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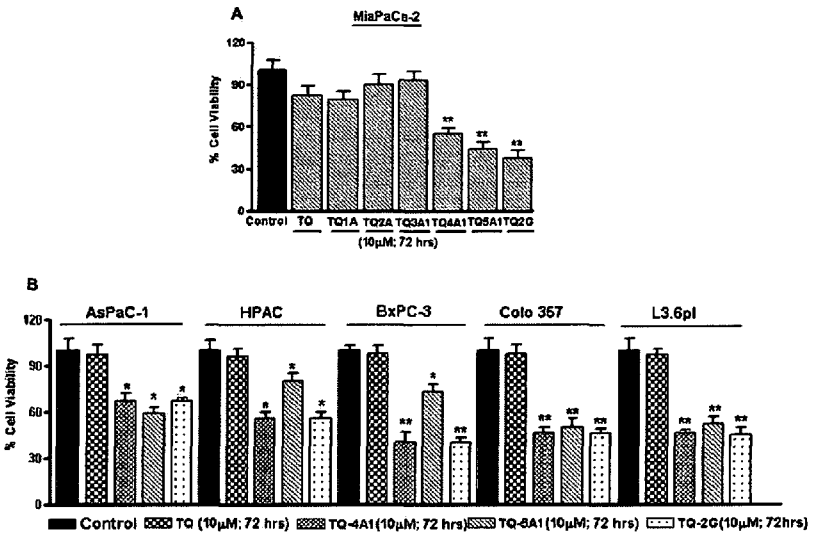
(54) Title: THYMOQUINONE ANALOGS FOR THE TREATMENT OF PANCREATIC CANCER

Fig. 2



(57) Abstract: Analogs of thymoquinone, an active compound extracted from *Nigella sativa* (black seed oil) are 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinones, are more potent than the naturally-occurring compound (e.g, have a lower IC_{50}) in terms of inhibition of cell growth, induction of apoptosis, and modulation of transcription fact NF-κB. Used in conjunction with conventional chemotherapeutic agents, such as gemcitibine or oxaliplatin, the analogs sensitize gemcitabine-resistant pancreatic cancer cells to gemcitabine-induced apoptosis. A preferred compound is 2,5-dichloro-3,6-bis[3-methoxyphenyl)amino]cyclohexa-2,5,-diene- 1,4-dione.

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Thymoquinone Analogs for the Treatment of Pancreatic Cancer

Government Rights

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Relationship to Other Application

This application claims the benefit of the filing date of United States Provisional Patent Application Serial Number Serial No. 61/341,185 filed March 26, 2010, Conf. No. 2604 (Foreign Filing License Granted) in the names of the same inventors as herein. The disclosure in the identified United States Provisional Patent Application is incorporated herein by reference.

Background of the Invention

FIELD OF THE INVENTION

This invention relates generally to synthetic analogs of thymoquinone, and more particularly, to thymoquinone analogs that have a lower IC_{50}, that are useful, *inter alia*, as anti-cancer agents, and methods of treating cancers, particularly pancreatic cancer, to prevent drug-resistance and/or to potentiate the effects of known chemotherapeutic agents.

DESCRIPTION OF THE PRIOR ART

Pancreatic cancer (PC) remains the fourth leading cause of cancer death in the United States. As an example, it was estimated that in 2009, 37,680 new cases of PC was diagnosed leading to 34,290 deaths. This dismal outcome in survival of patients diagnosed with PC is due, in part, to lack of early detection, the indolent nature of disease progression, and perceived *de novo* and acquired chemo-resistance to conventional cytotoxic agents, such as gemcitabine and oxaliplatin. Another factor that is believed to contribute to the aggressiveness of PC is enrichment of cancer stem cells (CSCs) during therapy. CSCs are highly resistant to conventional therapeutics and, thus, may contribute to the poor outcome.

There is, therefore, a need for improved chemotherapeutic agents that are not subject to the development of resistance and/or that can reverse the development of resistance, that potentiates the effects of conventional agents, and that inhibits growth and/or selectively targets CSCs.

There is, also, a need for chemotherapeutic agents that improve the survival outcome of patients diagnosed with PC without undesirable side effects normally associated with current therapies, such as toxicity and the development of drug-resistance.

