKp, CaMKKa, or AMPK and having kinase-inhibitory activity.

The disclosure provides for and encompasses additional aspects and embodiments, which will be apparent to those of skill in the art in light of the following description.

BRIEF DESCRIPTION OF THE DRAWINGS
Figure 1. Androgens increase CaMKKp levels in an AR-dependent manner. LNCaP or VCaP cells were treated for 24 h with vehicle or increasing concentrations of the synthetic androgen R1881 (A-0.1, 1, and 10 nM; B-0.01, 0.1, 1, and 10 nM). A, after treatment, cells were lysed, and RNA was isolated and reversed transcribed. The expression of CaMKKp was assessed using qPCR. B, after treatment, cells were subjected to western blot analysis and subsequent densitometry (top). CaMKKp protein levels were normalized to GAPDH loading control. A and B, results are expressed as fold induction over vehicle-treated cells + SE (n = 3). *, significant changes from vehicle-treated cells. C, LNCaP cells were transiently transfected with mock or Stealth siRNAs targeting a negative control (siLacZ) or CaMKKp (#1-3). Two days later, cells were treated for 24 h +/- 10 nM R1881. Whole-cell extracts were subjected to western blot analysis and densitometry (top) as described in B. *, significant changes from mock-transfected cells. D, LNCaP cells were transfected as described in C with mock or Stealth siRNAs targeting LacZ or AR and treated for 24 h. The expression of CaMKKp was assessed as in A using qPCR.

Figure 2. Validation of CaMKKp protein bands. For CaMKKp western blot analysis, a different monoclonal CaMKKp antibody (clone 1A11) was used than in Fig. 1. CaMKKp protein levels were normalized to GAPDH loading control. Results are expressed as fold induction over vehicle-treated cells + SE (n = 2). *, P < 0.05 indicates significant changes from mock-transfected cells.

Figure 3. CaMKKp levels are increased in prostate cancer samples. Independent microarrays were analyzed using the Oncomine resource. A, four separate studies determined that CaMKKp levels were elevated in prostate cancer samples (red) compared to normal prostate controls (blue). B, CaMKKp levels correlated with disease progression. C, CaMKKp levels are significantly higher in prostate cancer samples compared to other cancers (a-bladder, b-kidney, c-colon, d-breast, e-esophageal, f-liver, g-lung, h-ovarian, i-pancreatic, j-squamous cell lung). All changes in expression were at the P < 0.001 level.

Figure 4. The prostate expresses a different functional splice variant of CaMKKβ compared to brain. A, schematic of CaMKKp splice variants. B, RT-PCR using primers spanning specific exons (indicated in right schematic) was performed on cDNA generated from various tissues and cell lines. C, LNCaP or VCaP cells were treated for 24 h +/- 10 nM R1881. Cell lysates were then subjected to western blot analysis and subsequent densitometry (right).
Phospho-CaMKI (p-CaMKI) protein levels were normalized to total CaMKI. Results are expressed as fold CaMKI phosphorylation over vehicle-treated cells + SE (n = 3). *, significant changes from vehicle-treated cells.

[0043] **Figure 5.** The prostate expresses different splice variants of CaMKKp, compared to brain (expanded Fig. 4A and Fig. 4B). A, schematic of CaMKKp splice variants. B, RT-PCR using primers spanning specific exon-exon boundaries (indicated in right schematic) was performed on cDNA generated from various tissues and cell lines.

[0044] **Figure 6.** CaMKKp activity in androgen-mediated cell migration in prostate cancer cells. A, LNCaP cells were pretreated for 1 h with vehicle, 10 or 30 mM STO-609 prior to overnight treatment with vehicle, 100 pM or 10 nM R1881. Cell lysates were then subjected to western blot analysis and subsequent densitometry (right). Phospho-CaMKI (p-CaMKI) levels were normalized to total CaMKI. Results are expressed as fold induction/phosphorylation over double vehicle-treated cells + SE (n = 2). *, P < 0.05 indicates significant changes from vehicle-treated cells. #, P < 0.05 indicates significant changes from vehicle (no STO-609)-treated cells. B, VCaP cells were plated in 96-well plates and grown for 3 d. Cells were treated +/- 1 nM R1881 and +/- 30 mM STO-609 on d 3, d 5, and d 7. On d 10, cells were lysed and the relative number of cells was measured with the fluorescent DNA binding dye FluoReporter Blue. Each sample was performed in triplicate, and results from a representative experiment are shown. Results are expressed as relative cell number ± SE (n = 2). *, P < 0.05 indicates significant changes from vehicle (no R1881)-treated cells. C, VCaP cells were pretreated for 1 h +/- 30 mM STO-609 prior to overnight treatment +/- 10 nM R1881. Cells were then dissociated and reseeded into the top chamber for a Boyden dual chamber migration assay. Fresh medium with the corresponding treatments was added to the top and bottom chambers while either no chemoattractant or 5% FBS (serum) was added to the bottom chamber. After 16 h, migrated cells were fixed, stained with crystal violet and counted in three different microscopic fields and added together. The results are expressed as mean ± SE (n = 2). *, P < 0.05 indicates significant changes from vehicle (no R1881)-treated cells. D and E, densitometry results for western blots in Fig. 7C and Fig. 7D respectively. *, P < 0.05 indicates significant changes from vehicle-treated (D) or GAL4 control (E) cells. #, P < 0.05 indicates significant changes from control (siLacZ)-transfected cells (D).

[0045] **Figure 7.** CaMKKp activity in the androgen-mediated migration and invasion of prostate cancer cells. A, LNCaP cells were plated in 96-well plates and grown for 3 d. Cells were
treated +/- 1 nM R1881 and +/- 30 mM STO-609 on d 3, d 5, and d 7. On d 10, cells were lysed and the relative number of cells was measured with the fluorescent DNA binding dye FluoReporter Blue. Each sample was performed in triplicate, and results from a representative experiment are shown. Results are expressed as relative cell number ± SE (n = 2). *, significant changes from vehicle (no R1881)-treated cells. B, LNCaP cells were pretreated for 1 h +/- 30 mM STO-609 prior to overnight treatment +/- 10 nM R1881. Cells were then dissociated and reseeded into the top chamber for a Boyden migration or Matrigel extracellular matrix invasion assay. Fresh medium with the corresponding treatments was added to the top and bottom chambers while either no chemoattractant or 5% FBS (serum) was added to the bottom chamber. After 16 h, migrated cells were fixed, stained and counted in three different microscopic fields and added together. The results are expressed as mean ± SE (n = 3). *, significant changes from vehicle (no R1881)-treated cells. †, significant changes from vehicle (no STO-609)-treated cells. C top, LNCaP cells were transfected with indicated siRNAs. Two days after transfection, cells were treated +/- 10 nM R1881 and subjected to a Boyden migration assay as described in B. *, significant changes from vehicle-treated cells. †, significant changes from control (siLacZ)-transfected cells. C bottom, western blot to demonstrate CaMKKp knockdown. Quantification of these blots is presented in Fig. 6D. D right, LNCaP cells stably expressing either GAL4 (control) or CaMKKp were subjected to a migration assay as described in B using +/- 5% FBS as chemoattractant. The results are expressed as mean ± SE (n = 3). *, significant changes from LNCaP-GAL4 cells. D left, western blot confirming CaMKKp expression. Quantification of these blots is presented in Fig. 6E.

[0046] Figure 8. Androgen mediated prostate cancer cell migration and functional AR-mediated transcription. A and B, an example of the AR replacement strategy is shown. This method has the advantage of using cells with endogenous androgen signaling as opposed to the common reintroducing of AR into AR-negative cells, which often has artificial biological consequences. Here, cells that express endogenous AR, in this case LNCaPs, were retrovirally infected to create stable cell lines expressing a control (GAL4) or a v5-tagged version of AR (wild type or a DNA-binding domain mutant (C562S)) linked to an IRES-EGFP. Cells were then selected using 2 rounds of flow cytometry. Subsequently, EGFP-positive cells were transfected with chemical siRNAs targeting either a control sequence (siLacZ) or the 3'-UTR of AR (eliminates endogenous receptor). A, a western blot characterization of the resultant cell lines is
shown at the right using antibodies for v5 (recognizes only exogenous AR), AR (recognizes both exogenous and endogenous AR) or GAPDH (loading control). B, LNCaP cells used in the AR replacement experiments were also subjected to qPCR analysis using primers targeting the AR 3'UTR (monitors endogenous AR levels). The expression of AR was normalized to 36B4 levels and results are expressed as relative mRNA levels of AR compared to mock-transfected cells + SE (n = 2). *, P < 0.05 indicates significant changes from mock-transfected cells. C, cells were then subjected to a migration assay as described in Fig. 9. *, P < 0.05 indicates significant changes from vehicle-treated cells.

**Figure 9.** Identification of the ARE that regulates ΩαMKβ expression. A, LNCaP cells were pretreated for 1 h with vehicle or 1 mg/ml cycloheximide followed by vehicle or 10 nM R1881 for 24 h. CaMKKp or CXCR4 mRNA levels were quantified using qPCR. Results are expressed as fold induction over vehicle (no R1881)-treated cells ± SE (n = 3). *, significant changes from vehicle-treated cells. B, LNCaP cells were treated with vehicle (V) or 10 nM R1881 for 1 or 4 h. Cross-linked chromatin was immunoprecipitated with indicated antibodies. The precipitated DNA was amplified using primers spanning a region identified using ChIP on Chip data as a potential AR-binding site (indicated in top schematic) or a distal upstream region (negative control). The results are presented as percent input ± SE (n = 3). *, significant changes from IgG controls. C, various enhancer luciferase reporter constructs (depicted in top model) were transfected into LNCaP cells and treated overnight +/- 10 nM R1881. After treatment, cells were harvested and assayed for luciferase activity. Luciferase values were normalized to β-galactosidase control. Data are the mean relative light units (RLUs) + SEM for one representative experiment performed in triplicate (n = 3). *, significant changes from vehicle-treated cells. D, ΩαMKβ promoter constructs (depicted in top model) were transfected into LNCaP cells and then treated overnight with vehicle or 10 nM R1881. After treatment, cells were harvested and assayed for luciferase activity as in C. Emp Vec, empty vector.

**Figure 10.** Identification of the ARE that regulates ΩαMKβ expression. A, two ΩαMKβ enhancer (fragments D and E from Fig. 9C) luciferase reporter constructs were transfected into LNCaP cells and then pretreated for 30 minutes with vehicle or 10 mM Casodex followed by treatment overnight with vehicle or various concentrations of R1881 (0, 0.1, 1 and 10 nM). After treatment, cells were harvested and assayed for luciferase activity. Luciferase values were normalized to β-galactosidase control. Data are the mean relative light units (RLUs)
+ SEM for one representative experiment performed in triplicate (n = 3). *, P < 0.05 indicates significant changes from vehicle (no R1881)-treated cells. * P < 0.05 indicates significant changes from vehicle (no Casodex)-treated cells. B, VCaP cells were transfected, treated and assayed for luciferase activity as in A using the PSA enhancer and αMKKβ enhancer fragments D and E. C, αMKKβ enhancer deletion constructs were transfected into LNCaP cells and then treated and assayed for luciferase activity as in A (n = 2). Emp Vec, empty vector.

