(54) Title: STEROID HORMONE AND NON-Steroidal ANTI-INFLAMMATORY DRUG COMBINATIONS FOR INDUCING TUMOR CELL APOPTOSIS

(57) Abstract: A pharmaceutical composition is described, having at least one nonsteroidal anti-inflammatory drug at least one steroid hormone, a pharmaceutically acceptable carrier, and optionally, one or more excipients, wherein the at least one nonsteroidal anti-inflammatory drug and the at least one steroid hormone are present in amounts sufficient to induce tumor cell apoptosis. Also described is a method of inducing apoptosis of cancer cells in which therapeutically effective amounts of at least one nonsteroidal anti-inflammatory drug and at least one steroid hormone are administered to a subject. The nonsteroidal anti-inflammatory drug and steroid hormone may administered prophylactically to a subject having no measurable tumor burden, or may be administered to a subject having a detectable tumor.
STERIOD HORMONE AND NON-STEROIDAL ANTI-INFLAMMATORY
DRUG COMBINATIONS FOR INDUCING TUMOR CELL APOPTOSIS

CROSS REFERENCE TO A RELATED APPLICATION
This application relates to U.S. Provisional Serial No. 60/294,583, filed
on June 1, 2001, and which is incorporated in its entirety by reference herein.

BACKGROUND OF THE INVENTION

Field of the Invention
[0001] The invention relates to a combination therapy comprising steroid
hormones and non-steroidal anti-inflammatory drugs, both prescription and
non-prescription drugs, for inducing apoptotic-mediated death of hormone-
dependent cancer cells. The invention also relates to the use of non-
prescription NSAIDs such as acetaminophen, aspirin, naproxen and preferably
ibuprofen to inhibit cancer cells in vitro and in vivo, particularly human prostate
cancer cells.

Description of Related Art
[0002] Prostate cancer is one of the leading causes of cancer-related deaths
in men in developed economies, such as the United States. Because traditional
androgen-ablation therapy and chemotherapy have not resulted in a significant
survival benefit in patients with metastatic disease or advanced local disease,
new and more effective therapeutic approaches are needed.
[0003] Androgen ablation has been the mainstay clinical approach to treating
prostate cancer. Although this therapy produces significant palliation of
symptoms in patients with advanced prostate cancer, the proportion of tumors
that regress is actually relatively small. More important, however, is the fact that
this treatment regimen is doomed to failure in the long-term because it
promotes a hormone refractory disease, which is ultimately lethal for the
patient.
[0004] In one recent article, Prehn argued that declining rather than high
levels of androgens contribute to human prostate carcinogenesis, and that
androgen supplementation may lower the incidence of the disease. This view
is, of course, contrary to accepted medical opinion, wherein androgen is believed to induce proliferation and androgen ablation to induce apoptosis. However, Prehn points out that prostate cancer occurs coincident with decreasing rather than rising levels of androgen. While excess androgen stimulates secretory activity, it does not stimulate cell proliferation in vivo, at least in the normal prostate. In fact, high levels of androgen will be inhibitory to normal prostatic cells due to a mechanism mediated by the androgen receptor (AR), and excess androgen can inhibit prostate cancers under experimental conditions. Finally, the few clinical cases that have attempted treatment of prostate cancer with exogenous androgen have, in many cases, shown a significant regression of the cancer in response to such treatment.

There exists a growing body of evidence indicating nonsteroidal anti-inflammatory drugs (NSAIDs) may be effective in the prevention and treatment of hormone-dependent cancers, particularly prostate cancer. Historically, in vivo studies using rodents have indicated that NSAIDs can decrease the size of a prostatic tumor and suppress the metastasis of prostatic cancer. More recently, NSAIDs (i.e., NS398, Cayman Chemical Co., Ann Arbor, MI), sulindac (Sigma Chemical Co., St. Louis, MO), celecoxib (Searle Ltd., Caguas, Puerto Rico) have been shown to induce apoptosis in both androgen-sensitive and androgen-insensitive prostate tumor cell lines, and reduce the invasiveness of human prostatic tumor cells in vitro. Retrospective clinical studies have indicated a reduced risk of prostate cancer associated with regular use of NSAIDs.

Recent evidence has indicated that nonsteroidal anti-inflammatory drugs (NSAIDs) have potential in the treatment and prevention of hormone-related maladies, such as prostate cancer. The mechanism for the anti-carcinogenic effects of NSAIDs is a subject of ongoing investigation. One of the proposed mechanisms is inhibition of the production of cyclooxygenase-2 (COX-2), one of two isoforms of cyclooxygenase. COX-2 is induced by growth factors, cytokines, oncogenes, and tumor promoters, and is up-regulated in transformed cells and malignant tumors. Recently, it has been reported that there is an over-expression of COX-2 in human prostate adenocarcinomas. Liu
et al. reported that treatment with 100 μM of the selective COX-2 inhibitor NS398 induced apoptosis of LNCaP cells and caused a down-regulation in bcl-2 protein expression. Hsu et al. (J Biol Chem 275:11397 (2000)), reported that while a specific COX-1 inhibitor, piroxicam, had no effect on LNCaP cells, the specific COX-2 inhibitor celecoxib-induced apoptosis of LNCaP cells. However, Hsu et al. provided evidence to indicate that celecoxib induced apoptosis by blocking the anti-apoptotic kinase Akt, and that this apoptotic effect was independent of bcl-2.

[0007] Other recent studies indicate that NSAIDs may inhibit tumorigenesis by inhibiting the peroxisome proliferator-activated receptor beta (PPARβ), a downstream transcription mediator for prostaglandins and fatty acids. This suppression of PPARβ appears to be mediated in part by the ability of NSAIDs to directly inhibit the DNA binding activity of PPARβ.

[0008] While these and other studies suggest the use of NSAIDs in the treatment of prostate cancer, heretofore it was unknown which conditions inhibit or enhance the anti-carcinogenic effectiveness of NSAIDs against hormone-dependent cancer cells. Also, it was therefore unknown that common non-prescription NSAIDs very effectively prevent or inhibit cancer cells, especially hormonally-related cancers such as prostate cancer.

[0009] Also while it has been suggested that some NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) appear to be effective in the prevention and treatment of prostate cancer, very little information exists on the comparative effects of common non-prescription NSAIDs.

SUMMARY OF THE INVENTION

[0010] The present invention involves the discussion that a novel combination of steroid hormone and NSAID was found to enhance tumor cell death. Particularly, of hormonally-dependent cancer cells, in particular, dihydrotestosterone (DHT) was discussed to significantly enhances the ability of the NSAID indomethacin to induce apoptosis of androgen sensitive human prostate cancer cells, such as the cell line LNCaP. The ability of DHT to promote the apoptotic activity of indomethacin was affected by the
concentration of DHT in the incubation medium both before and following addition of indomethacin. DHT does not promote indomethacin induced apoptosis in at least one androgen insensitive prostate cancer cell line. However, DHT does promote indomethacin induced apoptosis in other androgen insensitive prostate cancer cells, although to a significantly lesser extent than for androgen sensitive LNCaP cells.

[0011] Progesterone, another steroid hormone that interacts with the androgen receptor, also was found to promote the apoptosis of LNCaP cells. Cell cycle analysis indicates that the concentrations of DHT capable of promoting indomethacin induced apoptosis also prompt LNCaP cells to shift from the S and G2-M stages of the cell cycle to the G1 stage of the cell cycle. Western blot analysis for the COX-2 protein indicated no change in this protein following treatment with concentrations of DHT which promote apoptosis.

[0012] The foregoing findings indicate NSAIDs may be most effective against prostate cancer and other androgen related cancer in the presence of high concentrations of androgen. Treatments based on this concept of employing NSAIDS with high concentrations of androgen run counter to traditional clinical approaches to prostate cancer, wherein androgen ablation is a common procedure.

[0013] In the present invention, the inventors evaluated the effects of widely used non-prescription NSAIDs on human prostate cancer cells in vitro. Specifically, using in vitro models of androgen-sensitive and androgen-insensitive human prostate cancer cells; we evaluated the effects of acetaminophen, aspirin, naproxen and ibuprofen on cell survival, cell cycle and the induction of apoptosis were compared. Also compared were the effects of these drugs to the selective cyclooxygenase-2 (COX-2) inhibitor, NS-398. It was found that ibuprofen is significantly more effective against human prostate cancer cells in vitro than the other tested non-prescription NSAIDs. As discussed in detail infra, MTT and analysis indicated that clinically relevant concentrations of ibuprofen significantly reduce the survival of LNCaP human prostate tumor cells. Also, TUNEL analysis demonstrated that the same amount of apoptosis of an androgen independent human prostate cancer cell
line (i.e., DU-145), but had little effect on normal mouse fibroblast cells (i.e., 3T3 cells). Cell cycle analysis of LNCaP cells indicated that ibuprofen causes LNCaP cells to shift from the S and G2/M stages of the cell cycle into the G0/G1 phases of the cell cycle. Another propionic acid NSAID, naproxen, had a similar but lesser overall effect as ibuprofen. Supra-pharmacological concentrations of aspirin and acetaminophen did not induce comparable levels of apoptosis in LNCaP cells as clinically relevant concentrations of ibuprofen. The selective COX-2 inhibitor NS-398 mirrored the effectiveness of ibuprofen against LNCaP cells in vitro. However, when the pharmacokinetics of selective COX-2 inhibitors and other NSAIDs reported to be effective against prostate cancer are taken into consideration, ibuprofen appears to be one of the most effective NSAIDs at clinically relevant concentrations. As discussed in detail infra, as their observations support the use of ibuprofen in future in vivo studies and in clinical trials designed to test the effectiveness of NSAIDs against human prostate cancer.