The "natural dietary agent" thymoquinone (TQ), derived from black seed (*Nigella sativa*) oil, has been shown to have therapeutic and chemoprotective potential. See, Banerjee, *et al.*, Antitumor Activity of Gemcitibine and Oxaliplatin is Augmented by Thymoquinone in Pancreatic Cancer, *Cancer Res.*, Vol. 69, No. 13, pages 5575-83 (2009). *Nigella sativa* is a spice that grows in the Mediterranean region and in Western Asian countries including India, Pakistan and Afghanistan. In folklore medicine, the seed has been reported to have therapeutic benefits in such diverse conditions as bronchial asthma, dysentery, headache, gastrointestinal problems, eczema, hypertension, and obesity.

The bioactive compound derived from *Nigella sativa* oil is TQ, which has been shown to exhibit anti-tumor activities including anti-proliferative and pro-apoptotic effects on cell lines derived from breast, colon, ovary, larynx, lung, myeloblastic leukemia and osteosarcoma. TQ has also been shown to inhibit hormone refractory prostate cancer by targeting androgen receptor and transcription factor E2F. The known therapeutic potential and mechanisms of action for TQ is summarized in Banerjee, *et al.*, Review of Molecular and Therapeutic Potential of Thymoquinone in Cancer, *Nutrition and Cancer*, Vol. 62, pages 938-946 (2010). Among the reported activities of TQ are antioxidant, anti-inflammatory and chemopreventive, antiproliferative, cell cycle regulation, apoptosis, and inhibition of angiogenesis and endothelial cell functions. Mechanistically, TQ appears to induce apoptosis in tumor cells by suppressing NF-κB, Akt activation and extracellular signal-regulated kinase signaling pathways, and to inhibit tumor angiogenesis.

Although a number of crucial genes are altered in PC, several important molecules stand out among them including the COX family of proteins which play critical roles in the initiation of cancer and the acquisition of resistance to chemotherapeutics in human PC. COX-2 is an enzyme that is up-regulated by growth factor and inflammatory cytokine-mediated activation of NF-κB in PC. Recent studies on breast, colon, and PC have shown that COX-2 plays a key role in aggressive tumor growth and metastases. It has been confirmed that down-regulation of the "master transcription factor," NF-κB, by natural agents, such as TQ, can lead to the sensitization of PC cells to cytotoxic conventional therapeutic agents, especially gemcitabine. Banerjee, *et al.*, *Cancer Res.*, Vol. 69, pages 5575-5583 (2009). However, the concentration of TQ required in this study may not be feasible for administration to human patients. There is, therefore, a need for novel synthetic analogs of TQ, that are more potent, that is, have a lower IC_{50} than the naturally-occurring TQ, as well as the proven biological activity of TQ, and preferably improved biological activity.

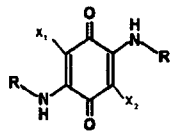
It is, therefore, an object of this invention to provide synthetic analogs of the natural dietary agent TQ that have better biological activity and lower IC_{50} than TQ, yet remain non-toxic.

It is another object of this invention to provide synthetic analogs of TQ that can be used alone, or in combination with conventional chemotherapeutic agents, to sensitize drug-resistant cells to chemotherapeutic treatment.

It is also an object of this invention to provide syththetic analogs of TQ that inhibit cell growth, induce apoptosis, and modulate transcription factor-NF-κB better than TQ and that sensitize gemcitabine- and oxaliplatin-induced apoptosis in MiaPaCa-2 (gemcitabine-resistant) PC cells, which was associated with downregulation of the anti-apoptotic and cell survival-related molecules , Bcl-2, Bcl-xL, survivin, XIAP, COX-2 and the associated Prostaglandin E_2.

Summary of the Invention

The foregoing and other objects are achieved by this invention which provides a 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of the general formula I:



wherein R is a saturated linear or branched lower alkyl, cycloalkyl, heterocycloalkyl, alcohol, alkoxy, aryloxy, or substituted or unsubstituted phenyl, phenyl(alkyl); and x_1 and x_2 are selectably H or a halide, preferably chlorine or fluorine. As used herein, the term "lower alkyl" refers to substituents having 1 to 6 carbons.