[0049] Figure 11. Androgen-mediated migration occurs through a CaMKKP-AMPK-dependent pathway. A, LNCaP cells were pretreated for 1 h +/- 30 mM STO-609 prior to overnight treatment +/- 10 nM R1881. Cell lysates were then subjected to western blot analysis and subsequent densitometry (right). CaMKP levels were normalized to GAPDH. Phospho-CaMKI (p-CaMKI) levels were normalized to total CaMKI. Phospho-AMPK (p-AMPK) levels were normalized to total AMPK. Results are expressed as fold induction/phosphorylation over double vehicle-treated cells + SE (n = 3). *, significant changes from vehicle-treated cells. B, LNCaP cells stably expressing either GAL4 or CaMKKP, were treated overnight +/- 10 nM R1881. Cell lysates were then subjected as in A to western blot analysis and densitometry (right). Results are expressed as fold induction/phosphorylation over LNCaP-GAL4 vehicle-treated cells + SE (n = 3). *, significant changes from LNCaP-GAL4 vehicle-treated cells. C and D, LNCaP cells were transfected with indicated siRNAs, treated and subjected to a migration assay (top) or western blot analysis (bottom) as in Fig. 7C. *, significant changes from control (siLacZ)-transfected cells. Quantification of the blots is presented in Fig. 12.

[0050] Figure 12. Androgen-mediated migration occurs through a CaMKKP-AMPK-dependent pathway. A, VCaP cells were treated for 24 h +/- 10 nM R1881. Cell lysates were then subjected to western blot analysis and subsequent densitometry (right). Phospho-CaMKI (p-CaMKI) levels were normalized to total CaMKI. Phospho-AMPK (p-AMPK) levels were normalized to total AMPK. Results are expressed as fold induction/phosphorylation over vehicle-treated cells + SE (n = 2). *, P < 0.05 indicates significant changes from vehicle-treated cells. B, selection of optimal AMPKα1 and α2 siRNAs. LNCaP cells were transfected as described in Fig. 11 with mock or Stealth siRNAs targeting LacZ (negative control) or AMPKα1 or α2. The expression of AMPK was assessed using qPCR and normalized to 36B4 levels. Results are expressed as fold induction over mock-transfected cells + SE (n = 2). *, P < 0.05 indicates significant changes from mock-transfected cells. C and D, densitometry results for
western blots in Fig. 11C and Fig. 11D, respectively. For AMPKa knockdown (C), siAMPKal-
# 1 and siAMPKa2-#l from B were selected since they produced the greatest knockdowns. *: P < 0.05 indicates significant changes from control (siLacZ)-transfected cells.

**Figure 13.** AMPK and androgen-mediated prostate cancer cell migration. A, LNCaP cells were pretreated for 1 h with vehicle or increasing concentrations of compound C (10 or 40 mM) prior to overnight treatment +/- 10 nM R1881 or 1 mM AICAR. Cells were then subjected to a migration assay as described in Fig. 7. The results are expressed as mean ± SE (n = 2). *: P < 0.05 indicates significant changes from double vehicle-treated cells. #: P < 0.05 indicates significant decreases from vehicle (no compound C)-treated cells. B, LNCaP cells were pretreated for 1 h with vehicle, 1, 10 or 40 mM compound C prior to overnight treatment +/- 10 nM R1881. Cell lysates were then subjected to western blot analysis and subsequent densitometry (top). ACC is a direct target of AMPK and thus, was used as a readout of AMPK catalytic activity. Phospho-ACC (p-ACC) levels were normalized to total ACC. Results are expressed as fold induction/phosphorylation over double vehicle-treated cells + SE (n = 2). *: P < 0.05 indicates significant changes from double vehicle-treated cells. C, LNCaP cells were treated overnight +/- 1 mM AICAR and then subjected to western blot analysis and densitometry (top) as in B. Phospho-AMPK (p-AMPK) levels were normalized to total AMPK. *: P < 0.05 indicates significant changes from vehicle-treated cells.

**DETAILED DESCRIPTION**

The inventors have identified Ca^{2+}/calmodulin-dependent protein kinase kinases (CaM KKs), such as CaM KKp, as viable targets for therapeutic intervention in various cancers such as, for example, prostate cancer, glioblastoma, and myeloid leukemia as well as other cancer types described herein. In a general sense, the disclosure provides an array of compounds and compositions that are active inhibitors of CaM KK and use of such compounds in methods relating to detection, determination of disease stage/progression, prognostic evaluation of hormone therapy, treatment of disease, identification of active agents against various cancers, as well as identification of CaM KK inhibitors, including inhibitors that are selective for a particular CaM KK. For purposes of illustration some particular aspects and embodiments are explicitly described herein, relating to Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaM KKp) which is shown (a) to be expressed in the prostate, (b) to be regulated by AR, (c) to correspond
to prostate cancer progression/disease stage and, accordingly, provides a therapeutic target for prostate cancer.

[0053] As used herein, the term "Ca²⁺/calmodulin-dependent protein kinase kinase" and/or "CaMKK" are used interchangeably herein and refer to a serine/threonine protein kinase that can phosphorylate and activate members of the Ca²⁺/calmodulin-dependent protein kinase (CaMK) family of enzymes as well as other protein substrates such as AMPK (e.g., SEQ ID NOs: 21-24). The terms encompass all of the various isoforms, orthologs, and splice variants of CaMKK proteins such as, for example, Ca²⁺/calmodulin-dependent protein kinase kinase β (CaMKKp, or CaMKK2) Ca²⁺/calmodulin-dependent protein kinase kinase α (CaMKKa, or CaMKKI), splice variants such as, for example, CaMKKp splice variants 1-7, CaMKKa splice variants 1-3, and the like (e.g., SEQ ID NOs: 1-20 and 25-46). Some embodiments relate to CaMKKp that comprises the amino acid sequence of SEQ ID NO:2, or a fragment thereof. The CaMKK amino acid sequences, such as CaMKKp, can be encoded by any appropriate polynucleotide molecule as determined by the genetic code and codon usage in any particular organism. In embodiments, CaMKKp is encoded by a polynucleotide comprising SEQ ID NO:1, or a fragment thereof. Some embodiments relate to CaMKKa that comprises the amino acid sequence of SEQ ID NO:16, or a fragment thereof. In some embodiments, CaMKKa like CaMKKp above, is encoded by any polynucleotide that can be envisioned by one of skill in the art and, in some embodiments, comprises SEQ ID NO:15, or a fragment thereof.

[0054] In some embodiments the disclosure relates to a CaMKK splice variant. Non-limiting examples of CaMKK splice variants include nucleotide sequences of SEQ ID NOs: 3, 5, 7, 9, 11, 13, 17, and 19. Some embodiments relate to "CaMKKp splice variant 2" and comprise a nucleotide sequence of SEQ ID NO:3, or a fragment thereof. Some embodiments relate to "CaMKKp splice variant 7" and comprise a nucleotide sequence of SEQ ID NO:13. In these embodiments, the splice variant proteins encoded by SEQ ID NO:3 and SEQ ID NO:13 are identical in sequence. Thus, embodiments of disclosure provide for a polynucleotide that encodes a CaMKKp splice variant protein comprising SEQ ID NO:4, or a fragment thereof. Similarly, the disclosure relates to polynucleotide sequences that encodes an amino acid sequence of any CaMKK or CaMK protein such as, for example those of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20. As noted above, CaMKKp splice variant 2 and splice variant 7 encode
for the same amino acid sequence; thus, in embodiments relating to a CaMKKp amino acid sequence encoded by splice variant 2 or splice variant 7, reference to an amino acid sequence encoded by either splice variant will also encompass the other (i.e., each term is interchangeable with and inclusive of the other when it relates to the encoded amino acid sequence).

[0055] Any sample can be used in the methods described herein. Embodiments provide for the use of a biological sample (e.g., tissue biopsy, cerebrospinal fluid, blood, sera, sputum, urine and/or tumor biopsies) from a subject with and/or without a cancer (which can be determined using standard clinical tests).

[0056] Embodiments of the disclosure relate to compounds that are inhibitors of a CaMK biochemical cascade. A CaMK biochemical cascade refers to a biochemical activation pathway that typically involves the phosphorylation of a first Ca^{2+}/calmodulin-dependent protein kinase (CaMK) by a second Ca^{2+}/calmodulin-dependent protein kinase (thus, a Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK)). The phosphorylated CaMK can subsequently phosphorylate a substrate. CaMK cascades are described in the literature. See, Corcoran, E.E., and Means, A.R., J Biol Chem, (Feb, 2, 2001); 276(5):2975-2978, incorporated herein by reference.

Methods of Treatment

[0057] In an aspect, the disclosure provides a method for treating cancer in a subject in need thereof comprising administering to the subject an effective amount of a Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) inhibitor. In embodiments, the method comprises administering an effective amount of a CaMKK inhibitor that is a selective inhibitor of CaMKKa and/or CaMKKp. In some embodiments the CaMKK inhibitor is a selective inhibitor of CaMKKa. In some embodiments the CaMKK inhibitor is a selective inhibitor of CaMKKp. The term "selective inhibitor," including "selective inhibitor of CaMKKp/a" or "CaMKKp/a selective inhibitor" relates to a compound (e.g., a small molecule or biological molecule) that has increased inhibitory activity for a target, for example, CaMKKp or CaMKKa, relative to the inhibitory activity for other CaMKs. For purposes of illustration, when describing embodiments comprising a selective inhibitor of CaMKKp, examples of "other" CaMKs include natural/physiological substrates of CaMKKp such as, for example, Ca^{2+}/calmodulin-dependent protein kinases (e.g., CaMKI and CaMKIV), CaMKs that are not substrates of CaMKKp (e.g., CaMKII and CaMKIII), AMP-activated protein kinase (e.g., AMPKal subunit and AMPKa2.
subunit) as well as other kinases that can phosphorylate such substrates (CaMKKa). Embodiments also relate to polypeptide fragments comprising a sequence that contains a portion of a CaMKKp substrate. In such embodiments, the fragment comprises an amino acid that can be phosphorylated. In some embodiments a selective inhibitor comprises a ratio of IC\textsubscript{50} concentrations (concentration inhibiting 50% of activity) wherein the ratio of the IC\textsubscript{50} concentration for one or more other CaMKs to the IC\textsubscript{50} concentration for CaMKKp is greater than 1. The ratio of IC\textsubscript{50} values can be readily determined from data obtained from one or more assay(s) (performed separately, in parallel or series) that is effective to measure activity or abundance of a CaMK or CaMKK (e.g., phosphorylation, mRNA transcription, protein expression, etc.), and can comprise any methods known in the art such as, for example those disclosed in U.S. Patent 7,105,312, which is incorporated herein by reference. The inhibitory activity can be assessed and demonstrated either in vivo and/or in vitro optionally in cell-based or cell-free assay systems.