[0014] Another preferred embodiment of the invention is a pharmaceutical composition comprising at least one nonsteroidal anti-inflammatory drug, at least one steroid hormone, a pharmaceutically acceptable carrier, and optionally, one or more excipients, wherein the at least one nonsteroidal anti-inflammatory drug and at least one steroid hormone are present in amounts sufficient to prevent and/or treat cancer, preferably by inducing tumor cell apoptosis. The least one nonsteroidal anti-inflammatory drug includes by way of example a member selected from the group consisting of ibuprofen, indomethacin, selective COX-2 inhibitors, and aspirin, and sildinac. The at least one steroid hormone of the composition may be dihydrotestosterone, progesterone, testosterone, estradiol, hydrocortisone or R1881 and fragments and derivatives thereof, and human agonists thereof.

[0015] In another preferred embodiment, the composition is capable of inducing cell death in cancerous cells from the prostate, breast, testicles, ovaries, uterus and colon. The composition may be any suitable formulation, including a powder, a solution, a gel, a syrup, a tablet or an implant. The formulation may permit delayed release of pharmaceutics over a period of time.
Such slow release formulations would serve to maintain titers of steroid hormone, NSAID, or both pharmaceutics for extended lengths of time.

[0016] Still another preferred embodiment is a method of inducing apoptosis of cancer cells comprising administering to a subject therapeutically effective amounts of at least one nonsteroidal anti-inflammatory drug and at least one steroid hormone. The at least one nonsteroidal anti-inflammatory drug may be indomethacin, aspirin, ibuprofen, selective COX-2 inhibitors, suldinac. The selective COX-2 inhibitor may comprise celecoxib. The at least one steroid hormone may be dihydrotestosterone, progesterone, testosterone, estradiol, hydroxycortisone, R1881 derivatives and fragments thereof, and agonists thereof. The at least one nonsteroidal anti-inflammatory drug and the at least one steroid hormone may be administered sequentially or concurrently.

[0017] In another preferred embodiment, the at least one nonsteroidal anti-inflammatory drug and the at least one steroid hormone may be administered via an injectable, oral, sublingual, topical or transdermal route. The at least one nonsteroidal anti-inflammatory drug and the at least one steroid hormone may be administered to a subject having a measurable tumor load. If desired, the one nonsteroidal anti-inflammatory drug and the at least one steroid hormone may be prophylactically administered to a subject having no measurable tumor load.

[0018] Still another preferred embodiment of the invention involves the treatment and/or prevention of cancers, especially hormonally-related cancers such as prostate, breast, ovarian, testicular or uterine cancer, by the administration of a prophylactically or therapeutically effective amount of a non-prescription NSAID selected from acetaminophen, aspirin, naproxen and ibuprofen, or a combination thereof, preferably ibuprofen.

[0019] In an especially preferred embodiment persons at risk of developing such hormonally-related cancers will be treated with a prophylactically effective amount of a non-prescription NSAID selected from acetaminophen, aspirin, naproxen and ibuprofen, especially ibuprofen.
BRIEF DESCRIPTION OF FIGURES

[0020] The invention will be further illustrated by the following Figures, wherein:

[0021] Figure 1a depicts a bar graph representing the percentage of apoptosis exhibited by LNCaP cells following incubation in different concentrations of DHT minus and plus NS-398;

[0022] Figure 1b depicts a bar graph representing the percentage of apoptosis exhibited by LNCaP cells following incubation in different concentrations of DHT minus and plus indomethacin;

[0023] Figure 2a depicts a scanning micrograph of LNCaP cells that have been treated for 24 hours with indomethacin (100 μM) in the presence of 10^{-9} M DHT;

[0024] Figure 2b depicts transmission electron of LNCaP cells that have been treated for 24 hours with indomethacin (100 μM) in the presence of 10^{-9} M DHT;

[0025] Figure 3a depicts a scanning electron micrograph of LNCaP cells that have been treated for 24 hours with indomethacin (100 μM) in the presence of 10^{-4} M DHT;

[0026] Figure 3b depicts a transmission electron micrograph of LNCaP cells that have been treated for 24 hours with indomethacin (100 μM) in the presence of 10^{-4} M DHT;

[0027] Figure 4 shows a graph depicting the percent (%) apoptosis seen in LNCaP cells pretreated with high concentrations of DHT (designated as pre^{-5} and pre^{-4}, respectively, in the X axis), versus LNCaP cells which were only post-treated with high concentrations of DHT (designated as post^{-5} and post^{-4}, respectively, in the X axis) prior to being exposed to DHT plus indomethacin (100 μM);

[0028] Figure 5 depicts the percentage of apoptosis seen in three cell lines (i.e. PC-3, DU-145 & LNCaP) following incubation for 48 hours in indomethacin (100 μM) without DHT (blue bars) and with DHT (10^{-4} M) (yellow bars); and
[0029] Figure 6 is a bar graph depicting the percentage apoptosis observed for LNCaP cells following incubation for 48 hours in a variety of steroid hormones in the presence and absence of 100 μm indomethacin.

[0030] Figure 7 MTT analysis of cell numbers (i.e., survival) of LNCaP cells following 48 hours of incubation in 1mM of acetaminophen (Acet), aspirin (Asp), naproxen (Nap), ibuprofen (Ibu) or 0.1mM of NS-398. Ibuprofen and the selective COX-2 inhibitor NS-398 produced the greatest reduction in number of viable cells. The error bars represent standard deviations.

[0031] Figure 8 This graph summarizes TUNEL analysis of LNCaP cells following 48 hours of incubation in selected concentrations acetaminophen (Acet), aspirin (Asp) naproxen (Nap), ibuprofen (Ibu) and NS-398. While aspirin and even acetaminophen induced apoptosis at supra-pharmacological concentrations (i.e., 5mM), ibuprofen and naproxen induced significant apoptosis at clinically relevant concentrations (i.e., 1mM). The error bars represent one standard deviation.

[0032] Figure 9 This figure represents a graph of TUNEL assays performed on DU-145 cells following 48 hours of incubation in the presence and absence (controls) of 1mM ibuprofen. Note that 1mM ibuprofen induces a similar increase in the percentage of apoptosis of DU-145 over controls as it does in LNCaP cells (See Figure 2). The error bar represents one standard deviation.

[0033] Figure 10 This graph summarizes the % apoptosis resulting from incubation of 3T3 cells in selected NSAIDs. Note that of the NSAIDs tested, acetaminophen and to lesser extent NS-398 induced a significant increase in apoptosis of 3T3 cells compared with controls (i.e., p<0.05). The error bars represent one standard deviation.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0034] The invention relates in part to the discovery of the synergistic ability of NSAIDs, such as NS-398 or indomethacin, to induce apoptosis of hormone sensitive human cancer cells in the presence of high concentrations of a steroid hormone. This observation represents the first demonstration that a naturally occurring steroid hormone can significantly affect the ability of NSAIDs to
induce apoptosis of cancer cells. Although there have been reports that chemotherapeutic drugs, such as the anthracyclines (e.g., doxorubicin) and vincristine, can work synergistically with NSAIDs to enhance the apoptosis of cancer cells, the generalized toxic side effects of these chemotherapeutic agents limit their usefulness.

[0035] The steroid hormone, DHT, however, appears to focus its apoptotic enhancing effects on proliferating cells having androgen receptors, thereby targeting a specific cell population (i.e., prostate cancer cells). In view of the dramatic nature of this synergistic effect between DHT and indomethacin, and the facts that both the onset of prostate cancer and current treatment regimens are associated with reduced levels of DHT, these observations have very important implications regarding future modalities of treatment and prevention of prostate cancer with NSAIDs.

[0036] Nonsteroidal anti-inflammatory drugs (i.e., NSAIDs) are expected to be strategic players in regimes for the prevention and treatment of many forms of cancer, including prostate cancer. A normally occurring steroid hormone (i.e., DHT) can potentiate the ability of the NSAID indomethacin to induce programmed cell death of a human prostatic cancer cell line (LNCaP). This synergistic effect of DHT in promoting indomethacin-induced apoptosis is very dramatic, with the percentage of apoptosis being affected by the concentration of DHT in the incubation medium both before and following addition of indomethacin. The percentage of apoptosis induced in LNCaP cells by combining high concentrations of DHT with NS-398 treatment is greater than that recently reported for several human colorectal adenoma and carcinoma cell lines which were treated for twice as long (i.e., 96 hours) with the same concentration (i.e., 100 μM) of NS-398. The latter is relevant in that NSAIDs have been proven to be effective against colorectal cancer in epidemiological, clinical and experimental animals studies.