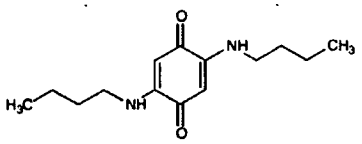
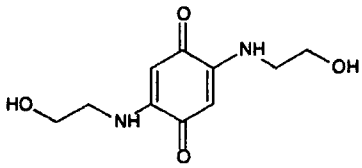
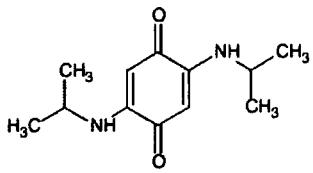
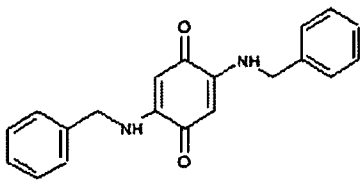
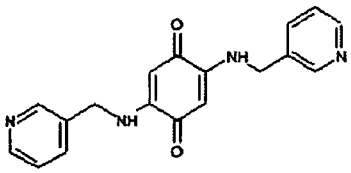
In a method of making embodiment of the invention, analogs of TQ, in accordance with the invention, were synthesized in a highly selective one-pot synthesis as shown in the reaction scheme of Fig. 1. In the illustrative example shown in Fig. 1, the starting compounds were 2-tert-butyl-5-methylcyclohexa-2,5-diene-1,4,-dione (TQ); p-benzoquinone (TQ2), and 2,6-di-t-butyl-benzoquinone (TQ3). It is to be understood that, the starting compounds can have substituents. In a particularly preferred embodiment, the starting compound is 2,3,5,6-tetrachloro-1,4-benzoquinone (not shown).

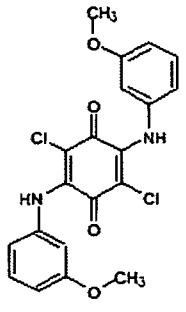
The starting compound, is dissolved in an appropriate solvent, such as methanol, and reacted with 2 Mol of a primary amine RNH_2 in the presence of air. The resulting 2,5-bis (alkyl/aryl amino) 1,4-benzoquinone product is filtered and re-crytallized. Advantageously, this reaction scheme induces minimal changes to the parent structure, and the product retains most of the original biological properties of the starting compound.

The chemical structure of illustrative analogs in accordance with the present invention are shown below in Table 1, with the code used herein in connection with the experiments and related figures, and IC_{50} in PC cells. Referring back to Fig. 1, the starting compound is p-benzoquinone (TQ2) for compounds TQ-1A, TQ-2A, TQ-3A1, TQ-4A1, and TQ-5A1. The R-moiety on the amine reactant (RNH_2) is shown on Fig. 1 as B, H, A, and G, respectively, for TQ-1A, TQ-2A, TQ-3A1, TQ-4A1. The TQ-5A1,

it is 3-methyl pyridine (not shown). Compound TA-2G had 2,3,5,6-tetrachloro-1,4-benzoquinone as a starting compound and the R-moiety F, as shown on Fig. 1.

TABLE 1

CODE	STRUCTURE	MOL WT	IC50
(BQBuNH2) **TQ-1A**		250	> 10 μM
(BQ Ethanolamine) **TQ-2A**		226	> 10 μM
(BQ Isopropylamine) **TQ-3A**		198	> 10 μM
TQ-4A1		318	< 10 μM
TQ-5A1		320	< 10 μM

TQ-2G		320	< 10 μM

In particularly preferred embodiments, the analogs of the present invention are 2,5-bis(benzylamino)cyclohexa-2,5-diene-1,4-dione (compound TQ-4A1; IC_{50} 7 μM); 2,5-bis[(pyridine-3-ylmethyl)amino]cyclohexa-2,5-diene-1,4-dione (compound TQ-5A1; IC_{50} 5 μM); and 2,5-dichloro-3,6-bis[3-methoxyphenyl)amino]cyclohexa-2,5,-diene-1,4-dione (compound TQ-2G; IC_{50} 5 μM). These compounds were more potent than the parental TQ, at equimolar concentrations (10 μM), when screened with the pancreatic cancer cell line MiaPaCA-2 (see, Fig. 2A).