[0058] In general, the CaMKK inhibitor, including a CaMKK selective inhibitor, can be any type of chemical or biological molecule that exhibits inhibitory activity against one or more CaMKK. Effective CaMKK inhibitors for use in the methods described herein can inhibit the kinase activity of a CaMKK or they can regulate the amount of a CaMKK in a cell. Accordingly, the CaMKK inhibitors can inhibit phosphorylation associated with a CaMK cascade, and/or regulate expression of a CaMKK (e.g., by inhibiting a CaMKK gene promoter, inhibiting CaMKK gene transcription, inhibiting CaMKK mRNA translation, and/or affect CaMKK mRNA stability).

[0059] In some embodiments of this aspect, the method includes at least one selective inhibitor of CaMKKp that comprises a compound of Formula III:

![Formula III](image-url)
wherein $R_1$, $R_2$, $R_3$, $R_4$, $R_5$, $R_7$, $R_8$, $R_9$, $R_{10}$, and $R_{11}$ are each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, cycloalkylalkenyl, cycloalkylalkynyl, heterocyclo, heterocycloalkyl, heterocycloalkenyl, heterocycloalkynyl, aryl, arylalkyl, arylalkenyl, arylalkynyl, heteroaryl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, alkoxy, halo, mercapto, azido, cyano, formyl, carboxylic acid, hydroxyl, nitro, acyl, aryloxy, alkylthio, amino, alkylamino, arylalkylamino, disubstituted amino, acylamino, acyloxy, ester, amide, sulfoxyl, sulfonate, sulfonic acid, sulfonamide, urea, alkoxyacylamino, aminoacyloxy, and, for the groups $R_7$ and $R_{7a}$, can optionally be taken together to form oxo; or a pharmaceutically acceptable salt or prodrug thereof. Such compounds are disclosed in U.S. patent application publication US 2010/0105716, incorporated herein by reference.

[0060] In further embodiments $R_7$ and $R_{7a}$ together form oxo ($C=O$).

[0061] In some embodiments, $R_7$ and $R_{7a}$ do not form oxo ($C=O$).

[0062] In some embodiments, $R_3$ is -COOH, -CH$_2$COOH, -CH$_2$CH$_2$COOH, or an ester thereof.

[0063] In some embodiments, $R_i$, $R_2$, $R_4$, $R_5$, $R_7$, $R_7a$, $R_8$, $R_9$, $R_{10}$, $R_{11}$ are all H.

[0064] In some embodiments, at least one, two, or three of $R_i$, $R_2$, $R_4$, $R_5$, $R_7$, $R_7a$, $R_8$, $R_9$, $R_{10}$, and $R_{11}$ is not H. Thus, in some embodiments, $R_i$ is not H; in some embodiments $R_2$ is not H; in some embodiments $R_3$ is not H; in some embodiments $R_4$ is not H; in some embodiments $R_5$ is not H; in some embodiments $R_6$ is not H; in some embodiments $R_7$ is not H; in some embodiments $R_9$ is not H; in some embodiments $R_{10}$ is not H; and/or in some embodiments $R_n$ is not H.

[0065] Compounds of Formula III, including further definitions of substituent terms and various formulations thereof are disclosed in U.S. Patent Application Publication No: 2010/0105716 as useful in methods of treating metabolic diseases/disorders including obesity, insulin resistance, hyperglycemia, diabetes, and the like. Synthetic routes and strategies for the compounds of Formula III are known in the art. The disclosure of US 2010/0105716 is incorporated herein by reference.

[0066] Embodiments of this aspect relate to a method comprising a selective inhibitor of CaMKKp of Formula I:

---

18
wherein $R_i$ is selected from CN, COOH, or COCl. Compounds of Formula I are described in U.S. Patent 2,820,037 for use as dye molecules, and is incorporated herein by reference.

Other embodiments of this aspect relate to a method comprising a selective inhibitor of CaMKKp of Formula II:

wherein $R_i$ and $R_2$ are independently selected from H, halo, alkyl, or haloalkyl; and $R_3$ is H, alkyl, or substituted alkyl, or three COOR$_3$ groups can be substituted at any location on the naphthalene ring. Compounds of Formula II are disclosed in Japanese Patent Application No. 2003-012516 as $\text{Ca}^{2+}$/calmodulin dependent kinase kinase (CaMKK) inhibitors; however the reference fails to disclose the use of these compounds as effective in methods relating to cancer. The disclosure of Japanese Patent Application No. 2003-012516 is incorporated herein by reference.

In other embodiments, the CaMKK inhibitor is a biological molecule, such as a polynucleotide having RNAi activity against a CaMKK or a substrate thereof, or an antibody that can specifically bind to a CaMKK or a substrate thereof.

Nucleic acids/RNAi

Embodiments of the disclosure relate to methods that include CaMKK inhibitors, wherein the inhibitors comprise nucleic acid molecules having inhibitory activity against one or more biological molecules involved in a CaMK cascade including CaMK enzymes such as, for example, CaMKI and/or CaMKIV as well as kinases for such molecules (CaMKKa, CaMKKp, etc.), other biological substrates of CaMKKs (e.g., AMPK), as well as other CaMKs (e.g., CaMKII and CaMKIII). In embodiments, the nucleic acid molecules can include decoy RNAs, dsRNAs, siRNAs, nucleic acid aptamers, antisense nucleic acid molecules, and enzymatic nucleic acid molecules that comprise a sequence that is sufficient allow for binding to a CaMK, AMPK, or CaMKK encoding nucleic acid sequence and inhibit activity thereof (i.e., are complementary to such encoding nucleic acid sequences).

In embodiments, the inhibitory nucleic acid molecule can bind to a target CaMK, AMPK, or CaMKK nucleic acid sequence under stringent binding conditions. The terms "stringent conditions" "stringent binding conditions" or "stringent hybridization conditions" refers to conditions under which a polynucleotide will hybridize to a target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). An example of stringent conditions include those in which hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1x SSC at 60°C to 65°C is performed. Amino acid and polynucleotide identity, homology and/or similarity can be determined using the ClustalW algorithm, MEGALIGN™ (Lasergene, Wis.).

Given a target polynucleotide sequence of a CaMK, CaMKK, or biological substrate thereof, an inhibitory nucleic acid molecule can be designed using motifs and targeted to a region that is anticipated to be effective for inhibitory activity, such as is known in the art.

Antibodies

Embodiments of the disclosure relate to methods that include CaMKK inhibitors, wherein the inhibitors comprise antibodies having specific binding activity against one or more biological molecules involved in a CaMK cascade including CaMK enzymes such as, for example, CaMKI and/or CaMKIV as well as kinases for such molecules (CaMKKa, CaMKKp,
etc.), biological substrates of CaMKKs (e.g., AMPK), and CaMKs that are not substrates of CaMKKs (e.g., CaMKII and CaMKIII).

[0076] Preparation of Antibodies

[0077] The antibodies described herein can be produced by any method known in the art, such as by immunization with a full-length CaMK or CaMKK, or fragments thereof. The antibodies can be polyclonal or monoclonal, and/or may be recombinant antibodies. In embodiments, antibodies that are human antibodies can be prepared, for example, by immunization of transgenic animals capable of producing a human antibody (see, for example, International Patent Application, Publication WO 93/12227).

[0078] Monoclonal antibodies (mAbs) can be produced by a variety of techniques, including conventional monoclonal antibody methodology, e.g., the standard somatic cell hybridization technique of Kohler and Milstein [Nature (1975); 256:495], and other techniques, e.g., viral or oncogenic transformation of B-lymphocytes.

[0079] Animal systems for preparing hybridomas include mouse. Hybridoma production in the mouse is very well established, and immunization protocols and techniques for isolation of immunized splenocytes for fusion are well known in the art. Fusion partners (e.g., murine myeloma cells) and fusion procedures are also known.

5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay, as well as U.S. Pat. No. 5,545,807
the disclosures of all of which are hereby incorporated by reference in their entirety.

[0081] Embodiments provide human monoclonal antibodies that are specific for and neutralize
biological activity of human CaMK and/or CaMKK polypeptides. Such antibodies can comprise
heavy and light chain amino acid sequences, the light and heavy chain variable regions, and any
combination (including all) hypervariable CDR regions, which are specific for and neutralize
CaMK and/or CaMKK polypeptides when they bind. Such antibodies can provide an effective
immunotherapy for CaMK and CaMKK associated diseases including various cancers, such
prostate cancer, glioma, glioblastoma, and myeloid leukemia. Such antibodies also provide a
useful reagent for the detection of a CaMK or a CaMKK in a biological sample.

[0082] In an embodiment, the antibodies target an epitope in a region of CaMK and/or
CaMKK located in the C-terminal portion. In some embodiments, the antibody recognizes and
binds specifically to an epitope in the C-terminal region of CaMKKp splice variants 2 and/or 7.

[0083] In some embodiments, the antibodies are of the IgGl, IgG2, IgG3, or IgG4 isotype. In
other embodiments, the antibodies of the invention are of the IgM, IgA, IgE, or IgD isotype. In
certain embodiments, the antibodies are cloned for expression in mammalian cells. In
embodiments, the antibodies can be a fragment of an antibody that retains specific binding
activity for a CaMK or CaMKK polypeptide and is effective to inhibit biological activity. Such
fragments are known in the art and include, for example, single-chain antibodies (scFV), Fab,
Fab’, Fab₂, and the like.

[0084] Any of the CaMKK inhibitors disclosed herein and which are useful in the methods
described herein can be provided as salts such as, for example, basic or acidic addition salts. The
selection and formation of such salts are within the ability of one skilled in the art. See, e.g.,
Remington: The Science and Practice of Pharmacy, 21st ed., Lippincott Williams & Wilkins, A

[0085] Further, embodiments of the disclosure provide for compositions or formulations
comprising any of the CaMKK inhibitors disclosed herein that can are suitable for
pharmaceutical use. Further, such formulations can be provided in suitable dosage forms. Such
compositions, formulations, and dosage forms are known to those of skill in the art. For
example, compounds of Formulas I-III can be provided as a composition or formulation and prepared in a dosage form as described U.S. patent application publication number US 2010/0105716, which is incorporated by reference herein. See, also, Remington: The Science and Practice of Pharmacy, 21st ed., Lippincott Williams & Wilkins, A Wolters Kluwer Company, Philadelphia, Pa (2005).

[0086] In an aspect, the disclosure provides a method for screening or identifying a compound having agonist or antagonist activity for CaMKK (including CaMKKp and/or CaMKKa) that includes contacting CaMKK and a substrate therefor, in the presence and absence of a test compound, under conditions that allow for CaMKK-dependent phosphorylation of the substrate; and determining, directly or indirectly, the level of phosphorylation of the substrate, wherein a reduction in phosphorylation of the substrate in the presence of the test compound is indicative of a CaMKK antagonist (for example, an anticancer agent) and an increase in phosphorylation of the substrate in the presence of the test compound is indicative of a CaMKK agonist. In embodiments, the CaMKK is CaMKKp.

[0087] In some embodiments of this aspect, the method identifies a compound that is selective for a specific CaMK relative to at least one other CaMK. In further embodiments, the method identifies a compound that is selective for CaMKKp relative to at least one other CaMK such as, for example, CaMKI, CaMKII, CaMKIII, CaMKIV, or CaMKKa. Yet further embodiments of the method provide identification of a compound that is selective for CaMKKp splice variant 2 or CaMKKp splice variant 7, relative to at least one other CaMKKp isoform, and relative to at least one other CaMK such as, for example, CaMKI, CaMKII, CaMKIII, CaMKIV, or CaMKKa.