[0037] It should be noted that although the concentrations of DHT used in the current studies were high, this hormone is very rapidly metabolized by LNCaP cells in vitro, with less than one-third of the initial DHT reported to be present in culture media following three hours of incubation. Therefore, the measurable
amount of DHT in culture media after 48 hours is significantly reduced relative to the amount that was originally added. This aspect of the hormone must be considered when selecting an effective amount of DHT for use in vivo, wherein the metabolized DHT would be replenished by re-circulating DHT.

[0038] Additional studies have shown that different NSAIDs vary in their ability to inhibit growth of prostate cancer cells. Also, investigations have shown that the ability of DHT to activate the androgen receptor can be significantly enhanced through the synergistic action of agents other than NSAIDs, although the clinical usefulness of combining such agents with androgen has yet to be determined.

[0039] Therefore, the amount of hormone required to work synergistically with differing NSAIDs, must be assessed for individual combinations of these medicaments in view of the type of hormone-dependent cancer cells targeted. Such assessments are well within the purview of a professional skilled in the art.

[0040] In the present invention, Western blot analysis of LNCaP cells treated with high concentrations of DHT indicated that this procedure causes no change in the expression of COX-2 in LNCaP cells. It may be possible, therefore, that DHT is not enhancing apoptosis through mechanisms similar to the way in which NSAIDs induce apoptosis. Also pertinent is the finding that the concentrations of DHT capable of promoting NSAID-induced apoptosis also shift exposed cells into the GO/G1 stage of the cell cycle. This is in line with similar observations by others, particularly studies that have shown a reduction in LNCaP growth rate is associated with high concentrations of DHT.

[0041] The present invention also encompasses the discovery that in addition to DHT, progesterone, but not hydrocortisone, promotes indomethacin-induced apoptosis. The finding that progesterone behaves in a manner similar to DHT may be explained potentially by the fact that LNCaP cells possess a mutated androgen receptor with a high affinity for progesterone. Furthermore, the presence of progesterone, like DHT, both stimulates and inhibits the growth of LNCaP. Observations that PC-3 cells do not respond to indomethacin-induced apoptosis in the presence of high concentrations of DHT also suggests that the
androgen receptor may be needed to elicit this synergistic response. Although DU-145 cells, which are believed to be androgen insensitive, when subject the same in vitro tests, exhibited increased apoptosis in the presence of DHT, the level of apoptosis exhibited was significantly less than that seen in LNCaP cells. The latter results potentially may be explained by studies indicating that DU-145 cells exhibit a reduced level of androgen receptor. Nevertheless, the foregoing observations indicate that the role of the androgen receptor in promoting the synergistic effect between DHT and NSAIDs may be a key to improved therapy for androgen-dependent pathologies, such as prostate cancer.

[0042] Also, another aspect of the invention relates to the discovery that certain non-prescription NSAIDs such as ibuprofen are significantly more effective in reducing the survival of human prostate cancer cells in vitro than the other non-prescription NSAIDs tested. Ibuprofen appears to accomplish this by inducing apoptosis and causing cancer cells to shift from mitotic to resting stages of the cell cycle. Ibuprofen proved effective against both androgen dependent (i.e., LNCaP) and androgen independent human prostate cancer cells (i.e., DU-145), with no effect on normal cells (i.e., 3T3 cells). These observations are in line with other investigations, which have reported that NSAIDs are effective in reducing the survival of both androgen dependent and independent human prostate cancer cells, but have little effect on normal cell lines (Hsu et al., J Biol Chem 275:11397 (2000)), (Kamijo et al., Int J Urol 8: S35 (2001)), (Lim et al., Biochem Pharm 58:1097 (1999)), and (Liu et al. Cancer Res 58:4245 (1998)). Of particular significance, however, is that ibuprofen is able to induce these effects at clinically relevant concentrations. Following oral administration of maximum clinically acceptable dosages of ibuprofen (i.e., up to 3200 mg/day), pharmacokinetic studies indicate that the levels of ibuprofen shown to be effective in this study (i.e., 0.5 to 1 mM ibuprofen) would be present in the blood (Konstan et al., N Engl J Med. 332:848 (1995)), (Lockwood et al., Clin Pharmacol. Ther. 34:97 (1983)) and (Moote, CA (Clin Drug Invest 11:1 (1996)). Symptoms of ibuprofen overdose are unlikely after ingestion of up to 100 mg/kg body weight, and are usually not
life-threatening unless more than 400 mg/kg is ingested (Smolinske et al., Drug Saf 5: 252 (1992)). Relevant to patients with prostate cancer is that advanced age has very little effect on the pharmacokinetics of ibuprofen (Albert et al., Am J Med 77:47 (1984)). Also, ibuprofen is one of the least damaging of the non-selective cyclooxygenase inhibitors to the gastric mucosa (Warner et al., Proc Nat Acad Sci 96:7563 (1999)), and patients can be maintained on high doses of ibuprofen for years without serious adverse effects (Konstan et al., N Engl Med 332:848 (1995)). Since this circulating ibuprofen would be effective against distant metastatic sites as well as the localized cancer, additional studies of ibuprofen and its analogs are warranted in order to determine the possible clinical significance of these observations, especially in view of the poor prognosis of patients who have developed androgen independent metastatic disease.

[0043] Also, we found significant decreases in both cell survival and apoptosis by 48 hours following incubation in either ibuprofen or naproxen. In recent years, selective COX-2 inhibitors (i.e., rofecoxib, celecoxib) have become popular as the "next generation of NSAIDs" due to their ability to selectively inhibit cyclooxygenase-2 while not affecting the protective "housekeeping" effects of cyclooxygenase-1 (i.e., maintenance of gastrointestinal tract lining and renal function) (Taketo et al., Nat. Cancer Inst 90: 1609 (1998)) and (Vane et al., Ann. Rev. Pharmacol. Toxicol 38:97 (1998)). Therefore, the present invention, we compared the effectiveness of an experimental selective COX-2 inhibitor (i.e., NS-398, which is not for use in humans) with other widely used non-prescription NSAIDs. Our finding that 0.1mM of NS-398 reduces the number of viable LNCaP cells by about 28% after two days of incubation, is similar to the results of Liu et al. (Liu et al., Cancer Res 58:4245 (1998)), who reported an approximately 32% decrease in the number of viable LNCaP cells following incubation of these cells for two days in the same concentration of NS-389. These researchers also reported that NS-398 induced apoptosis is associated with a down-regulation of Bcl-2 protein expression, thereby indicating a possible mechanism of action of NS-398. Kamijo et al. (Kamijo et al. Int J Urol 8:S35 (2001)) compared the effects of NS-398 with a clinically
available selective COX-2 inhibitor, Etodolac, on LNCaP and PC-3 human prostate cancer cells. Using the MTT analysis, they reported a time and dose dependent response of both selective COX-2 inhibitors in reducing survival of these prostate cancer cells, while not affecting a normal prostate stromal cell line (PRSC). They also found that NS-398 reduced survival more than Etodolac, despite the fact that Etodolac is a more effective cyclooxygenase-2 inhibitor than NS-398. Recently, Hsu et al., (Hsu et al. J Biol Chem 275:11397 (2000)) reported that the selective COX-2 inhibitor celecoxib is more effective against LNCaP and PC-3 human prostate cancer cell lines than piroxicam (from the oxicam family of NSAIDs), as well as the selective COX-2 inhibitors NS-398, rofecoxib and DuP697. Using trypan blue exclusion to evaluate cell viability, they reported that concentrations of celecoxib as small as 10uM would result in a reduction in cell viability. However, pharmacokinetic studies indicate even supratherapeutic concentrations of this drug (e.g., 800 mg/day) result in less than 10 uM of this drug in the blood (Davies et al., Clin Pharmacokinet 38:225 (2000)). These researchers also provided evidence that the action of celecoxib is not associated with Bcl-2 expression, but rather involves blocking the activation of the anti-apoptotic kinase Akt (i.e., protein kinase B). Therefore, when considering the data reported thus far, it unexpectedly appears that a less expensive non-prescription NSAID (i.e., ibuprofen) might be one of the most useful NSAIDs against prostate cancer, and potentially other cancers, especially hormonally-related cancer because more effective blood serum levels are obtainable following administration of maximum dosages.

[0044] The importance of comparing clinically relevant dosages of ibuprofen with other NSAIDs is illustrated by a recent in vivo study that compared the effectiveness of celecoxib with ibuprofen in a model of breast cancer (Haris et al., Cancer Res 60:2101 (2000)). In this study, female rats were placed on diets containing equal concentrations of celecoxib or ibuprofen (i.e., 1500 mg/kg) and then were treated with dimethylbenz(a)anthracene to induce breast tumors. While both NSAIDs produced striking reductions in the incidence, multiplicity, and volume of breast tumors relative to control groups, the mean blood serum levels at the end of the experiments were 5.1 ug/ml for celecoxib and 8.0 ug/ml
for ibuprofen. Although these investigators concluded that celecoxib appeared more effective than ibuprofen, the blood serum level of celecoxib represented more can be achieved with maximum dosages of this NSAID in man, while the blood serum level of ibuprofen was dramatically less than can be achieved following acceptable high dosages of this drug in man (Konstan et al., N Engl J Med 332: 848 (1995)), (Lockwood et al, Clin Pharmacol. Ther 34: 97 (1983)) and (Moote et al., Clin Drug Invest 11:1 (1996)). Therefore, contrary to the investigators’ conclusions, the results of the present invention suggest that ibuprofen may be more effective against human breast cancer when clinically relevant dosages are taken into consideration.