In a further composition of matter aspect of the invention, a formulation comprises a therapeutically effective amount of a compound in accordance with the present invention in a delivery vehicle, or pharmaceutically-acceptable carrier. The compound may be the free drug or a pharmaceutically acceptable salt thereof. The term pharmaceutically acceptable salt includes, at least, the commonly used alkali metal salts used to form addition salts of free acids or free bases.

In a particularly preferred embodiment, the formulation includes 2,5-dichloro-3,6-bis[3-methoxyphenyl)amino]cyclohexa-2,5,-diene-1,4-dione or 2,5-bis[(pyridine-3-ylmethyl)amino]cyclohexa-2,5-diene-1,4-dione.

As used herein, the term "therapeutically-effective" refers to an amount of the compound that produces an ameliorating effect in the treatment and/or prevention of cancer, or other targeted disease, and is not toxic to the patient, and preferably does not produce excessive adverse side effects.

It is contemplated that the compounds in accordance with the present invention can be formulated for delivery in any route of administration. For oral administration,

the pharmacologic agent(s) can be delivered dry in the form of a tablet or capsule, or as a liquid solution or suspension. Oral drug delivery forms are well-known and typically include, conventional additives, such as binders and fillers, disintegrants, lubricants, and the like. For intravenous, intramuscular, subcutaneous, or intraperitoneal administration, the active pharmacologic agent may be combined with a sterile aqueous solution, such as saline or dextrose, preferably isotonic. Of course, a liquid injectable formulation can include other components, such as excipients, anti-oxidants, buffers, osmolarity adjusting agents, and the like, as are known in the art. In addition to conventional drug delivery approaches, it is within the contemplation of the invention that the pharmacologic agents of the present invention can be administered in targeted delivery media, such as in microparticle and nanoparticle formulations.

The analogs of the present invention can be used alone, or in combination, with other therapeutic agents, including anti-cancer agents, which may include known cytotoxic agents, such as genistein, celecoxib, gemcitabine, 5-flurouracil and oxaliplatin. The analogs of the present invention may be administered to a subject in need of treatment as a anti-cancer agent, or administered in conjunction with other chemotherapeutic agents to prevent drug-resistance and/or potentiate the effects of the known chemotherapeutic agents. The analogs can be used as an adjuvant, before, after, or concurrent with the administration of other medications or treatment therapies, such as radiation therapy.

In a specific preferred method of treating embodiment, there is provided a method of inhibiting the growth of chemo-resistant pancreatic cancer cells, of the type that are enriched in cancer stem-like cells, in a subject having pancreatic cancer, comprising administering to the subject a therapeutically effective amount of a compound according to the invention either alone, or preferably in combination or conjunction with gemcitibine and/or oxaliplatin.

In another further method of treating, there is provided a method of sensitizing pancreatic cancer cells and gemcitabine-resistant pancreatic cancer cells to the cytotoxic effects of gemcitabine for the prevention of tumor progression and/or treatment of pancreatic tumors in a subject comprising administering to the subject a therapeutically

effective amount of a pharmaceutical formulation comprising a compound in accordance with the invention to prevent drug-resistance and/or potentiate the effects of gemcitibine. Of course, the analogs of the present invention may have sensitizing and/or potentiating effects against other chemotherapeutic agents, and type of cancers, and this description is not meant to be limiting.

Brief Description of the Drawing

Comprehension of the invention is facilitated by reading the following detailed description, in conjunction with the annexed drawing, in which:

Fig. 1 is an illustrative one-pot chemical synthesis scheme for making the 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone analogs of the present invention;

Fig. 2A is a graphical representation of cell viability of pancreatic cancer MiaPaCa-2 cells assessed by an MTT assay following treatment for 72 hours with TQ or the analogs shown in Table 1 at a concentration of 10 μM;

Fig. 2B is a graphical representation of cell viability of various pancreatic cancer cells lines assessed by MTT assay following treatment for 72 hours with TQ or analogs TQ-4A1, TQ-5A1, and TQ-2G at a concentration of 10 μM;