[0088] Embodiments of these methods provide compounds having selective antagonist activity for a CaMK wherein the CaMK-dependent phosphorylation of the substrate is reduced by about 4-fold or more in the presence of the compound compared to phosphorylation in the absence of the compound (e.g., about 4-fold to about 100-fold or more).

[0089] Merely for purposes of illustration of an embodiment of this aspect, an assay system can comprise calmodulin (CaM), calcium, CaMKKp, and a substrate (such as a synthetic peptide that can be phosphorylated by CaMKKp such as from either AMPK or CaMKIV). The assay can further comprise evaluation of the test compound(s) that involves AMPK as the enzyme and a peptide from acetyl-CoA-carboxylase (ACC) as the substrate. In particular, assay conditions
that allow for phosphorylation are provided (e.g., any appropriate buffer system) and further includes one or the other of CaMKKa and CaMKKp (i.e., run in parallel), a calcium salt (e.g., CaCl₂), a phosphate source (e.g., ATP, optionally comprising radiolabeled ³²P), calmodulin (CaM, e.g., from bovine), and two substrates (one that can be phosphorylated by both CaMKKa and CaMKKp, while the other can only be phosphorylated by one or the other of CaMKKa or CaMKKp). A non-limiting example of a substrate that can be phosphorylated by both CaMKKa and CaMKKp includes CaMIV, or a peptide fragment thereof (for example, Lys- Lys- Lys-Glu-His-Gln-Val-Leu-Met-Lys-Thr-Val-Cys-Gly-Thr-Pro-Gly-Tyr). A non-limiting example of a substrate that can be phosphorylated by CaMKKp and not CaMKKa includes AMPK, or a peptide fragment thereof (for example, Ala-Lys-Pro-Lys-Gly-Asn-Lys-Asp-Tyr-His-Leu-Gln-Thr-Cys-Cys-Gly-Ser-Leu-Ala-Tyr-Arg-Arg-Arg). Any substrate, including fragments thereof can be used in these methods, as long as the substrate can be phosphorylated. Differences between the amount of phosphorylation of the substrates can be used to evaluate the substrate specificity and selectivity of a candidate test compound. Concentrations of the various assay components can vary widely, but are usually in the range of 1 nM to 500 μM (for active reagents including proteins and substrates, phosphate source(s) and test compounds) and in the mM range for other assay components (calcium and magnesium salts/cofactors, reducing agents, buffer systems, etc.). Incubation time and temperature can also be varied depending on the particular activity and sensitivity of the assay components. In embodiments, the temperature can range from about 4°C to about 30°C, and the incubation time can be on the order of minutes (e.g., 10 minutes) to hours (e.g., 1 hrs, 1.5 hrs, 2 hrs, 2.5 hrs, 3 hrs, 3.5 hrs, etc.). In embodiments, a selective inhibitor will inhibit CaMKKp activity to a greater extent than it will inhibit CaMKKa activity. In some embodiments the selective inhibitor will inhibit CaMKKp activity about anywhere from about 3-100 fold or more, relative to CaMKKa activity (e.g., about 10-20 fold, about 20-30 fold, about 40-50 fold, about 50-60 fold, about 60-70 fold, about 70-80 fold, about 80-90 fold, about 90-100 fold, or over 100 fold).

[0090] Embodiments of the disclosure provide for detection of CaMK, such as CaMKKa and/or CaMKKa in circulating tumor cells (CTCs). CTCs are known in the art and comprise cells that have detached from a primary tumor and circulate in the bloodstream. It is thought that CTCs may indicate potential for metastasis and spread of a primary tumor to different tissues.

[0091] In an aspect, the disclosure also provides a method for treating conditions or diseases associated with abnormal AMP-activated protein kinase (AMPK) activity, which includes increased phosphorylation of AMPK, by administering an effective amount of at least one
compound that inhibits CaMKKp to a subject having such a condition or disease. Diseases characterized by abnormal AMPK activity include, but are not limited to, various cancers including prostate cancer.

[0092] As used herein, the term "subject" is intended to include human and non-human animals. Exemplary human subjects include a human patient having a disorder, e.g., a disorder described herein, such as cancer, or a normal subject. The term "non-human animals" includes all vertebrates, e.g., non-mammals (such as chickens, amphibians, reptiles) and mammals, such as non-human primates, domesticated and/or agriculturally useful animals (such as sheep, dogs, cats, cows, pigs, etc.), and rodents (such as mice, rats, hamsters, guinea pigs, etc.).

[0093] "Treatment" or "treat" refers to both therapeutic treatment and prophylactic or preventative measures. Those subjects in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented.

[0094] The terms "treating" and "treatment" refer to both therapeutic treatment and prophylactic or preventative measures. Those subjects in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented. When used with reference to a disease or a subject in need of treatment the terms accordingly include, but are not limited to, halting or slowing of disease progression, remission of disease, prophylaxis of symptoms, reduction in disease and/or symptom severity, or reduction in disease length as compared to an untreated subject. In embodiments, the methods of treatment can abate one or more clinical indications of the particular disease being treated. Certain embodiments relating to methods of treating a disease or condition associated with activation of a substrate in a CaMK cascade (CaMKI, CaMKIV, AMPK) and comprise administration of therapeutically effective amounts of a compound that inhibits CaMKKp, as well as pharmaceutical compositions thereof. In embodiments, the method of treating can relate to any method that prevents further progression of the disease and/or symptoms, slows or reduces the further progression of the disease and/or symptoms, or reverses the disease and/or clinical symptoms associated with expression of CaMKKp or kinase activity thereof.

[0095] In embodiments, the methods are used to treat cancer in a subject, wherein the subject is a mammal. Yet further embodiments relate to methods wherein the mammal is a human.
Aspects of the disclosure provide a method of inhibiting CaMKKp in a cell, including a cell within a subject, comprising contacting the cell with a compound in an amount effective to inhibit CaMKKp activity. In embodiments, the method provides for inhibiting CaMKKp activity in a cell in a subject, wherein the method includes administering to the subject a compound, or a pharmaceutically acceptable salt thereof, according to Formula I in an amount effective to inhibit CaMKKp activity in the cell in the subject. Both the activity of CaMKKp and AMPK can be monitored by any method familiar to those of skill in the art. In some embodiments CaMKKp and/or AMPK activity can be monitored by clinical evaluation of the symptoms or stage of a disease associated with abnormal CaMKKp and/or AMPK activity. In embodiments, the disease is cancer. In further embodiments, the cancer is glioma, glioblastoma, carcinoma, or leukemia. In some embodiments, the cancer is prostate cancer, cancer of the blood or bone marrow, or cancer of the brain/CNS.

In these embodiments, "inhibiting" or "inhibition" of CaMKKp means that there is a measurable decrease in the activity of CaMKKp in the presence of a compound (e.g., through contacting/administration), relative to the activity of CaMKKp in the absence of the compound. As described above, the decrease in CaMKKp activity can arise from direct inhibition of kinase activity by, for example, binding of a small molecule inhibitor of Formulas I-III to the active site of CaMKKp. A decrease in CaMKKp activity can also arise from inhibition of expression of a CaMKKp gene via antisense inhibition, gene silencing, disruption or degradation of CaMKKp mRNA via RNAi (e.g., siRNA). CaMKKp expression can also be modulated indirectly by manipulating the activity or expression of a regulator of CaMKKp, such as androgen receptor (AR) or proteins involved in CaMKKp splicing activity such as Fox2/RTA-1, using any agent having such activity. In embodiments, CaMKKp can be inhibited by about 10% to about 100% or more (e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, etc.) relative to a control. In some embodiments compounds can inhibit CaMKKp (e.g., IC_{50}) at concentrations from about 0.1 nM to about 500 μM, (e.g., about 0.1 nM to about 250 μM, about 0.5 nM to about 200 μM, about 1.0 nM to about 100 μM, about 10 nM to about 50 μM, or about 100 nM to about 10 μM, and the like).

In some embodiments the therapeutically effective amount is an amount sufficient to stop or slow the progression of the cancer. In further embodiments, the therapeutically effective
amount is an amount sufficient to reduce the number of cancer cells in the subject (i.e., killing of cancer cells). Methods for monitoring the proliferation of cancer cells and progress of cancer in a subject (e.g., tumor size, cell counts, biochemical markers, secondary indications, etc.) are known in the art.

[0099] In various embodiments of the method, the cancer is associated with the activity of CaMKKp. Non-limiting examples of cancer that are associated with CaMKKp activity include carcinoma, melanoma, leukemia, myeloid leukemia, glioma, and glioblastoma. In embodiments, the cancer is leukemia, cancer of the prostate or cancer of the brain/central nervous system. In further embodiments, the cancer is prostate cancer.

[0100] In some embodiments, the method of treatment is used as a co-therapy such as, for example, administration in conjunction with radiation, surgery, or other chemotherapeutics. In some embodiments, the method includes administration of a therapeutically effective amount of a compound that inhibits CaMKKp in combination with an additional anti-cancer agent. A wide variety of anti-cancer (i.e., anti-neoplastic) agents are known in the art and include, for example alkylating agents, antimetabolites, natural antineoplastic agents, hormonal antineoplastic agents, angiogenesis inhibitors, differentiating reagents, RNA inhibitors, antibodies or immunotherapeutic agents, gene therapy agents, small molecule enzymatic inhibitors, biological response modifiers, and anti-metastatic agents.

[0101] In embodiments, the method comprises treating prostate cancer in a subject who is in need of treatment, where the method includes administering to the subject an effective amount of a CaMKK inhibitor in combination with a second treatment. In such embodiments, the second treatment can include such non-limiting examples as surgery, radiation, and chemotherapy. In further embodiments, the method comprises co-administration of an effective amount of a CaMKK inhibitor and a second agent effective against prostate cancer such as, for example, anti-androgens, Selective Androgen Receptor Modulators (SARMs), Selective Androgen Receptor Degraders (SARDs), CYP17 inhibitors, suphhatase inhibitors, Src inhibitors, anti-estrogens, estrogens, Selective Estrogen Receptor Modulators (SERMs), Selective Estrogen Receptor Degraders (SERDs), ERb antagonists, aromatase inhibitors, vaccine-based therapeutics such as sipuleucel-T (Provenge®), and the like. In further embodiments the method comprises administration an effective amount of a CaMKK inhibitor and an active agent selected from MDV3100 (an androgen receptor antagonist from Medivation Inc., San Francisco, CA); ARN-
509 (an androgen receptor antagonist from Aragon Pharmaceuticals, San Diego, CA); bicalutamide (Casodex® a non-steroidal anti-androgen from AstraZeneca); or flutamide (Eulexin® a non-steroidal anti-androgen from Schering-Plough).