Previously, (Rotem et al., Prostate 42:172 (2000)) had evaluated the effects of aspirin on LNCaP cells. In their study, Rotem et al. observed that aspirin decreased cell proliferation of prostate cancer cells, but observed no effect of aspirin on cell death. Our data regarding survival of LNCaP cells following incubation in 1mM of aspirin mirrored the results of this group. Our results on cell cycle analysis also indicated, like Rotem et al., no statistically significant effects from incubation in 1mM of aspirin. Although Rotem et al. also evaluated a higher dosage of aspirin (i.e., 2mM), they reported no increase in cell death compared with the control group using the trypan blue exclusion procedure (Rotem et al., Prostate 42:172 (2000)). When we evaluated a suprapharmacological concentration of aspirin (i.e., 5mM), however, the TUNEL procedure reveals a significant increase in apoptosis of LNCaP cells when compared with controls. Therefore, contrary to what had been reported, aspirin also may be used to promote cancer cell death.

The mechanisms associated with the effectiveness of NSAIDs against cancer cells remain an area under investigation. One of the proposed mechanisms is inhibition in the production of cyclooxygenase-2 (COX-2), one of two isoforms of cyclooxygenase. COX-2 is induced by growth factors, cytokines, oncogenes, and tumor promoters, and is up regulated in transformed cells and malignant tumors (Herschman, et al., Annu Rev Biochem 60: 281 (1991)) and (Mitchell et al, Proc Natl Acad Sci USA 90:11693 (1994)). In the present invention, the effectiveness of the non-prescription NSAIDs correlated
with their ability to inhibit COX-2 activity. That is, ibuprofen > naproxen > aspirin > acetaminophen in inhibiting COX-2 activity (Kawai et al., Eur J Pharmacol 347:87 (1998)) and Warner et al., Proc Natl Acad Sci 96: 7563 (1999)). However, there is accumulating evidence indicating that effectiveness of NSAIDs against cancer cells is due to cyclooxygenase-2 independent mechanisms. As noted above, sulindac sulfone is effective against prostate cancer cells despite not having cyclooxygenase-2 inhibitory ability (Lim et al., Biochem Pharm 58: 1097 (1999)). NSAIDs still have anti-neoplastic effects when used against COX-1 and 2 null mouse embryo fibroblasts cells (Zhang et al., J Exp Med 190: 451 (1999)). The concentrations of NSAIDs that inhibit growth are often 10 to 100 times higher than are required to inhibit cyclooxygenase activity (He et al., Cell 9: 335 (1999)). Finally, while a number of studies have indicated an over expression of COX-2 in prostate cancer (Gupta et al., Prostate 42:73 (2000)), Kirschenbaum et al., Urology 56:671 (2000)), Kirchenbaum et al., Urology 58:127 (2001)) and Yoshimura et al. Cancer 89: 589 (2000)) a recent study has not supported these findings and has indicated that prostate cancer cells lines (i.e., LNCaP, DU-145, PC-3 and TSU) do not express detectable levels of COX-2 protein under basal conditions (Zha et al., Cancer 61:8617 (2001)). These and other studies have provided evidence for number of alternative mechanisms to explain the anti-cancer effects of NSAIDs. As noted above, (Liu et al., Cancer Res 58:4245 (1998)) have indicated the possible involvement of Bcl-2, while (Hsu et al., Cancer Res 58: 4245 (1998)) have indicated the possible involvement of Akt. Others have suggested the mechanisms underlying the anti-cancer action of NSAIDs may involve the expression of the apoptotic proteins Bax and Bcl-xl (Zhang et al., Science 290:989 (2000)), inhibiting the expression of peroxisome proliferator-activated receptor beta (i.e., PPAR-beta) (He et al., Cell 99: 335 (1999)), induction of arachidonic acid elevation leading to the production of ceramide (Cao et al., Proc Natl Acad Sci 97: 11280 (2000)) and Chan et al., Proc Natl Sci 95: 681 (1998)) or inhibiting the transcription factor NF-kappaB by blocking the phosphorylation and degradation of the NF-kappaB inhibitor IkBa (Palayoor et al., (1999)). Although the present invention does not establish the mechanistic
aspects of NSAIDs, the fact that high concentrations of ibuprofen were found to be increasingly effective despite the fact that these concentrations were well above those required to inhibit cyclooxygenase activity (Warner et al., Proc Nat Acad Sci 96:7563 (1999)), argues for a non-COX-2 mechanism for this NSAID as well.

**[0047]** Although acetaminophen is considered a relatively weak NSAID (Botting et al., Clin Infect Dis 312:S202 (2000)), it was tested because of its widespread use and in view of recent evidence indicating its effectiveness against brain (Casper et al., Neurooncol 46:215 (2000)) and ovarian cancers (Moysich et al., Cancer Epidemiol Biomarkers Prev 10:903 (2001)). In our study, acetaminophen was the least effective of the NSAIDs against human prostate cancer cells in vitro. The toxicity of acetaminophen to normal mouse fibroblasts was an unexpected finding. Acetaminophen is well-known to be toxic to hepatocytes due to generation of the toxic metabolite N-acetyl-p-benzoquinoneimine, leading to depletion of intracellular glutathione, alteration of redox potential and ultimately cell death. However, acetaminophen has other toxic effects including inhibition of ribonucleotide reductase (Hongso et al., Mutagenesis 5:475 (1990)) and nucleotide excision repair (Hongso et al., Mutagenesis 9:93 (1994)). Recently, Rocha et al. (Rocha et al., PNAS 98:5317 (2001)) reported that acetaminophen is toxic to inner medullary collecting duct cells in vitro, implicating it in the papillary necrosis which results from chronic ingestion of combinations of NSAIDs. Despite the toxicity of acetaminophen to 3T3 cells, ibuprofen and aspirin did not produce statistically significant increases in the apoptosis of 3T3 cells. Although NS-398 showed a statistically significant increase in apoptosis when compared with controls, this increase was not dramatic. As noted previously, others have reported that most NSAIDs that have been found to decrease the survival of prostate cancer cells appear to have little or no effect on normal cell lines (Hsu et al., J Biol Chem 275:11397 (2000)), (Kamijo et al., Int J Urol 8:S35 (2001)), (Lim et al. Biochem Pharm 58: 1097 (1999)) and (Liu et al., Cancer Res 58: 4245 (1998)). Nevertheless, the variable effects of NSAIDs on 3T3 cells seen in the present
study, underlines the specificity of the toxic effects of different NSAIDs to different cell lines.

[0048] It should be added, that in addition to their ability to inhibit proliferation and induce apoptosis of prostate cancer cells, NSAIDs also appear to inhibit the metastasis of these cells (Attiga et al., Proc Am Assoc Can Res 41: 131 (2000)) and angiogenesis associated with prostate tumors (Liu et al., Clin Exp Metastasis 17: 687 (1999)) and Masferrer et al., Cancer Res 60: 1306 (2000)). Attiga et al. (Attiga et al., Proc Am Assoc Can Res 41: 131 (2000)) reported that both ibuprofen and NS-398 inhibit human prostate cancer cells (i.e., DU-145 and PC-3) from passing through Matrigel. This inhibition was associated with a decrease in the secretion of metalloproteinases, key enzymes in the proteolysis of Matrigel during invasion. Liu et al. (Liu et al., Clin Exp Metastasis 17: 687 (1999)) have presented data indicating that NS-398 inhibits the angiogenesis of human prostate tumors induced in mice by injection of PC-3 cells. These researchers provided evidence that this anti-angiogenesis effect may be due to a down regulation of tumor vascular endothelial growth factor (VEGF) expression (Liu et al., Clin Exp Metastasis 17: 687 (1999)).

[0049] Previously, NSAIDs have been reported to work synergistically with other chemotherapeutic drugs such as the anthracyclines (e.g., doxorubicin) and vincristine to enhance the apoptosis of cancer cells (Duffy et al., Eur J Cancer 34: 1250 (1998)) and (Roller et al., Biochem Biophys Res Commun 259:600 (1999)). Also, ibuprofen has been shown to have a combined antitumor effect when used with radiation on human prostate cancer cells (Palayoor et al., Clin Cancer Res 4: 763 (1998)). Since this enhanced radiation response requires ibuprofen concentrations that are higher than those reported to inhibit prostaglandin synthesis, Palayoor et al. also hypothesized that other molecular mechanisms might be responsible for this effect (Palayoor et al., Clin Cancer Res 4: 763 (1998)). However, this was only hypothesized, as this group did not contact a separate analysis of the effective different NSAIDs including ibuprofen as herein.

[0050] In summary, while recent studies have suggested that NSAIDs can decrease the risk of prostate cancer and may be useful in the treatment of this
disease, the present *in vitro* investigation suggests that, ibuprofen exhibits unexpected results in treating and preventing cancers, especially hormonally-dependent cancers such as prostate cancer.