Fig. 3A are the flow cytometry scans of Annexsin-V-FITC/Propidium Iodide stained MiaPaCa-2 cells following 48 hours of exposure to TQ or analogs TQ-4A1, TQ-5A1, and TQ-2G at a concentration of 10 μM;

Fig. 3B is a graphical representation of the flow data shown in Fig. 3A;

Fig, 3C is a graphical representation of apoptosis as determined by histone-DNA ELISA in MiaPaCa-2 cells following 48 hours of exposure to TQ or analogs TQ-4A1, TQ-5A1, and TQ-2G at a concentration of 10 μM;

Figs. 4A1-4A5 are flow cytometry scans for DNA cell cycle analysis showing the distribution of cells in G0/G1, G2/M, and S phases following treatment with TQ and analogs of the present invention;

Fig. 4B is a graphical representation of the data from Figs. 4A1 to 4A5 in the form of a histogram showing the % distribution of the cells in each phase;

Fig. 5A is a series of Western immunoblots showing the effect of TQ and the three analogs on the expression of apoptosis-related proteins in whole cell lysates prepared from treated MiaPaCA-2 cells;

Fig. 5B is a graphical representation of caspase-3 activity in the treated cell lysates;

Fig. 6A is a gel shift assay showing down-regulation of NF-κB DNA binding activity in nuclear extracts of MiaPaCa-2 cells that had been treated with TQ and the analogs;

Fig. 6B is a graphical representation of PGE_2 expression in BxPC-3 cells following exposure to TQ and the analogs;

Figs. 6C and 6D are graphical representations in the form of histograms depicting the COX-1 and COX-2 enzyme activity of BxPC-3 cells following treatment with TQ and the three analogs;

Figs. 7A to 7D are graphical representations of cell viability of MiaPaCa-3 cells assessed by MTT assay following pre-treatment for 48 hours with TQ or the analogs TQ-4A1, TQ-5A1, and TQ-2G at a concentration of 10 μM and subsequent exposure to suboptimal doses of gemcitibine and oxaliplatin for 36 hours; and

Fig. 8 is a series of graphical representations showing the % apoptosis, as determined by histone-DNA ELISA, caused by sensitization of MiaPaCa-2 cells by pretreatment with the analogs of the present invention followed by gemcitabine or oxaliplatin.

Detailed Description

I. Synthesis of TQ Analogs

The analogs of TQ in accordance with the present invention were synthesized by a one-pot synthesis technique, as described above, that was exceptionally selective and led only to the production of 2, 5-bis (alkyl/aryl-amino) 1,4-benzoquinones of the amine used as a reactant.

II. Experimental Methods/Data

The analogs were tested in a pancreatic cell line for growth inhibition, induction of apoptosis, and modulation of the transcription factor NF-κB.

MTT Assay for Cell Viability

A standard MTT assay was used to measure the effect of TQ and the analogs shown on Table 1 on viability of pancreatic cancer MiaPaCa-2 cells. The cells were seeded into culture plates ($3x10^3$ cells/well) and treated with TQ and each of the analogs of Table 1 at a concentration of 10 μM. The formazan formed by metabolically viable cells was dissolved in isopropanol, and the absorbance was measured at 595 nm using a plate reader. The data was plotted as percent viable cell relative to control (growth medium). The results are shown in Fig. 2A in which analogs TQ-4A1, TQ-5A1, and TQ-2G were shown to be more effective than the parental TQ.

Similar MTT assays were conducted in pancreatic cancer cell lines having different molecular signatures, specifically BxPC-3, AsPC-1, Colo 357, L3.6pl and HPAC cells. Fig 2B shows the results for TQ and the most effective analogs, TQ-4A1, TQ-5A1, and TQ-2G. These analogs suppressed cell viability in almost all PC cell lines tested with ≤50% loss of viability at 10 μM concentration after 72 hours of treatment.

Since the other analogs were less effective than TQ-4A1, TQ-5A1, and TQ-2G, the following studies were conducted using these three analogs to demonstrate the effects of these compounds on cell cycle and apoptosis in gemcitibine-resistant MiaPaCa-2 cells.