[00102] In some embodiments, the method of treatment can be used an adjuvant therapy (i.e., additional treatment) such as, for example, when compounds of any of Formulas I-III, or pharmaceutical compositions thereof, are administered after surgery or other treatments (e.g., radiation, hormone therapy, or chemotherapy). Accordingly, in such embodiments, the method of adjuvant therapy encompasses administering the compounds of Formula I-III to a subject following a primary or initial treatment, and can be administered either alone or in combination with one or more other adjuvant treatments, including, for example surgery, radiation therapy, or systemic therapy (e.g., chemotherapy, immunotherapy, hormone therapy, or biological response modifiers). Those of skill in the art will be able to use statistical evidence to assess the risk of disease relapse before deciding on the specific adjuvant therapy. The aim of adjuvant treatment is to improve disease-specific and overall survival. Because the treatment is essentially for a risk, rather than for provable disease, it is accepted that a proportion of patients who receive adjuvant therapy will already have been effectively treated or cured by their primary surgery. Adjuvant therapy is often given following surgery for many types of cancer including, for example, colon cancer, lung cancer, pancreatic cancer, breast cancer, prostate cancer, and some gynecological cancers.

[00103] Some embodiments of the method relate to neoadjuvant therapy, which is administered prior to a primary treatment. Effective neoadjuvant therapy is commonly characterized by a reduction in the number of cancer cells (e.g., size of the tumor) so as to facilitate more effective primary treatment such as, for example, surgery.

[00104] The term "cancer" refers to or describes the physiological condition in mammals that is typically characterized by unregulated cell growth. Some non-limiting examples of cancer include carcinoma, melanoma, lymphoma, blastoma, sarcoma, germ cell tumors, and leukemia or lymphoid malignancies. Non-limiting examples of cancers that fall within these broad categories include squamous cell cancer (e.g., epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian
cancer, liver cancer, bladder cancer, cancer of the urinary tract, hepatoma, breast cancer, colon
cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland
carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic
carcinoma, anal carcinoma, melanoma, multiple myeloma and B-cell lymphoma, brain, as well
as head and neck cancer, and associated metastases.

[00105] The term "cancer" also encompasses cell proliferative disorders which are associated
with some degree of abnormal cell proliferation, and includes tumors. "Tumor" as used herein,
refers to any neoplasm or neoplastic cell growth and proliferation, whether malignant or benign,
and all pre-cancerous and cancerous cells and tissues. In embodiments disclosed above, the
cancer can be prostate cancer, cancer of the brain/CNS (glioma, glioblastoma, etc.), or leukemia
(myeloid leukemia).

[00106] Administration of an effective amount of an inhibitor of CaMKK such as, for example a
compound of Formula I-III, such as STO-609 for example, to a subject may be carried out by
any means known in the art including, but not limited to intraperitoneal, intravenous,
intramuscular, subcutaneous, or transcutaneous injection or oral, nasopharyngeal or transmucosal
absorption. Such administration encompasses the administration of a CaMKK inhibitor
formulated as a pharmaceutical composition. Delivery (administration route) also includes
targeted delivery wherein the CaMKK inhibitor is only active in a targeted region of the body
(for example, in the prostate and/or cancerous tissues), as well as sustained release formulations
in which the CaMKK inhibitor compound is released over a period of time in a controlled
manner. Sustained release formulations and methods for targeted delivery are known in the art
and include, for example, use of liposomes, drug loaded biodegradable microspheres, drug-
polymer conjugates, drug-specific binding agent conjugates and the like. Pharmacologically
acceptable carriers are well known to those of skill in the art. Determination of particular
pharmaceutical formulations and therapeutically effective amounts and dosing regimen for a
given treatment is within the ability of one of skill in the art taking into consideration, for
example, patient age, weight, sex, ethnicity, organ (e.g., liver and kidney) function, the extent of
desired treatment, the stage and severity of the disease and associated symptoms, and the
tolerance of the patient for the treatment.

Kits
[00107] In an aspect, the disclosure relates to kits. Such kits can be used in methods of identifying a cancer that can be responsive to a method of treatment comprising administration of a CaMKK inhibitor, methods of identifying a compound as an inhibitor of CaMKK, methods of evaluating efficacy of a therapeutic regimen comprising administration of a CaMKK inhibitor, and the like. Kits can also include appropriate buffer systems and reagents, such as substrates of one or more CaMKKs, phosphate-donating groups (optionally radiolabeled phosphate-donating groups such as $^{32}$P-ATP), a calmodulin and a calcium source, typically a calcium salt, and molecules that can detect the presence of a CaMK or CaMKK (e.g., antibodies). Kits also include instructions for use.

[00108] It will be understood that any numerical value recited herein includes all values from the lower value to the upper value. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended, and all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application.

[00109] Also, it is to be understood that the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use herein of terms such as "comprising," "including," "having," and variations thereof is meant to encompass the items listed thereafter and equivalents thereof as well as additional items. "Comprising" encompasses the terms "consisting of" and "consisting essentially of." The use of "consisting essentially of means that the composition or method may include additional ingredients and/or steps, but only if the additional ingredients and/or steps do not materially alter the basic and novel characteristics of the claimed composition or method.

[00110] All patents, publications and references cited herein are hereby fully incorporated by reference.

[00111] While the following examples provide further detailed description of certain embodiments of the invention, they should be considered merely illustrative and not in any way limiting the invention, as defined by the claims.

EXAMPLES

Cell culture and RNA. The LNCaP and VCaP human prostate carcinoma cell lines were obtained from ATCC and maintained as recommended. All experiments were performed with cells of passage less than 25. These cells were authenticated by morphological inspection and mycoplasma testing by the ATCC. Furthermore, their response to androgens was authenticated using growth and reporter gene assays. RNA from placenta, skeletal muscle, cerebellum, whole brain and normal prostate was from Clontech (Mountain View, CA). RNA from glioblastoma cell lines was a generous gift from Valerie Curtis (Duke University, Durham, NC).

RNA isolation, cDNA preparation, and quantitative and standard reverse transcription (RT)-PCR. RNA isolation, cDNA preparation and quantitative RT-PCR (qPCR) were performed as previously described using 36B4 as a control (12). Standard RT-PCR was performed using the Advantage GC 2 Polymerase Mix and PCR Kit (Clontech). All qPCR and RT-PCR primers used in this study are listed in Table 1.

Western blot analysis. Western blots were performed as previously described (12) with the exception that a modified radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris (pH 8.0), 200 mM NaCl, 1.5 mM MgCl$_2$, 1% Triton X-100, 1 mM EGTA, 10% glycerol, 50 mM NaF, 2 mM Na$_3$VO$_4$ and protease inhibitors] was used. Results shown are representative blots. For each sample, protein levels were determined by densitometry using the ImageJ software (NIH) and normalizing to indicated controls.

Small interfering RNA (siRNA) transfection of human prostate cells. Stealth siRNA (Invitrogen) transfections were performed as previously described (5). The sequences of all siRNAs used in this study are listed in Table 1.

Chromatin immunoprecipitation (ChIP). ChIP was performed as previously described (4). All primers used for ChIP qPCR analysis are listed in Table 1.

Transient transfections and reporter gene assays. Transient transfections and reporter gene assays were performed as previously described (4).

Cell proliferation assay. Proliferation assays were performed as previously described (12) by measuring the cellular DNA content using the FluoReporter Blue Fluorometric double-stranded DNA Quantitation Kit (Invitrogen) as per the manufacturer's protocol.

Migration and invasion assays. Boyden dual chamber migration assays were performed as previously described (4). Invasion assays were performed the same as migration
assays except that inserts were layered with 100 ml of Matrigel extracellular matrix (BD Biosciences) prior to reseeding of cells.

[00121] **Statistical analysis.** Data were analyzed using one-way ANOVA and post hoc Dunnett's test with GraphPad Prism, Version 4 (GraphPad Software, Inc.). Unless otherwise noted, significance was determined at the $P < 0.05$ level.

[00122] **Chemicals.** Methyltrienolone (R1881) was purchased from PerkinElmer (Waltham, MA) and dissolved in ethanol. Bicalutamide (Casodex) was provided as a gift from P. Turnbull (GlaxoSmithKline, Research Triangle Park, NC) and resuspended in a 1:1 mixture of ethanol and dimethylsulfoxide (DMSO). Cycloheximide was obtained from Sigma (St Louis, MO) and dissolved in DMSO. Compound C (in DMSO) was from Calbiochem (San Diego, CA). STO-609 was purchased from Tocris (Ellisville, MO) and resuspended in 100 mM NaOH. 5- aminomidazole-4-carboxamide 1-b-D-ribo-furanoside (AICAR) was from Enzo Life Sciences (Plymouth Meeting, PA) and dissolved in water.

[00123] **Antibodies.** The CaMKK antibody used, unless otherwise specified, was from BD Biosciences (Palo Alto, CA). The CaMKKp (clone 1A1 1) antibody was from Abnova (Walnut, CA). The v5 antibody was purchased from Invitrogen (Carlsbad, CA). The GAPDH and AR antibodies have previously been described (1). Phospho-CaMKI (T177), CaMKI and Lamin A antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-AMPKa (T172), AMPKα, phospho-acetyl-CoA carboxylase (S79), and acetyl-CoA carboxylase antibodies were from Cell Signaling Technology (Danvers, MA).

[00124] **Plasmids.** The CMV-Pgal and PSA-Luc plasmids were previously described (2). The pGL4.14 (lacks both basal promoter and enhancers) and pGL4.26 (lacks enhancer, but contains basal promoter) vectors were obtained from Promega (Madison, WI). MSCV-GWb-GAL4(DNA-binding domain (DBD))-IRES-EGFP, MSCV-GWb-CaMKKp-IRES-EGFP, MSCV-GWb-v5-ARwt-IRES-EGFP and MSCV-GWb-v5-AR(C562S)-IRES-EGFP were created using the Invitrogen Gateway recombinase subcloning system according to the manufacturer's instructions. To do this, GAL4(DBD), CaMKKp, v5-ARwt or v5-AR(C562S) were shuttled from pENTR-GAL4(DBD), pENTR-v5-ARwt, pENTR-v5-AR(C562) or pOTB7-CaMKKp prostate splice variant (American Type Culture Collection (ATCC), Manassas, VA) to MSCV-IRES-EGFP that was converted to a Gateway destination vector. The pGL4.14-CaMKKp promoter construct was created by PCR amplifying a 2.1 kb genomic sequence that encompassed
the ΩαΜΚΚβ transcriptional start site through the potential AR binding site identified using ChIP on Chip (previously described (3)). This fragment was then cloned into the pGL4.14 vector using NheI and HindIII restriction sites. Subsequent deletion constructs were created by PCR amplifying smaller fragments that were cloned into pGL4.26 using NheI and HindIII restriction sites. Finally, the pGL4.14-CaMKKp promoter-ARE deletion construct was created from the original pGL4.14-CaMKKp promoter construct using the ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All primers used for the creation of constructs are listed in Supplementary Table 1. All sequences were confirmed using restriction digests and sequencing.