[0051] With respect to future treatment regimens, the observations discussed herein indicate that NSAIDs may be most effective against hormone-dependent pathologies alone or in the presence of excess androgen. Therefore, a treatment regimen involving the acute administration of androgen together with NSAIDs may be a reasonable alternative to or adjunct therapy for such pathologies, particularly prostate cancer, especially in view of the lack of success of androgen ablation.

[0052] In addition to androgen-dependent cancer cells, other hormone-dependent cancer cells are contemplated as being susceptible to combination treatments comprising steroid hormones and NSAIDs. Cancer cells exhibiting growth dependencies on progesterone, testosterone, estradiol, hydroxycortisone, and R1881 (methyltrienolone), for example, are also candidates for combination treatments comprising steroid hormones and NSAIDs. Evidence exists linking a substantially reduced rate of breast cancer to women with a history of extended intake of ibuprofen. Thus, including one or more steroid hormones in a chemotherapeutic regimen comprising at least one NSAID is contemplated for treating such pathologies as testicular cancer, ovarian cancer, breast cancer, uterine cancer, colon cancer, and the like.

[0053] Clinical use of combined steroid hormone and NSAID therapies to induce cell death is envisioned to embrace a number of formulations and routes of administration. Patients with measurable tumor loads may be dosed with an amount of steroid hormone known to raise serum titers to levels predetermined to have maximal synergistic effects with NSAIDs against the types of tumor cells being targeted. For example, a patient suffering from prostate cancer may be dosed with an amount of DHT capable of raising serum titers to a range of $10^{-6}$ to $10^{-7}$ molar. Likewise, NSAIDs may be administered to patients receiving dosages of steroid hormones, the NSAIDs being administered in an amount having a therapeutic effectiveness against hormone-dependent cells, the effectiveness known to be synergistically enhanced by these steroid hormones.
Typical dosages of NSAIDs for use in combination with steroid hormones, such as androgens, may range from 0.1 μM to 5 mM.

[0054] Patients having no measurable tumor load may be dosed with the inventive combination of a steroid hormone and an NSAID to prophylactically prevent tumor development. Such treatment would be useful in, for example, prophylactic treatment of metastatic disease after a primary tumor has been excised from a patient. Similarly, patients susceptible to new primary tumor development, such as breast cancer patients, would benefit from prophylactic administration of a composition having at least one steroid hormone and at least one NSAID.

[0055] Steroid hormones may be administered to a patient prior to administration of one or more NSAIDs, together with the NSAIDs, or both. Optionally, the NSAID may be administered prior to the hormone. Compositions containing effective amounts of one or more steroid hormones, one or more NSAIDs, optional excipients, and a pharmaceutical carrier may be used to target hormone-dependent cancer cells for apoptotic-induced cell death. Non-hormone-dependent cells may also be susceptible to such compositions as well, particularly those for which hormones are partial agonists.

[0056] Pharmaceutically acceptable compositions containing therapeutically effective amounts of one or more steroid hormones and one or more NSAIDs may be administered by a clinically relevant route, including intraperitoneal, intrathecal, intravenous intramuscular and subcutaneous injection. Oral, sublingual, topical and transdermal routes of administration are also acceptable.

[0057] Compositions containing effective amounts of one or more steroid hormones and one or more NSAIDs may be formulated as powders, solutions, gels, syrups, tablets, implants, and the like. Such formulation may be engineered to deliver hormones and NSAIDs to target cells in vivo over a period of time. For example, a steroid, an NSAID, or both may be packaged within a liposome in a composition for delayed release over a period of time in a patient.
[0058] Suitable non-steroidal anti-inflammatory compounds selected from the
 group consisting of aspirin, a non-steroidal anti-inflammatory acetic acid, a
 fenamate, include these sodium salicylate, acetaminophen, phenacetin,
 ibuprofen, ketoprofen, indomethacin, flurbiprofen, naproxen, piroxicam,
 tebufelone, etodolac, nabumetone, tenidap, alcofenac, antipyrine,
 amimopryrine, dipyrone, animopyrone, phenylbutazone, clofezone,
 oxyphenbutazone, prexazone, apazone, benzydamine, bucolome, cinchopen,
 clonixin, ditrazol, epirizole, fenoprofen, floctafenin, flufenamic acid, glaphene,
 indoprofen, meclofenamic acid, mfenamic acid, niflumic acid, salidifamides,
 sulindac, suprofen, tolmetin, nabumetone, tiaramide, proquazone, bufexamac,
 fiumizole, tinoridine, timegadine, dapsone, difunisal, benorylate, fosfosyal,
 fenclofenac, etodolac, fentiazac, tilomisole, carprofen, fenbufen, oxaprozin,
 tiaprofenic acid, pirprofen, feprazone, piroxicam, sudoxicam, isoxicam,
 celecoxib, Vioxx.RTM. and tenoxicam.

[0059] The invention is further illustrated by the following non-limiting
 Examples.

EXAMPLES

EXAMPLES 1-6

Materials and Methods

[0060] Cell Cultures: The LNCaP, PC-3 and DU-145 cell lines were
 purchased from the American Type Culture Collection (Rockville, MD). The
 LNCaP cell line is an androgen responsive human prostate adenocarcinoma
 which was isolated from a biopsy of a lymph node aspirated from a patient with
 a confirmed diagnosis of metastatic prostate carcinoma. The PC-3 and DU-145
 cell lines are androgen insensitive human prostate cancer cell lines. The cells
 were grown in flasks containing 55 fetal calf serum-supplemented Delbecco's
 Modified Eagle's Medium (DMEM from Mediatech, Herndon, VA), were seeded
 onto six-well cluster dishes. Culture dishes were kept in a humidified
 atmosphere of 10% CO₂ in air at a temperature of 37°C. Once the cells had
 grown to approximately 50% confluence, they were washed with phosphate
buffered saline and the medium changed to phenol red-free medium containing 5% dextran-coated charcoal treated (i.e., steroid-depleted) fetal calf serum plus different concentrations of DHT.

[0061] **TUNEL Analysis of Apoptotic Cells:** The cells were preserved (i.e., fixed) for TUNEL assay by the addition of 10% neutral formalin (2 ml per well). The fixed cells were harvested by scraping an analyzed using an *in situ* apoptosis detection kit (ApopTag from Intergen co, Purchase, NY) according to the manufacturer's instructions (TUNEL method). Labeled cells were examined and counted using a OM-2 Olympus microscope equipped for fluorescence. Cell counts were undertaken blindly by two independent observers and statistical analysis performed using ANOVA. Significance between groups was determined using Student's t test with a p value < 0.05 being considered statistically significant.

[0062] **Electron Microscopic Analysis:** For transmission electron microscopy, the cells were fixed overnight in 2% phosphate buffered glutaraldehyde, post-fixed for 45 minutes in 1% phosphate buffered osmium tetroxide, dehydrated and embedded in Spurr medium. Ultrathin sections (20-50 nm) were mounted on grids, stained with uranyl acetate and lead citrate, and examination with a JEOL transmission electron microscope operating at 60kV. For scanning electron microscopy, the cells were fixed overnight in 2% phosphate buffered glutaraldehyde, post-fixed in 1% phosphate buffered osmium tetroxide, dehydrated in acetone, dried using critical point method, mounted on stubs, coated with a thin layer of palladium/gold in a sputter coater (Hummer X), and viewed and photographed using a Hitachi Model 570 Scanning Electron Microscope operating at 15 kV.

[0063] **Cell Cycle Analysis:** The cells were washed twice in PBS, trypsinized, 1-2 x 106 cells were pelleted in triplicate by centrifugation and resuspended in 100uL citrate buffer (40 mM trisodium citrate-2H2O, 250 mM sucrose, and 5% DMSO, pH 7.6). Nuclei were prepared for flow cytometric cell cycle analysis using the method of Vindelov et al. (15), with propidium iodide as the stain for nucleic acid. Cell cycle analysis was performed using the FACStar Plus
fluorescence-activated ModFit cell cycle analysis program (Verity Software House, Topsham, Main). Histograms represent 20,000 events.

[0064] **Western Blot Analysis:** Protein was obtained from the treated cells by incubation in lysis buffer consisting of PBS, 1% Igepal CA-630 (Sigma Chemical Co., St. Louis, MO), 2 μg/ml aprotinin (Sigma Chemical Co., St. Louis, MO), and 2 μg/ml leupeptin (Sigma Chemical Co., St. Louis, MO). Each protein sample (50 μg) was run on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose (Amersham Life Sciences, Inc., Arlington Heights, IL). The nitrocellulose was blocked in 5% non-fat milk in PBS for 1 hour, rinsed twice with TTBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5, 0.1% SDS), incubated overnight at 4°C with a goat polyclonal anti-COX-2 antibody (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TTBS. The blots were washed twice for 5 minutes each in TTBS and incubated with a horseradish peroxidase conjugated anti-goat or anti-mouse IgG (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) in TTBS for 1 hour at room temperature, rinsed twice in TTBS for 5 minutes each and finally for 5 minutes in TBS. Immunoreactivity was visualized with Opti-4CN (BioRad, Richmond, CA).