Quantification of Apoptosis

An Annexin V-FITC assay kit (BD Biosciences, San Jose, CA) was used, according to the manufacturer's protocol, to evaluate apoptosis by flow cytometric analysis of Annexin V-FITC/propidium iodide stained samples of MiaCaPa-2 cells following 48 hours of treatment by TQ and the three analogs TQ-4A1, TQ-5A1, and TQ-2G (10 μM). This assay, which is considered to be a highly specific indicator of apoptosis, showed the percentage of apoptotic cells to be 6%, 7% 19% and 48%, respectively.

Similar trend towards apoptosis are shown in Fig. 3C, which is a graphical representation of the absorbance at 455 nm of the histone/DNA complex produced in an

ELISA assay of MiaPaCa-E cells following 48 hrs of treatment with TQ and each of the analogs TQ-4A1, TQ-5A1, and TQ-2G at a concentration of 10 μM.

Cell Cycle Progression

MiaPaCa-2 cells were treated with equimolar concentrations (10μM) of TQ or the analogs TQ-2G, TQ4A1 and TQ-5A1 for 72 hrs . After treatment, the cells were collected by trypsinization, washed with cold PBS and subsequently fixed by incubating the cells in 450 μl of ice-cold ethanol 1 h at 4°C. The fixed cells were then centrifuged for 5 min, and the resulting pellet was washed twice with cold PBS, re-suspended in 500 μl of PBS, and incubated with RNase (20 μg/ml, final concentration) for 30 min at 37°C. The cells were then chilled over ice for 10 min, stained with propidium iodide (50 μg/ml final concentration) for 1 h, and analyzed by flow cytometry. The results are shown on Fig. 4A and Fig. 4B

The untreated MiaPaCa-2 cells (control; Fig. 4A1) demonstrate a pattern wherein most cells are in the G0/G1 phase (57%), a lower G2/M phase (10%) and S phase (32%). The alterations in the cell cycle distribution of MiaPaCa-2 cells treated for TQ and the selective analogs are shown in Fig. 4A2-4A5. Only the analog TQ-5A1 caused significant G2/M phase cell cycle arrest (65.73 % versus 10.28 % in the control). The analog TQ-2G resulted in a minor increase in G2/M phase arrest (16% versus 10.28% in control). However, the proportion of G0/G1 phase cells decreased significantly after 72 hrs of treatment. Interestingly, neither TQ, nor the analog TQ-4A1, at the concentration tested exhibited any major effect on cell cycle progression. Fig. 4 B is a histogram showing the % distribution of cells in the G0/G1, G2-M, and S phases of the cell cycle. Fig. 4B graphically demonstrates that the analogs TA-5A1 and TQ-02G operate on cell cycle progression by different mechanisms.

Inhibition of Anti-Apoptotic Molecules

Using MiaPaCa-2 cells as a representative model to investigate the molecular effects of TQ and its analogs, a Western blot analysis was conducted to ascertain the expression of apoptosis-related proteins in whole cell lysates prepared from MiaPaCa- cells that had been treated equimolar concentrations (10μM) of TQ and the analogs for 72 h. About 30-40 mg total protein from the cell lysates was separated on SDS-

PAGE, electrotransferred, and probed with the anti-bodies specified on Fig. 5A. β-actin protein was used as the loading control.

Referring to Fig. 5A, expression of the anti-apoptotic protein Bcl-2 was significantly inhibited by the analogs TQ-5A1 and TQ-2G indicating that the apoptosis-inducing effects of these analogs could be due, in part, to up-regulation of the Bax/Bcl-2 protein ratio, which is a critical determinant of the induction of apoptosis. Expression of other anti-apoptotic molecules, Bcl-xL, survivin and XIAP, relative to the control, were down-regulated in cells that had been exposed to the analogs TQ-2G, TQ4A1 and TQ-5A1.

The Western blots also revealed that, at equivalent concentrations, treatment by the analogs, but not TQ, resulted in the appearance of PARP, and the cleaved active component of caspase-3. Therefore, caspase-3 activity was measured by colorimetric assay according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Referring to Fig. 5B, caspase-3 activity was significantly elevated. An upstream event in the activation of the caspase cascade is the release of cytochrome c from mitochondria. TQ has been reported to induce the release of cytochrome-c, which suggests that the induction of apoptosis by TQ, and its analogs, is mediated, in part, by the mitochondrial pathway.