[00125] **Creation of stable cell lines.** To create LNCaP-GAL4, LNCaP-CAMKβLNCaP-v5-ARwt and LNCaP-v5-AR(C562S) cells, parental cells were infected with retrovirus expressing MSCV-GWb-GAL4(DBD)-IRES-EGFP (negative control), MSCV-GWb-CaMKKp-IRES-EGFP, MSCV-GWb-v5-ARwt-IRES-EGFP or MSCV-GWb-v5-AR(C562S)-IRES-EGFP. EGFP positive cells were then selected through two rounds of cells sorting using flow cytometry and expression levels were confirmed by western blot and/or qPCR.

**Table 1.** Primers and siRNA sequences used in these studies

<table>
<thead>
<tr>
<th>Primer/siRNA</th>
<th>Sequence (SEQ ID NO)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>qPCR primers</strong></td>
<td></td>
</tr>
<tr>
<td>36B4</td>
<td>Forward: 5'-GGACATGTGGCTGGCAATAA-3' (SEQ ID NO:48)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGGCCCGAGACCAGTTTGT-3' (SEQ ID NO:49)</td>
</tr>
<tr>
<td>CaMKKβ</td>
<td>Forward: 5'-TCCAGACCCAGCCGGACATAG-3' (SEQ ID NO:50)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CAGGGGTCAGGATTGGTTTC-3' (SEQ ID NO:51)</td>
</tr>
<tr>
<td>CXC4</td>
<td>Forward: 5'-TGGCCCTTATCTGGCGCTGTAT-3' (SEQ ID NO:52)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AGGAGTCGTGATGATCCCAA-3' (SEQ ID NO:53)</td>
</tr>
<tr>
<td>AR 3'UTR</td>
<td>Forward: 5'-CCATGGCCACCTCAGACTTT-3' (SEQ ID NO:54)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-ACTGGGCACTATGAGGATCA-3' (SEQ ID NO:55)</td>
</tr>
<tr>
<td>AMPKα1</td>
<td>Forward: 5'-CTCAGTTCTGGGAGAAGATGG-3' (SEQ ID NO:56)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCAGTGCAATTGTCATGGTGCC-3' (SEQ ID NO:57)</td>
</tr>
<tr>
<td>AMPKα2</td>
<td>Forward: 5'-ATGGAATATGTGCTGGAGGTG-3' (SEQ ID NO:58)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TGCTTCAGGTCGATGAAC-3' (SEQ ID NO:59)</td>
</tr>
<tr>
<td><strong>CaMKKβ enhancer ChIP primers</strong></td>
<td></td>
</tr>
<tr>
<td>distal upstream control</td>
<td>Forward: 5'-GCACAGTTGGCACAAGTGAACATG-3' (SEQ ID NO:60)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCTTTGAGATGGGCTGTGCTGTTTGGAAC-3' (SEQ ID NO:61)</td>
</tr>
<tr>
<td>CaMKKβ enhancer</td>
<td>Forward: 5'-AACAGGAAAGGACCAACCTG-3' (SEQ ID NO:62)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AACCCTTCTCAGACGGCATG-3' (SEQ ID NO:63)</td>
</tr>
<tr>
<td><strong>CaMKKβ enhancer and promoter reporter gene primers</strong></td>
<td>Forward: 5'-CGCTTAGGAGGAGATCCGAGCAATA-3' (SEQ ID NO:64)</td>
</tr>
<tr>
<td>promoter</td>
<td>Reverse: 5'-CAAAAGCTTTTGAGGCGATGAATCTGTTGAGCAATCAG-3' (SEQ ID NO:65)</td>
</tr>
<tr>
<td>Primer/siRNA</td>
<td>Sequence (SEQ ID NO)</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>fragment A enhancer</td>
<td>Fwd: 5'-CGCTAGCAGGGTGGTGTGAGCATCAAATA-3' (SEQ ID NO: 103)</td>
</tr>
<tr>
<td>fragment B enhancer</td>
<td>Fwd: 5'-ACCAUGAGUAGUAGAAGCCAC-3' (SEQ ID NO: 101)</td>
</tr>
<tr>
<td>fragment C enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 106)</td>
</tr>
<tr>
<td>fragment D enhancer</td>
<td>Fwd: 5'-GACCTAGGAGGAGGGGAGG-3' (SEQ ID NO: 107)</td>
</tr>
<tr>
<td>fragment E enhancer</td>
<td>Fwd: 5'-CGCTAGTAGTCCTGACTGAGATTT-3' (SEQ ID NO: 108)</td>
</tr>
<tr>
<td>fragment F enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 109)</td>
</tr>
<tr>
<td>fragment G enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 110)</td>
</tr>
<tr>
<td>fragment H enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 111)</td>
</tr>
<tr>
<td>566 bp enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 112)</td>
</tr>
<tr>
<td>487 bp enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 113)</td>
</tr>
<tr>
<td>312 bp enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 114)</td>
</tr>
<tr>
<td>233 bp enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 115)</td>
</tr>
<tr>
<td>152 bp enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 116)</td>
</tr>
<tr>
<td>90 bp enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 117)</td>
</tr>
<tr>
<td>69 bp enhancer</td>
<td>Fwd: 5'-CCUCAUUAAUUUAUUCCUCUGG-3' (SEQ ID NO: 118)</td>
</tr>
<tr>
<td>CaMKKP RT-PCR primers</td>
<td>F(1-6) 5'-ACCTGTATCCCAAGACCTTCGGA-3' (SEQ ID NO: 98)</td>
</tr>
<tr>
<td>R(1-6) 5'-CGATCTCGGATCTCTGAGCTTA-3' (SEQ ID NO: 99)</td>
<td></td>
</tr>
<tr>
<td>F(7) 5'-TGAGCGGAGGACCGAGCGAGCTG-3' (SEQ ID NO: 100)</td>
<td></td>
</tr>
<tr>
<td>R(1-7) 5'-TCAAGGAGCTTCTGCTGAGCTTA-3' (SEQ ID NO: 101)</td>
<td></td>
</tr>
<tr>
<td>F1 5'-AGCTAGGAGAATCTGAGCTTA-3' (SEQ ID NO: 102)</td>
<td></td>
</tr>
<tr>
<td>R1 5'-AGCTAGGAGGACCGAGCGAGCTG-3' (SEQ ID NO: 103)</td>
<td></td>
</tr>
<tr>
<td>R2 5'-ACCTGAGCTGAGCTTA-3' (SEQ ID NO: 104)</td>
<td></td>
</tr>
<tr>
<td>siRNA sequences</td>
<td>CaMKKP #1 5'-GGACCAUGUAUCAUGUGUGUGGAGC-3' (SEQ ID NO: 105)</td>
</tr>
<tr>
<td>CaMKKP #2 5'-GCUAGAUCUUUGUGUAGAGCAUGA-3' (SEQ ID NO: 106)</td>
<td></td>
</tr>
<tr>
<td>CaMKKP #3 5'-CAGGUAUGGUAGWAGAGCCGAGCC-3' (SEQ ID NO: 107)</td>
<td></td>
</tr>
<tr>
<td>AR 3'UTR 5'-CAGAUGUGUCUGACCCUUAACG-3' (SEQ ID NO: 108)</td>
<td></td>
</tr>
<tr>
<td>AMPKal #1 5'-CCGAAUCAUAGUACAGUACAGUAC-3' (SEQ ID NO: 109)</td>
<td></td>
</tr>
<tr>
<td>AMPKal #2 5'-CCGAAUCAUAGUACAGUACAGUAC-3' (SEQ ID NO: 110)</td>
<td></td>
</tr>
<tr>
<td>AMPKal #3 5'-CCGAAUCAUAGUACAGUACAGUAC-3' (SEQ ID NO: 111)</td>
<td></td>
</tr>
<tr>
<td>AMPKal #1 5'-CCGAAUCAUAGUACAGUACAGUAC-3' (SEQ ID NO: 112)</td>
<td></td>
</tr>
<tr>
<td>AMPKal #2 5'-CCGAAUCAUAGUACAGUACAGUAC-3' (SEQ ID NO: 113)</td>
<td></td>
</tr>
</tbody>
</table>
**Table 1:**

<table>
<thead>
<tr>
<th>Primer/siRNA</th>
<th>Sequence (SEQ ID NO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMKI #1</td>
<td>5'-GGAGATACAGCTCTAGATAAGAATA-3' (SEQ ID NO: 114)</td>
</tr>
<tr>
<td>CaMKI #2</td>
<td>5'-CCATAGGTGTCATCGCCTACATCTT-3' (SEQ ID NO: 115)</td>
</tr>
</tbody>
</table>

[00126] Example 1: Androgens increase αMKKβ mRNA and protein levels in an AR-dependent manner.

[00127] In an effort to identify novel prostate cancer therapeutics, we have focused on defining key regulators downstream of AR action that contribute to prostate pathobiology and that may be amenable to pharmacological exploitation. As a first step in this process, we analyzed the expression level of mRNAs encoding targetable signaling molecules using microarray data derived from androgen-treated LNCaP prostate cancer cells (13). These studies suggested that one such candidate, CaMKKp, was upregulated by androgens. To confirm the significance of this observation, CaMKKp mRNA levels were analyzed by qPCR following treatment with the synthetic androgen R1881. In both LNCaP and VCaP prostate cancer cell lines, CaMKKp mRNA levels increased in a dose-dependent manner (Fig. 1A). Further, western immunoblot analysis revealed a corresponding dose-dependent increase in CaMKKp protein levels in both cell lines (Fig. 1B). The specificity of the antibodies used in this study was verified using three siRNAs targeting CaMKKp mRNA (Fig. 1C). In addition, analogous immunoblot results were obtained using a second antibody (clone 1A11) directed against CaMKKp (Fig. 2). Finally, androgen-mediated induction, but not the basal expression, of CaMKKp mRNA was abrogated in cells in which AR expression was inhibited using a validated siRNA (4) directed against the AR mRNA (Fig. 1D). Taken together, these data demonstrate that androgens, acting through AR, increase both CaMKKp mRNA and protein levels in multiple cellular models of prostate cancer.

[00128] Example 2: Functionally active splice variants of CaMKKβ are expressed in response to androgens in the prostate.

[00129] Given that AR increases CaMKKp levels in multiple cellular models of prostate cancer, we next determined if its expression correlated with the development of prostate cancer in human samples. Analysis of the clinically annotated prostate cancer data sets accessible through Oncomine revealed that αMKKβ expression increases with grade (14-17) (Fig. 3A and Fig.
Interestingly, this analysis also revealed that ΟαΜΚΚβ was consistently overexpressed in prostate tumors, but not other malignancies (Fig. 3C) (18).

The full-length CaMKKp protein is encoded by an mRNA composed of 18 exons. Interestingly, the majority of commercially available CaMKKp antibodies target the C-terminus of the protein that is absent in some functionally active splice variants (19). Thus, given that the expression of CaMKKp in the prostate has not been reported previously, we hypothesized that the prostate, and prostate cancers, may express a functionally important splice variant(s) of CaMKKp that was not recognized by the most commonly used antibodies. To test this hypothesis, we performed RT-PCR analysis using primers spanning various exon boundaries to examine the splice variant repertoire in the normal prostate and in prostate cancer cells. In this manner, it was demonstrated that unlike in brain, which expresses a longer variant, both normal prostate and prostate cancer cells predominantly express shorter variants of CaMKKp (Fig. 4A, Fig. 4B, and Fig. 5). The variants found are equivalent to the previously described CaMKKp splice variants 2 and 7 that lack exon 16 (of note, splice variants 2 and 7 make identical protein products) (19). Interestingly, these shorter variants were also found in brain tumors (Fig. 4B). A complete analysis of the additional variants expressed in the prostate/prostate cancer is described in Fig. 5. Phosphorylation of the classical CaMKKp target CaMKI was observed in both androgen-treated LNCaP and VCaP cells (Fig. 4C), indicating that the CaMKKp variant expressed in prostate cancer cells is functionally active.