**Example 1**

[0065] DHT was found to promote the ability of indomethacin to induce apoptosis of LNCaP cells. Figures 1a and 1b summarize the extent of apoptosis which occurs when LNCaP cells are preincubated in the presence of 10^{-7} M DHT for 48 hours, and then incubated in either low or high concentrations of DHT plus NS-398 (100 μM, as shown in Figure 1a) or indomethacin (100 μM, as shown in Figure 1b) for 48 hours. Figures 1a and 1b depict bar graphs representing the percentage of apoptosis exhibited by LNCaP cells following incubation in different concentrations of DHT minus and plus NS-398 or indomethacin. The error bars represent one standard deviation.

[0066] When incubated in media containing NS-398 or indomethacin plus 10^{-9} M DHT, LNCaP cells exhibited 10% and 2% apoptosis, respectively (Figures 1a and 1b). When incubated in NS-398 or indomethacin in the
presence of higher concentrations of DHT, the extent of apoptosis significantly increased when compared with control samples (p>0.05). When incubated in NS-398 or indomethacin in the presence of higher concentrations of DHT, the extent of apoptosis significantly increased, when compared with controls (p>0.05). Although the foregoing results were seen in LNCaP cells cultured in phenol red-free medium containing 5% dextran-coated charcoal treated (i.e., steroid-depleted) fetal calf serum stripped serum, it should be noted that similar results were seen in LNCaP cells cultured in non-stripped serum plus media containing phenol red.

**Example 2**

[0067] Electron microscopic analysis confirmed the apoptosis of LNCaP cells discussed in Figure 1. As is illustrated in Figures 2a through 3b, electron microscopy reveals that LNCaP cells exposed to high concentrations of DHT plus indomethacin undergo dramatic ultrastructural alterations. When incubated in indomethacin (100 μM) plus a low concentration of DHT (10^{-6} M), LNCaP cells appeared as irregularly shaped, flattened, elongate cells, with extended cell processes, irregularly shaped nuclei, dispersed chromatin, and a normal complement of cytoplasmic organelles (2a, 2b). Figures 2a and 2b depict scanning (2a) and transmission electron (2b) micrographs of LNCaP cells that were treated for 24 hours with indomethacin (100 μM) in the presence of 10^{-6} DHT. The LNCaP cells appeared as elongate flattened, spindle-shaped cells with extended cell processes, euchromatic nuclei (N), and a normal complement of cytoplasmic organelles. Magnification was as follows: for 2a, x 425, and for 2b, x 4,300.

[0068] When exposed to indomethacin (100 μM) plus high concentrations of DHT (10^{-4}), however, LNCaP cells were transformed into small, rounded cells with rounded nuclei containing highly condensed clumps of chromatin (3 a, 3b). Figures 3a and 3b depict scanning (3a) and transmission electron (3b) micrographs of LNCaP cells that were treated for 24 hours with indomethacin (100 μM) in the presence of 10^{-4} DHT. The LNCaP cells transformed into smaller rounded cells, with nuclear condensation characteristic of cells in late
stages of apoptosis (large arrows). An apoptotic body (small arrow) is also evident. Magnification: for 3a, x 425, and for 3b, x 5,400.

**Example 3**

[0069] Prior exposure of LNCaP cells to high concentrations of DHT promotes the apoptotic effects of indomethacin. The effects of incubation for 48 hours in either no DHT or high concentrations of DHT prior to the addition of indomethacin to the incubation media are summarized in Figure 4, wherein error bars represent one standard deviation. When LNCaP cells were cultured in the absence of DHT for 48 hours and then cultured in the presence of high concentrations of DHT plus indomethacin (designated as “post”), the mean percentage of apoptosis was 0.5% and 2% in $10^{-5}$ and $10^{-4}$ M DHT, respectively. However, when LNCaP cells were incubated in $10^{-4}$ M DHT for 48 hours (designated as “pre”) and subsequently incubated in high DHT plus indomethacin, the mean percentage of apoptosis increased to 9% and 91% in $10^{-5}$ and $10^{-4}$ M DHT, respectively.

[0070] These results indicate that prior exposure of LNCaP cells to high concentrations of DHT predisposes these cells to subsequent indomethacin induced apoptosis. Note that the latter values are significantly higher than those seen when LNCaP cells were cultured for 48 hours in media containing $10^{-7}$ DHT for 48 prior to being incubated in high concentrations of DHT ($10^{-4}$ M) plus indomethacin. See Figure 1. Also, unlike the latter studies, pre-incubation and post-incubation in high concentrations of DHT resulted in an increase in the percentage of apoptosis from exposure from exposure to DHT alone ($p<0.005$). It therefore appears that long term exposure of LNCaP cells to very high concentrations of DHT can, in itself, induce some apoptosis independent of the effects of indomethacin.

**Example 4**

[0071] DHT has either no effect or a reduced effect in promoting apoptosis in androgen-insensitive prostate cancer cell lines. In this series of experiments, two cell lines which are believed to be androgen insensitive (i.e., PC-3 and
DU-145), were subjected to same conditions of DHT (i.e., $10^{-4}$ M) for 48 hours prior to being exposed to indomethacin (100 $\mu$M) plus or minus $10^{-4}$ M DHT. DHT did not promote indomethacin induced apoptosis in the PC-3 cell line ($p>0.05$) as shown in Figure 5. Figure 5 depicts the percentage of apoptosis seen in three cell lines (i.e. PC-3, DU-145 & LNCaP) following incubation for 48 hours in indomethacin (100 $\mu$M) without DHT (blue bars) and with DHT ($10^{-4}$M) (yellow bars). Note that in the presence of DHT, PC-3 cells do not exhibit any increase in apoptosis ($p>0.05$), DU-145 cells exhibit a small, but statistically significant increase in apoptosis ($p<0.05$), and LNCaP cells exhibit a dramatic increase in apoptosis ($p<0.005$). The error bars represent one standard deviation.

[0072] Interestingly, exposure to DHT plus indomethacin did induce a statistically significantly increase in the % apoptosis of DU-145 cells ($p<0.05$). However, the percentage of apoptosis seen in the DU-145 cells was significantly less than that seen in similarly treated LNCaP cells ($p<0.005$).

**Example 5**

[0073] Other steroid hormones that interact with the androgen receptor on LNCaP cells also promote indomethacin induced apoptosis. In this series of experiments, the addition of hydroxycortisone or progesterone to the incubation media was evaluated for promotion of indomethacin induced apoptosis in a manner similar to DHT. The results indicate that the addition of high concentrations (i.e., $10^{-5}$) of hydroxycortisone does not increase the percentage of indomethacin induced apoptosis of LNCaP cells when compared with cells cultured in indomethacin plus $10^{-9}$ DHT alone ($p>0.05$), as shown in Figure 6. The addition of $10^{-6}$ progesterone, however, did result in a small but statistically significant increase in the percentage of apoptosis of LNCaP cells when compared with samples incubated in progesterone alone and samples incubated in indomethacin plus $10^{-9}$ DHT ($p<0.05$). Furthermore, the increased apoptosis seen by the addition of progesterone was not statistically different from that seen following the addition of an equivalent amount of DHT.
Example 6

[0074] High concentrations of DHT cause a significant shift in the cell cycle of LNCaP cells. Cell cycle analysis of LNCaP cells 24 Hours following incubation in $10^{-4}$, $10^{-5}$ or $10^{-9}$ M DHT indicated that within 24 hours of incubation in the concentrations of DHT, which promoted apoptosis (i.e., $10^{-4}$), the LNCaP cells exited from the S and G2/M phases of the cell cycle and accumulated in the GO/G1 phases of the cell cycle. See Table 1.

TABLE 1.
Exemplary date is contained in Table 1.

<table>
<thead>
<tr>
<th>DHT Concentration Molar</th>
<th>$10^{-9}$</th>
<th>$10^{-5}$</th>
<th>$10^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI:</td>
<td>62.16</td>
<td>66.22</td>
<td>86.97</td>
</tr>
<tr>
<td>G2-M:</td>
<td>13.89</td>
<td>11.72</td>
<td>8.24</td>
</tr>
<tr>
<td>%S:</td>
<td>23.95</td>
<td>22.07</td>
<td>4.79</td>
</tr>
</tbody>
</table>

[0075] Western blot analysis indicates no increase in COX-2 expression occurs in LNCaP following 48 hours of incubation in high concentrations of DHT. When LNCaP cells were evaluated following 24 hours of incubation in $10^{-4}$, $10^{-5}$ or $10^{-9}$M DHT, Western blots revealed no apparent change in the amount of COX-2 expressed by these cells 2. The addition of indomethacin to the incubation media resulted in a decrease in the expression of COX-2.

Example 7

[0076] Effects of DHT and Indometacin on cell cycle is LNCaP cells.