Inhibition of Activation of NF-κB

An electromobility shift assay (EMSA) was conducted to investigate whether TQ and the analogs could abrogate constitutively expressed NF-κB *in vitro* in MiaPaCa-2 cells. The cells were treated for 48 hours with TQ and the analogs (10μM). Nuclear extracts were prepared from treated samples, and EMSA was performed by incubating 10μg of nuclear extract with IRDye™-700 labeled NF-κB oligonucleotide as described in Banerjee, *et al.*, *id.* The DNA-protein complex that formed was visualized by an infrared imaging system. For loading control, 10μg of nuclear protein from each sample was subjected to Western immunoblotting for retinoblastoma protein.

The DNA binding activity of NF-κB is shown on the gel shift assay of Fig. 6A. The analogs TQ-2G, TQ-4A1, and TQ-5A1 all caused down-regulation of the DNA

binding effect of NF-κB in MiaPaCa-2 cells. This is consistent with down-regulation of the transcriptional target genes of NF-κB, such as Bcl-2 family of anti-apoptotic proteins, survivin and XIAP.

Suppression of COX Activity and PGE_2 Synthesis in BXPC3 Cells

The parent compound TQ had been shown to inhibit COX-2 protein expression in HPAC pancreatic cancer cells. Banerjee, *et al.*, *id.* Based on preliminary computer modeling data, TQ docks into the active site of COX-2 with a binding energy of -7.68 Kcal/mole. Therefore, TQ Inhibition of COX-2 and Prostaglandin E2 (PGE_2) is considered a promising chemotherapeutic target for the treatment, and reversal of the chemo-resistance phenotype.

BxPC-3 cells were used to determine the effects of TQ, and its analogs, on the production of PGE_2 because BxPC-3 cells have a higher basal expression of COX-2 enzyme than MiaPaCa-2 cells. The BxPC-3 cells were treated with either TQ or one of the analogs for 24 hrs. The conditioned media was collected, centrifuged, and analyzed.

Fig. 6B is a graphical representation of PGE_2 expression (pg/106 cells) in BxPC-3 cells as assayed by a PGE_2 high-sensitivity immunoassay kit (R&D Systems, Minneapolis MN). Cells exposed to the analogs TQ-5A1 and TQ-2G showed a significant decrease in PGE_2 secretion.

COX-1 and COX-2 activities were determined using a COX (ovine) Inhibitor Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instructions. Figs. 6C and 6D are histograms depicting COX-1 and COX-2 enzyme activity, respectively, in BxPC-3 cells after treatment with TQ and its analogs. The results demonstrate that TQ, and the analogs, are effective in inhibiting COX-1 and COX-2 enzyme activity at the stated concentration (10 μM).

Sensitization of PC Cells to Chemotherapeutic Agents

Studies were conducted to ascertain whether pretreatment of gemcitabine-resistant MiaPaCa-2 cells by the analogs of the present invention would make the cells more sensitive to gemcitabine and oxaliplatin as compared to pre-treatment of the cells with the parental TQ.

Cells were pretreated with equimolar concentrations (10 μM) of TQ, or the analogs TQ-2G, TQ-4A1 and TQ-5A1,for 48 hrs. Then, the pretreated cells were incubated for 36 hours with suboptimal doses of either gemcitabine (0.5 μM) or oxaliplatin (6 μg/ml). Cell viability was determined by MTT assay and the results are shown in Figs. 7A to 7D (*$p < 0.05$, **$p < 0.001$ relative to control).

Pretreatment of cells with TQ followed by either gemcitabine or oxaliplatin treatment caused cell killing of 22% and 39%, respectively. These results do not differ significantly from cell killing by monotherapy regimens (gemcitabine at 13% or oxaliplatin at 37%). In sharp contrast, the analogs, and particularly TQ-2G and TQ-5A1, caused significant loss of cell viability.