Example 3: CaMKKi is necessary and sufficient for AR-mediated prostate cancer cell migration and invasion.

Given that the expression of CaMKKp is upregulated by androgens and is elevated in prostate cancer, we next wanted to assess its potential role(s) in processes of pathological importance in this disease. As a first step, we evaluated the ability of the CaMKK antagonist STO-609 to inhibit the androgen-mediated cellular growth of prostate cancer cells. However, at a concentration that suppressed CaMKKp activity (Fig. 6A), this drug had no significant effect on LNCaP and VCaP cell number over the seven-day period of this assay (Fig. 7A and Fig. 6B).

In addition to proliferation, androgens increase the migration of prostate cancer cells (4, 20). Since CaMKKp has recently been implicated in cell migration during neuronal development
(21, 22), we next asked whether CaMKKp is involved with AR-mediated prostate cancer cell migration and/or invasion. Using Boyden dual chamber migration assays, treatment with the CaMKK antagonist STO-609 blocked the androgen-mediated migration of both LNCaP (Fig. 7B, top) and VCaP prostate cancer cells (Fig. 6C). STO-609 also inhibited androgen-mediated invasion of LNCaP cells through a Matrigel extracellular matrix (Fig. 7B, bottom). Furthermore, knockdown of CaMKKp suppressed, while its overexpression increased, both basal and androgen-stimulated cell migration (Fig. 7C, Fig. 7D, Fig. 6D, and Fig. 6E). These findings highlight a heretofore-unrecognized role for CaMKKp in prostate cancer cell migration and invasion.

[00134] Example 4: Definition of the molecular mechanism for AR-mediated CaMKKβ mRNA expression.

[00135] Using a knockdown/replacement strategy, it was demonstrated that expression of wild-type AR, but not a transcriptionally inactive DNA binding mutant (C562S), was able to complement the knockdown of endogenously expressed AR in an LNCaP cell migration assay (Fig. 8). Further, at a concentration that inhibits the expression of secondary androgen target genes (ex. CXCR4 (4)), cycloheximide treatment did not block the R1881-mediated increase in CaMKKp mRNA levels (Fig. 9A). Together, these data indicate that CaMKKp is a primary AR target gene.

[00136] By mining our previously published ChIP on Chip data (23), we identified a putative AR binding region located ~2.3 kb upstream of the OαMKKβ transcriptional start site (Fig. 9B, top). No other AR binding was detected within the OαMKKβ gene or within 100 kb in either direction of the gene. The validity of this AR-binding site was confirmed using ChIP assays, which showed that AR was recruited to this region of the promoter within one hour following R1881 treatment (Fig. 9B, bottom). Given these data, we focused on characterizing the functionality of the putative ARE identified. To this end, we cloned overlapping regions of CaMKKβ's 5' upstream region and tested their ability to confer androgen responsiveness to an enhancerless luciferase reporter gene. In this manner, we determined that a construct incorporating a fragment, -2231 to -1632 (D), and an overlapping fragment, -2019 to -1632 (E), contained an AR-dependent enhancer (Fig. 9C). Both fragments D and E demonstrated androgen responsiveness in a dose-dependent manner that was suppressed by the antiandrogen Casodex.
(Fig. 10A). Similar results were obtained in VCaP cells (Fig. 10B). Deletion analysis further narrowed down the androgen-responsive region to a 79 bp stretch of DNA that included a sequence, GTAACAtgaTGTTAAA, that resembled the consensus androgen response element (ARE) AGAACAnnnTGTTCT (Fig. 10C). Deletion of the 15 bp ARE in the full-length CaMKKp promoter construct (-2231 to +83) completely abolished the androgen responsiveness (Fig. 9D). Thus, in the context of prostate cancer cells, OαMKKβ is a direct target of AR.

[00137] Example 5: Androgens promote prostate cancer cell migration through an AR-OαMKKβ-AMPK signaling axis.

[00138] CaMKI, CaMKIV and, more recently, AMPK have been shown to be downstream targets of CaMKKp (24). Since CaMKIV is not expressed in the prostate (data not shown), we tested whether AR-CaMKKp signaling led to increased CaMKI and/or AMPK signaling. Western blot analysis revealed that androgens increased the phosphorylation of both CaMKI and AMPK at their CaMKKp activation loop target sites (T177 and T172 respectively) in both LNCaP and VCaP cells, an effect that was reversed by pretreatment with STO-609 (Fig. 11A and Fig. 12A). Interestingly, we found that overexpression of CaMKKp alone was sufficient to increase the phosphorylation/activity of AMPK, but not CaMKI (Fig. 11B). These findings indicated that AMPK, rather than CaMKI, could be regulating cell migration because CaMKKp overexpression alone was also sufficient to increase migration (Fig. 7D). To verify this, we used our most efficacious siRNAs (Fig. 12B) to knockdown both isoforms of the catalytic subunit of AMPK (Fig. 11C, bottom and Fig. 12C) or CaMKI (Fig. 11D, bottom and Fig. 12D). In this manner, it was demonstrated that loss of AMPK, but not CaMKI, resulted in decreased prostate cancer cell migration (Figs. 11C and Fig. 11D). In support of these findings, cotreatment of cells with the AMPK antagonist compound C, at a concentration that inhibited its kinase activity, completely abolished androgen-mediated cell migration (Fig. 13A and Fig. 13B). Conversely, treatment of LNCaP cells with the AMP mimetic AICAR alone was sufficient to increase cell migration (Fig. 13A and Fig. 13C). These data highlight a central role for AMPK in prostate cancer cell migration. Definition of the mechanism(s) by which AMPK interfaces with the cellular processes responsible for migration and invasion is currently under investigation.

[00139] REFERENCES
The following references are incorporated herein by reference in their entireties.


CLAIMS

We claim:

1. A method of diagnosing prostate cancer in a subject comprising:
   a) obtaining a sample from the subject;
   b) determining an amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject; and
   c) comparing the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit from the sample from the subject to an amount of the CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in a control sample;

   wherein the subject is diagnosed as having prostate cancer when the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject is greater than the amount in the control sample.

2. The method of claim 1, wherein the determining comprises detecting the amount of mRNA expression.

3. The method of claim 1, wherein the determining comprises detecting the amount of at least one protein of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the samples.

4. The method of claim 1, wherein the sample from the subject comprises prostate tissue.
5. The method of claim 3, wherein the detecting comprises an antibody that specifically binds to a C-terminal portion of the amino acid sequence encoded by CaMKKp, CaMKKp splice variant 2, or CaMKKp splice variant 7.

6. A method for determining disease stage in a subject having prostate cancer, the method comprising:
   a) obtaining a sample from the subject;
   b) determining an amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject; and
   c) comparing the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject to an amount of the CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in a control sample;

   wherein the disease stage of prostate cancer is determined by the difference in the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject and the amount in the control sample.

7. A method for predicting the likelihood of success of hormone-based therapeutic treatment of a subject having prostate cancer, the method comprising:
   a) obtaining a sample from the subject;
   b) determining an amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject; and
   c) comparing the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit from the sample from the subject to
an amount of the CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in a control sample;

wherein the subject is predicted to respond to hormone-based therapeutic treatment when the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject is greater than the amount in the control sample.

8. A method for early detection of prostate cancer in a subject comprising:
   a) obtaining a sample from the subject;
   b) determining an amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject; and
   c) comparing the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit from the sample from the subject to an amount of the CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, AMPK, and AMPK a1 subunit in a control sample;

   wherein early detection of prostate cancer is made when the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the sample from the subject is greater than the amount in the control sample.

9. A method for identifying a selective inhibitor of CaMKKp comprising,
   (a) contacting CaMKKp or an active fragment thereof with a test compound;
   (b) determining a biological activity of CaMKKp after contacting with the test compound;
   (c) determining a control level of biological activity of CaMKKp in the absence of the test compound;
   (d) determining an IC50 value for the test compound;
(e) repeating (a) - (d) with a Ca\textsuperscript{2+}/calmodulin-dependent protein kinase, or an active fragment thereof, that does not comprise CaMKKp;

wherein when the IC\textsubscript{50} value for the test compound on CaMKKp biological activity is lower than the IC\textsubscript{50} value for the test compound on the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase that does not comprise CaMKKp, the test compound is identified as a selective inhibitor of CaMKKp.

10. The method of claim 8 or claim 9, wherein the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase, or an active fragment thereof, that does not comprise CaMKKp is selected from the group consisting of CaMKKa, CaM Ki, CaM KII, CaM KIII, and CaM KIV.

11. A method for identifying a selective inhibitor of CaMKKp comprising,

(a) contacting CaMKKp or an active fragment thereof with a test compound;

(b) determining a biological activity of CaMKKp in response to the contacting with the test compound;

(c) determining a control level of biological activity of CaMKKp in the absence of the test compound;

(d) comparing the biological activity of CaMKKp in the presence of the test compound relative to the biological activity of CaMKKp in the absence of the test compound to determine a percent inhibition;

(e) repeating (a) - (d) with a Ca\textsuperscript{2+}/calmodulin-dependent protein kinase, or an active fragment thereof, that does not comprise CaMKKp;

wherein the test compound is identified as a selective inhibitor of CaMKKp when the percent inhibition of the test compound on CaMKKp biological activity is greater than the percent inhibition of the test compound on the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase that does not comprise CaMKKp.

12. A method of screening a test compound for CaMKKp antagonist activity comprising:
i) contacting CaMKKp and a substrate therefor, in the presence and in the absence of the
test compound, under conditions such that CaMKKP-dependent phosphorylation of
said substrate can be effected, and
ii) determining the level of phosphorylation of said substrate resulting from step (i) and
comparing said level with a level of phosphorylation of said substrate in the absence
of said test compound, wherein an increase in phosphorylation of said substrate in
the presence of said test compound indicates that said test compound is a
CaMKKp antagonist.

13. The method according to claim 12 wherein the substrate is a peptide substrate.

14. The method according to claim 12 wherein the CaMKKp and the substrate are present in
a cell free system.

15. The method according to claim 12 wherein the CaMKKp and the substrate are present in
a cell.

16. The method according to claim 12 wherein, in step (i), the CaMKKp and the substrate
are contacted with the test compound in the presence of calmodulin and calcium under
conditions such that allow for CaMKKP-dependent phosphorylation of the substrate.

17. A method of screening a test compound for its anti-cancer activity comprising:
i) contacting CaMKKp and a substrate therefor in the presence of the test compound,
under conditions that allow for CaMKKP-dependent phosphorylation of the
substrate, and
ii) determining the level of phosphorylation of the substrate resulting from step (i) and
comparing the level with a level of phosphorylation of the substrate obtained in the
absence of the test compound, wherein a reduction in the level of phosphorylation
of the substrate in the presence of the test compound indicates that the test
compound has said anti-cancer activity.
18. The method according to claim 17 wherein said substrate is a peptide substrate.