[0077] Cell cycle analysis of LNCaP cells following 48 hours of incubation in media containing different concentrations of DHT or indomethacin was also effected. As shown in Table 2, the results obtained indicate that high concentrations of DHT or indomethacin cause LNCaP cells to shift from the S/G2-M phases of the cell cycle into the GO/G1 phases of the cell cycle. Cell cycle differences between cells incubation in $10^{-9}$ versus $10^{-4}$ DHT and indomethacin are all statistically significant from controls (i.e., P < 0.005).
Table 2. Effects of Dihydrotestosterone and Indomethacin on Cell Cycle in LNCaP Cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>%GO/G1 (SD)</th>
<th>%G2/M (SD)</th>
<th>%S (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO DHT</td>
<td>82.61 (0.33)</td>
<td>10.73 (0.09)</td>
<td>6.67 (0.28)</td>
</tr>
<tr>
<td>10⁻⁹ DHT</td>
<td>78.46 (0.40)</td>
<td>12.89 (0.21)</td>
<td>8.65 (0.19)</td>
</tr>
<tr>
<td>10⁻⁴ DHT</td>
<td>89.38 (0.730)</td>
<td>8.26 (0.68)</td>
<td>2.39 (0.17)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>87.25 (0.71)</td>
<td>8.59 (0.75)</td>
<td>4.16 (0.45)</td>
</tr>
</tbody>
</table>

SD: Standard Deviation

Table 2. Cell cycle analysis of LNCaP cells following 48 hours of incubation in media containing different concentrations of DHT or indomethacin. The results indicate that high concentrations of DHT or indomethacin cause LNCaP cells to shift from the S/G2-M phases of the cell cycle into the GO/G1 phases of the cell cycle. Cell cycle value differences between cells incubation in 10⁻⁹ versus 10⁻⁴ DHT and indomethacin are all statistically significant (i.e., P<0.05).

EXAMPLES 8-12

Materials and Methods

[0078] **Cell Culture:** The LNCaP, DU-145 and 3T3 cell lines were purchased from the American Type Culture Collection (Rockville, MD). The LNCaP cell line is an androgen-responsive human prostate adenocarcinoma, which was isolated from a biopsy of a lymph node aspirated from a patient with a confirmed diagnosis of metastatic prostate carcinoma (19). The DU-145 cell line is an androgen insensitive human prostate cancer cell line originally isolated from the brain of a patient with metastatic carcinoma of the prostate (52). The 3T3 cell line is a contact inhibited embryonic mouse fibroblast cell line first established in 1962 by Todaro and Green (54). All cells were grown in flasks containing 10% fetal calf serum -supplemented Delbecco’s Modified Eagle’s Medium (DMEM from Mediatech, Herndon, VA), and were seeded onto six-well cluster dishes. LNCaP cells were grown in the presence of 10⁻⁶ DHT (maintenance level). Culture dishes were kept in a humidified atmosphere of 10% CO₂ in air at a temperature of 37°C. All NSAIDs that were added to the culture media (i.e., acetaminophen, acetylsalicylic acid, ibuprofen, naproxen,
and NS-398) were purchased through the Sigma Chemical Company (St. Louis, MO.).

[0079] **MTT Analysis for Cell Proliferation:** Cellular proliferation was determined with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay according to the procedure provided by the manufacturer (Roche Diagnostics Corporation, Indianapolis, IN) and as reported by Mosmann (38). The principle of the assay is that MTT, a nontoxic pale yellow substrate, is taken up by living cells to yield a dark blue formazan product. The process requires active mitochondria; thus, dead cells will not form formazan. The formazan formed is read spectrophotometrically (570 nm absorbance) and is directly proportional to the number of viable cells. Since no media or cells are removed from the culture wells during the MTT analysis, there is no loss of cells (i.e., dead or floating) as a result of these procedures. For this assay, cells were seeded in 96-well plates and allowed to incubate for 48 hours. Media containing selected concentrations of the different NSAIDs were then added to the wells and the cells incubated for an additional 48 hours. Following incubation in NSAIDs, 10 ul of labeling reagent (MTT) was added to each well, and the cells further incubated for an additional 4 hours at 37°C. The cells were then solubilized by incubation overnight at 37°C in 100 ul of a solubilization solution (10% sodium dodecyl sulfate in 0.01 M HC1). The absorbance at 570 nm was determined in a microtiter plate reader (Bio-Rad Laboratories, Richmond, CA). The results are expressed as the percentage of viable cells compared with non-treated controls.

[0080] **TUNEL Analysis of Apoptotic Cells:** The cells were preserved for TUNEL assay by the addition of 10% neutral formalin to each of the six-well plates (2 ml per well). The fixed cells were harvested by scraping and analyzed using an *in situ* apoptosis detection kit (ApopTag from Intergen Co, Purchase, NY) according to the manufacturer's instructions. Labeled cells were examined and counted using an OM-2 Olympus microscope equipped for fluorescence. Two independent observers undertook cell counts blindly and statistical
analysis was performed using ANOVA. Significance between groups was
determined using Student's t test with a p value < 0.05 being considered
statistically significant.

[0081] **Cell Cycle Analysis:** The cells were washed twice in PBS and
trypsinized. 1-2 x 10^6 cells were pelleted in triplicate by centrifugation and
resuspended in 100uL citrate buffer (40 mM trisodium citrate-2H_2O, 250 mM
sucrose, and 5% DMSO, pH 7.6). Nuclei were prepared for flow cytometric cell
cycle analysis by members of the Vincent T. Lombardi Cancer Research
Center Flow Cytometry Core Facility (Georgetown University Medical Center,
Washington, D.C.) using the method of Vindelov et al. (57), with propidium
iodide as the stain for nucleic acid. Cell cycle analysis was performed using
the FACStar Plus fluorescence-activated cell sorter (Becton Dickinson
Immunocytochemistry Systems, Mountain View, California) equipped with the
ModFit cell cycle analysis program (Verity Software House, Topsham, Main).

**Example 8**

*Ibuprofen Suppresses the Proliferation of LNCaP Cells:*

[0082] Where tested as described above, except for acetaminophen, all the
NSAIDs tested produced a significant reduction in the proliferation of LNCaP
cells (p<0.05). However, incubation in 1mM ibuprofen resulted in significantly
greater suppression of proliferation than aspirin or naproxen and was similar to
incubation in 0.1mM of NS-398 (See Figure 7).

**Example 9**

*At Clinically Relevant Concentrations, only the Propionic Acid Category of
NSAID Induced Significant Apoptosis of LNCaP Cells:*

[0082] The percentage of apoptosis resulting from incubation of LNCaP cells
in selected NSAIDs for 48 hours is summarized in Figure 8. Only supra-
pharmacological concentrations (i.e., 5mM) of aspirin and acetaminophen
induced statistically significant increases in apoptosis of LNCaP cells relative to
controls (p<0.05). The propionic acid NSAIDs ibuprofen and naproxen,
however, induced 100% apoptosis at 5 mM, and a significant level of apoptosis at 1mM. NS-398 also induced significant apoptosis at a concentration of 0.1 mM. Additional studies indicated that 48 hours of incubation in the presence of 2 mM ibuprofen also induced 100% apoptosis, while concentrations of ibuprofen as low as 0.5 mM caused statistically significant levels of apoptosis (p< 0.05) (The latter data is not shown in Figure 8).

**EXAMPLE 10**

*Ibuprofen Induces a Similar Percentage of Apoptosis in an Androgen Independent Human Prostate Cancer Cell Line:*

**[0083]** In this study, we evaluated the extent of apoptosis of DU-145 cells following 48 hours of incubation in 1mM of ibuprofen (See Figure 9). Because of its greater effectiveness against androgen dependent human prostate cancer cells (i.e., LNCaP cells), this study focused on ibuprofen. Compared with controls, 1mM of ibuprofen induced a similar increase in apoptosis of DU-145 cells (i.e., approximately 29% more than controls) as it did in LNCaP cells (i.e., approximately 28% more than controls).

**EXAMPLE 11**

*Of the NSAIDs Tested, Only Acetaminophen Induced Extensive Apoptosis of Normal Fibroblastic Cells (i.e., 3T3 Cells):*

**[0084]** In this study, we evaluated the ability of the different NSAIDs to induce apoptosis of a rapidly proliferating non-tumorigenic cell line derived from a different tissue type (i.e., 3T3 cells). When 1mM of selected NSAIDs were added to the medium of non-confluent normal fibroblastic 3T3 cells and incubated for 48 hours, only acetaminophen induced extensive apoptosis (See Figure 10). The effect of acetaminophen at 1 mM was impressive with over 40% of the cells being apoptotic. Even lower concentrations of acetaminophen (0.1mM) induced a statistically significant level of apoptosis of the 3T3 cells (data not shown). Of the rest of the NSAIDs tested, only NS-398 showed a statistically significant increase in percentage apoptosis when compared with controls (i.e., P<0.05).
Example 12

Ibuprofen Causes LNCaP Cells to Shift from the S and G2/M Stages of the Cell Cycle into the G0/G1 Phases of the Cell Cycle:

[0085] We used flow cytometry to determine the effect of selected NSAIDs on the cell cycle of LNCaP cells following 48 hours of incubation. As can be seen in Table I, ibuprofen (1mM) and NS-398 (0.1mM) induced the largest statistically significant shift of LNCaP cells out of the S and G2/M stages of the cell cycle and into the G0/G1 stage of the cell cycle relative to control.

Table 3. Effects of NSAIDs on Cell Cycle in LNCaP Cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>% G0/G1 (SD)</th>
<th>%G2/M (SD)</th>
<th>%S(SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63.43 (1.73)</td>
<td>16.32 (0.68)</td>
<td>20.1 (1.38)</td>
</tr>
<tr>
<td>Aspirin 1 mM</td>
<td>67.76 (2.77)</td>
<td>14.23 (0.76)</td>
<td>17.86 (2.39)</td>
</tr>
<tr>
<td>Aspirin 0.1 mM</td>
<td>66.99 (1.97)</td>
<td>16.21 (0.71)</td>
<td>16.78 (1.32)</td>
</tr>
<tr>
<td>Tylenol 1 mM</td>
<td>61.81 (0.20)</td>
<td>14.68 (0.41)</td>
<td>23.44 (0.24)</td>
</tr>
<tr>
<td>Tylenol 0.1 mM</td>
<td>62.08 (1.86)</td>
<td>16.14 (0.82)</td>
<td>21.79 (1.05)</td>
</tr>
<tr>
<td>Ibuprofen 1 mM</td>
<td>82.00 (0.68)*</td>
<td>9.20 (0.62)*</td>
<td>8.80 (1.01)*</td>
</tr>
<tr>
<td>Ibuprofen 0.1mM</td>
<td>66.83 (4.83)*</td>
<td>15.60 (0.50)</td>
<td>17.57 (4.35)</td>
</tr>
<tr>
<td>NS 398 0.1 mM</td>
<td>82.27 (2.05)*</td>
<td>11.17 (0.22)*</td>
<td>6.56 (2.07)*</td>
</tr>
</tbody>
</table>

[0086] Table 3 Cell cycle analysis of LNCaP cells following 48 hours of incubation in media containing different NSAIDs. The results indicate that high concentrations of ibuprofen (i.e., 1 mM) and NS-398 (i.e., 0.1 mM) cause LNCaP cells to shift from S/G2-M phases of the cell cycle into the G0/G1 phases of the cell cycle. SD: Standard Deviation

*Cell cycle value differences between cell incubation in Ibuprofen (1mM) and NS-398 (0.1 mM) are all statistically significant from controls (i.e., P <0.005).
[0087] While the invention has been illustrated by the preferred embodiments discussed herein, it will be apparent to one of skill in the art that modifications can be made for practicing the invention without departing from the scope of the invention as claimed.
What is claimed:

1. A method of treating or preventing a cancer in a subject in need of such treatment or prevention comprising administering a combination of a steroid hormone and a non-steroidal anti-inflammatory drug (NSAID), wherein said hormone and NSAID are administered separately, or in combination, in either order.

2. The method of claim 1 wherein said steroid hormone is selected from the group consisting of dihydrotestosterone, progesterone, testosterone, estradiol, and hydroxycortisone.

3. The method of claim 1 wherein said NSAID is selected from the group consisting of sodium salicylate, acetaminophen, phenacetin, ibuprofen, ketoprofen, indomethacin, flurbiprofen, diclofenac, naproxen, piroxicam, tebufelone, etodolac, nabumetone, tenidap, alclofenac, antipyrine, amimopyrine, dipyrone, animopyrone, phenylbutazone, clofazone, oxyphenbutazone, prexazone, apazone, benzydamine, bucolome, cinchopen, clonixin, ditrazol, epirizole, fenoprofen, floctafeninl, flufenamic acid, glaphenine, indoprofen, meclofenamic acid, mefenamic acid, niflumic acid, salidifamides, sulindac, suprofen, tolmetin, nabumetone, tiaramide, proquazone, bufexamac, flumizole, tinoridine, timegadine, dapsone, difunisal, benorylate, fosfosal, fenclofenac, etodolac, fentiazac, tiomisole, carprofen, fenbufen, oxaprozin, tiaprofenic acid, pirprofen, feprazone, piroxicam, sudoxicam, isoxicam, celecoxib, Vioxx.RTM., tenoxicam, ketoprofen, and naproxen.

4. The method of claim 3 where said NSAID is a selective COX-2 inhibitor.

5. The method of claim 1 wherein said NSAID is selected from the group consisting of indomethacin, aspirin, ibuprofen, suldinac, and celecoxib.

6. The method of claim 1 wherein the NSAID is ibuprofen.
7. The method of claim 1 wherein the cancer is a hormonally related cancer.

8. The method of claim 7 wherein said cancer is selected from the group consisting of prostate, breast, testicular, ovarian and uterine cancer.

9. The method of claim 8 wherein the cancer is prostate cancer.

10. The method of claim 1 wherein the NSAID is administered by a mode selected from injection, oral, sublingual, typical and transdermal.

11. The method of claim 1 wherein the hormone is administered by a mode selected from the group consisting of injection, oral, sublingual, topical and transdermal.

12. The method of claim 1 wherein the NSAID is ibuprofen and the hormone is dihydrotestosterone.

13. The method of claim 1 wherein the NSAID and the hormone are administered orally.

14. The method of claim 1 which is used to treat a subject at increased risk of developing prostate, breast, uterine, ovarian or testicular cancer because of family history, age, and/or previous occurrence or a marker that is associated with said cancer.

15. The method of claim 14 wherein said cancer is prostate cancer.

16. The method of claim 15 wherein said treatment reduces the expression of prostate surface antigen.

17. The method of claim 1 wherein ibuprofen is administered daily.
18. The method of claim 17 wherein said dosage ranges from 100 to 3200 mg/day.

19. The method of claim 17 wherein said dosage does not exceed 3200 mg/day.

20. A composition adopted for treatment or prophylaxis of a hormonally-related cancer comprising the combination of
   (i) an NSAID; and
   (ii) a steroidal hormone.

21. The composition of claim 20 wherein said hormone is selected from dihydrotestosterone, progesterone, testosterone, estradiol, and hydroxycartisone.

22. The composition of claim 20 wherein said NSAID is selected from sodium salicylate, acetaminophen, phenacetin, ibuprofen, ketoprofen, indomethacin, flurbiprofen, diclofenac, naproxen, piroxicam, tebufelone, etodolac, nabumetone, tenidap, alcofenac, antipyrine, amimopyrine, dipyrone, animopyrone, phenylbutazone, clofezone, oxyphenbutazone, prexazone, apazone, benzydamine, bucolome, cinchopen, clonixin, ditrazol, epirizole, fenoprofen, floctafeninl, flufenamic acid, glaphenine, indoprofen, meclofenamic acid, mefenamic acid, niflumic acid, salidifamides, sulindac, suprofen, tolmetin, nabumetone, tiaramide, proquazone, bufexamac, flumizole, tinoridine, timegadine, dapsone, diflunisal, benorylate, fosfosal, fenclofenac, etodolac, fentiazac, tilomisole, carprofen, fenbufen, oxaprozin, tiaprofenic acid, pirprofen, feprazone, piroxicam, sudoxicam, isoxicam, celecoxib, Vioxx.RTM., tenoxicam, ketoprofen, and naproxen.

23. The composition of claim 20 wherein said NSAID is a selective COX-2 inhibitor.
24. The composition of claim 20 wherein said NSAID is ibuprofen.

25. The composition of claim 20 wherein said NSAID is selected from the group consisting of indomethacin, aspirin, ibuprofen, sulindac and celecoxib.

26. The composition of claim 20 which is adopted for oral administration.

27. The composition of claim 20 which is adopted for transdermal administration.
% APOPTOSIS OF PC-3, DU-145 AND LNCaP CELLS FOLLOWING 48 HOURS INCUBATION IN INDOMETHACIN (100 uM) MINUS AND PLUS DHT

![Graph showing % apoptosis of PC-3, DU-145, and LNCaP cells following incubation with and without DHT.](image)

**FIG. 5**
% APOPTOSIS OF LNCaP CELLS FOLLOWING INCUBATION FOR 48 HOURS IN DIFFERENT STEROID HORMONES MINUS AND PLUS INDOMETHACIN

FIG. 6
SURVIVAL OF LNCaP CELLS FOLLOWING INCUBATION IN SELECTED NSAIDs

FIG. 7

SUBSTITUTE SHEET (RULE 26)
% APOPTOSIS OF LNCaP CELLS FOLLOWING INCUBATION IN SELECTED NSAIDs

**FIG. 8**

SUBSTITUTE SHEET (RULE 26)
% APOPTOSIS OF DU-145 CELLS FOLLOWING INCUBATION IN 1mM IBUPROFEN

FIG. 9
% APOPTOSIS OF 3T3 CELLS FOLLOWING INCUBATION IN SELECTED NSAIDs

FIG. 10

SUBSTITUTE SHEET (RULE 26)
INTERNATIONAL SEARCH REPORT

INTERNATIONAL APPLICATION NO.
PCT/US02/17193

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 31/19, 31/56, 31/215
US CL : 514/170, 557, 531
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/170, 557, 531

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>US 6,080,426 A (AMEY ET AL) 27 June 2000 (27.06.2000), see calim 38.</td>
<td>20, 22, 24-26</td>
</tr>
</tbody>
</table>

[] Further documents are listed in the continuation of Box C.  
[ ] See patent family annex.

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29 July 2002 (29.07.2002)

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