19. The method according to claim 17 wherein the CaMKKp and the substrate are present in a cell free system.

20. The method according to claim 17 wherein the CaMKKp and the substrate are present in a cell.

21. The method according to claim 17 wherein, in step (i), the CaMKKp and the substrate are contacted with the test compound in the presence of calmodulin and calcium under conditions such that CaMKKp-dependent phosphorylation of the substrate can be effected.

22. A method of screening a test compound for therapeutic effect against prostate cancer comprising:
   i) contacting CaMKKp and a substrate therefor in the presence of the test compound, under conditions that allow for CaMKKp-dependent phosphorylation of the substrate, and
   ii) determining the level of phosphorylation of the substrate resulting from step (i) and comparing that level with a level of phosphorylation of the substrate obtained in the absence of the test compound, wherein an increase in the level of phosphorylation of the substrate in the presence of the test compound indicates that the test compound has activity against prostate cancer.

23. The method according to claim 22 wherein the substrate is a peptide substrate.

24. The method according to claim 22 wherein the CaMKKp and the substrate are present in a cell free system.

25. The method according to claim 22 wherein the CaMKKp and the substrate are present in a cell.
26. The method according to claim 22 wherein, in step (i), the CaMKKp and the substrate are contacted with the test compound in the presence of calmodulin and calcium under conditions such that allow for CaMKKp-dependent phosphorylation of the substrate.

27. A method of treating prostate cancer in a subject, comprising administering to the subject an effective amount of a compound that inhibits activity of at least one of CaMKKp, CaMKKp splice variant 2, or CaMKKp splice variant 7.

28. The method of claim 27, wherein the compound is selected from an inhibitory RNA or an antibody that specifically binds to a C-terminal portion of CaMKKp, CaMKKp splice variant 2, or CaMKKp splice variant 7.

29. The method of claim 27, wherein the compound has Formula III:

![Formula III](image)

wherein R₁, R₂, R₃, R₄, R₅, R₆, R₇, R₈, R₉, R₁₀, and R₁₁ are each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, cycloalkylalkenyl, cycloalkylalkynyl, heterocyclo, heterocycloalkyl, heterocycloalkenyl, heterocycloalkynyl, aryl, arylalkyl, arylalkenyl, aryalkynyl, heteroaryl, heteroaryalkyl, heteroaryalkenyl, heteroaryalkynyl, alkoxy, halo, mercapto, azido, cyano, formyl, carboxylic acid, hydroxyl, nitro, acyl, arloxy, alkylthio, amino, alkylaminocarbonyl, alkylaminocarbonyl, disubstituted amino, acylamino, acyloxy, ester, amide, sulfoxyl, sulfonyl, sulfonate, sulfonic acid, sulfonamide, urea, alkoxycarbonylaminocarbonyl, and aminoacyloxy; or a pharmaceutically acceptable salt or prodrug thereof.
30. The method of claim 29, wherein the compound is STO-609.

31. The method of any of claims 27-30 wherein the method further comprises administering an effective amount of a second agent selected from anti-androgens, Selective Androgen Receptor Modulators (SARMs), Selective Androgen Receptor Degraders (SARDs), CYP17 inhibitors, suphatase inhibitors, Src inhibitors, anti-estrogens, estrogens, Selective Estrogen Receptor Modulators (SERMs), Selective Estrogen Receptor Degraders (SERDs), ERb antagonists, aromatase inhibitors, and a vaccine.

32. The method of claim 31, wherein the second agent is selected from MDV3100, ARN-509, bicalutamide, and flutamide.

33. A method of inhibiting proliferation of a prostate cancer cell, comprising contacting the cell with an effective amount of an inhibitor of at least one of CaMKKp, CaMKKp splice variant 7, or CaMKKp splice variant 2 or any combination thereof.

34. A method of inhibiting proliferation of a prostate cancer cell in a subject, the method comprising administering to the subject an effective amount of an inhibitor of at least one of CaMKKp, CaMKKp splice variant 7, or CaMKKp splice variant 2 or any combination thereof.

35. The method of claims 33-34 wherein the inhibitor comprises an inhibitory RNA.

36. The method of claims 33-34 wherein the inhibitor comprises a compound of Formula III:

\[
\text{Formula III}
\]
wherein $R_1, R_2, R_3, R_4, R_5, R_7, R_8, R_9, R_{10}$, and $R_{11}$ are each independently selected from the group consisting of H, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, cycloalkylalkenyl, cycloalkylalkynyl, heterocyclo, heterocycloalkyl, heterocycloalkenyl, heterocycloalkynyl, aryl, arylalkyl, arylalkenyl, arylalkynyl, heteroaryl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, alkoxy, halo, mercapto, azido, cyano, formyl, carboxylic acid, hydroxyl, nitro, acyl, aryloxy, alkylthio, amino, alkylamino, arylalkylamino, disubstituted amino, acylamino, acyloxy, ester, amide, sulfoxyl, sulfonate, sulfonic acid, sulfonamide, urea, alkoxyacylamino, and aminoacyloxy; or a pharmaceutically acceptable salt or prodrug thereof.

37. The method of claims 33-35 wherein the inhibitor comprises an antibody that specifically binds human CaMKKp or the amino acid sequence encoded by CaMKKp splice variant 2.

38. The method of claim 37, wherein the antibody specifically binds to the C-terminal region of human CaMKKp or CaMKKp splice variant 2/7.

39. A method of treating prostate cancer in a subject, the method comprising administering to the subject an effective amount of an inhibitor of AMPK or AMPKa subunit.

40. The method of claim 39, wherein the inhibitor is an inhibitory RNA.

41. A method of inhibiting proliferation of a prostate cancer cell comprising contacting the cell with an effective amount of an inhibitor of AMPK, AMPKa subunit, or AMPKa2 subunit, or any combination thereof.

42. A method of inhibiting proliferation of a prostate cancer cell in a subject, the method comprising administering to the subject an effective amount of an inhibitor of AMPK, AMPKa subunit, or AMPKa2 subunit, or any combination thereof.

43. The method of claims 40-43 wherein the inhibitor is an inhibitory RNA.
44. A method of determining the efficacy of therapy in a patient being treated for prostate cancer, the method comprising:

   a) obtaining a series of samples from the subject;

   a) determining an amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 subunit in the series of samples from the subject, where the samples are taken from the subject at different time points during the therapy; and

   b) comparing the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 from the series of samples from the subject;

wherein when the amount of at least one of CaMKKp, CaMKKp splice variant 2, CaMKKp splice variant 7, phosphorylated AMPK, phosphorylated AMPKal subunit, and phosphorylated AMPKa2 in the series of samples is about the same or increases the therapy is determined to be not effective.

45. A method of inhibiting androgen-mediated migration of a prostate cancer cell comprising contacting the cell with an effective amount of an inhibitor of at least one of CaMKKp, CaMKKp splice variant 7, or CaMKKp splice variant 2 or any combination thereof.

46. A method of inhibiting androgen-mediated invasion of a prostate cancer cell comprising contacting the cell with an effective amount of an inhibitor of at least one of CaMKKp, CaMKKp splice variant 7, or CaMKKp splice variant 2 or any combination thereof.

47. The method of claims 45-46, wherein the inhibitor comprises a compound of Formula III:

48. A nucleic acid molecule comprising a sequence that binds under stringent conditions to a region that is about 2.3 kb upstream (5') relative to a CaMKKp transcriptional start site.
49. The nucleic acid molecule of claim 46 wherein the region is from about -223 to about -1632 upstream (5') relative to a CaMKKp transcriptional start site.

50. The nucleic acid molecule of claim 48 wherein the region is from about -2019 to about -1632 upstream (5') relative to a CaMKKp transcriptional start site.

51. The nucleic acid molecule of claim 50 comprising the sequence 5' - GTA ACA TGA TGT AAA - 3'.

52. The nucleic acid molecule of claim 48, wherein the nucleic acid molecule is selected from decoy RNA, dsRNA, a nucleic acid aptamer, an antisense nucleic acid molecule, and an enzymatic nucleic acid molecule.

53. A nucleic acid molecule comprising a double stranded siRNA that down-regulates expression of a CaMKKp gene via RNA interference (RNAi), wherein: a) each strand of the siRNA molecule is independently about 18 to about 28 nucleotides in length; and b) one strand of the siRNA molecule comprises a sequence that binds under stringent conditions to a CaMKKp RNA of the CaMKKp gene and directs cleavage of the CaMKKp RNA via RNA interference.

54. The nucleic acid molecule of claim 53 comprising the siRNA sequences of SEQ ID NOs. 105-113.
Figure 1
Figure 2

![Graph showing normalized fold induction with Vehicle and R1881 comparisons for CaMKKβ(1A11) and GAPDH with siLacZ and siCaMKKβ treatments.]
**Figure 3**

**A**

**CAMKK2**
Calcium/calmodulin-dependent protein kinase kinase 2, beta

<table>
<thead>
<tr>
<th>Class</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Normalized expression units

**Box Plot - Description**

**Normal Prostate**

**Prostate Cancer**

1) Lapointe et. al.
2) Welsh et. al.
3) Yu et. al.
4) Varambally et. al.

**B**

**CAMKK2**
Calcium/calmodulin-dependent protein kinase kinase 2, beta

<table>
<thead>
<tr>
<th>Sub Class</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Normalized expression units

**Box Plot - Description**

**Normal Prostate**

**Prostate Cancer**

Metastatic Prostate Cancer

Yu et. al.

**C**

**CAMKK2**
Calcium/calmodulin-dependent protein kinase kinase 2, beta

<table>
<thead>
<tr>
<th>Sub Class</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1</td>
</tr>
<tr>
<td>b</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td></td>
</tr>
<tr>
<td>d</td>
<td></td>
</tr>
<tr>
<td>e</td>
<td></td>
</tr>
<tr>
<td>f</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td></td>
</tr>
<tr>
<td>i</td>
<td></td>
</tr>
<tr>
<td>j</td>
<td>2</td>
</tr>
</tbody>
</table>

Normalized expression units

**Box Plot - Description**

**Various Cancers**

**Prostate Cancer**

Su et. al.
Figure 4
Figure 7

A) Relative cell #

B) Migration

C) Number of cells migrated/well

D) Invasion

---

Figure 7
**Figure 8**

**A**  
Complementation assay design

- LNCaP  
  - GAL4  
  - v5-ARwt  
  - v5-AR(C562S)

- siControl  
- siAR 3'UTR

+ siRNA

- perform assay

**B**

Bar chart showing relative mRNA levels of endogenous AR:

- GAL4
- v5-ARwt
- v5-AR(C562S)

- R1881:
  - Mock
  - siLacZ
  - siAR 3'UTR

**C**

Graph showing number of cells migrated/well:

- GAL4
- GAL4 (siLacZ)
- v5-ARwt
- v5-AR(C562S)

- Vehicle
- R1881
Figure 10
Figure 12
Figure 13