In combination with gemcitabine, the loss of viable cells with the analogs TQ-2G, TQ-4A1 and TQ-5A1 was 45%, 78% and 69%, respectively, relative to the untreated control ($p<0.001$). The corresponding data for gemcitabine alone, ranged between 13-23% of cell killing. Similarly, the loss of viable cells upon treatment with the analogs followed by oxaliplatin, ranged between 57% (TQ-4A1+Oxaliplatin compared to 38% with oxaliplatin alone), 89% (TQ-5A1+ Oxaliplatin compared to 51% with oxaliplatin alone), and 76% (TQ-2G+ oxaliplatin compared to 34% with oxaliplatin alone).

The foregoing demonstrates that therapeutic regimens in which the analogs, and particularly the TQ-2G and TQ-5A1 analogs, are administered with oxaliplatin as well as gemcitabine, would be advantageous.

Sensitization of MiaPaCa-2 Cells to Apoptosis
by Gemcitibine and Oxaliplatin

Using a Histone DNA-ELISA assay, it was demonstrated that pretreatment of MiaPaCa-2 cells with the analogs TQ-4A1, TQ-5A1 and TQ-2G, followed by the cytotoxic chemotherapeutic drugs Gemcitibine and oxaliplatin induces more apoptosis (~50% more; $p<0.001$) than no pretreatment. The results are shown on Fig. 8 which confirms the superior sensitizing effect of the TQ analogs (TQ-2G, TQ4A1 and TQ-5A1).

TQ and its analogs are non-toxic to animals

TQ administered at doses of 3 mg/mouse/day for 21 days did not cause any signs of apparent toxicity to mice. See, Banerjee, *et al.*, *id.*

One of the analogs of the present invention, TQ-2G, was tested in SCID mice at doses up to 50 mg/kg i.v. and up to 700 mg/kg by oral gavage. Based on animal body weight and overall well being, no evidence of any severe toxicity as observed. There were no signs of aversion to food intake or diarrhea during the window of treatment. Furthermore, no macroscopic evidence of necrosis or hemorrhage in any visceral organs was observed following treatment. Therefore, the TQ analogs of the present invention are non-toxic. Thus, the analogs may be used alone, or in combination therapy, without added toxicity.

In view of the foregoing, it is clear that the analogs of the present invention have the ability to sensitize gemcitabine and oxaliplatin-induced apoptosis in a gemcitabine resistant cell line, MiaPACA-2. This effect has been associated with down-regulation of NF-κB, and the anti-apoptotic and cell survival-related molecules such as Bcl-2, Bcl-xl, survivin, and XIAP. Moreover, the analogs inhibit the production and secretion of PGE2 in high COX-2-expressing PC cells (BxPC-3). The analogs have a much lower IC50 (< 10 μM) than the parent TQ (~ 25 μM).

The chemo-sensitizing effect of the analogs is more potent that the parent TQ in sensitizing PC cells to the action of gemcitibine and oxaliplatin. This action has been associated with the down-regulation of anti-apoptotic molecules, such as Bcl-2, Bcl-xl, survivin, and XIAP. Moreover, the analogs exhibited increased caspase-3 activity which is associated with increased apoptosis of PC cells.

The effects of the analog TQ-5A1 on cell cycle, particularly the strong G2/M cell cycle arrest, suggests that this analog would be useful for combination therapy using another mode of treatment such as radiation.

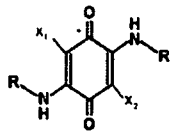
Although the disclosure has been directed to the use of the novel TQ analogs in connection with pancreatic cancer, TQ has been shown to exhibit anti-tumor activities including anti-proliferative and pro-apoptotic effects on cell lines derived from breast, colon, ovary, larynx, lung, myeloblastic leukemia and osteosarcoma. TQ

has also been shown to inhibit hormone refractory prostate cancer by targeting androgen receptor and transcription factor E2F. Therefore, it is specifically contemplated that the analogs of the present invention, and the pharmaceutical compositions and methods of treating with the same, could be used in connection with the treatment of other types of cancer.

Although the invention has been described in terms of specific embodiments and applications, persons skilled in the art may, in light of this teaching, generate additional embodiments without exceeding the scope or departing from the spirit of the invention described and claimed herein. Accordingly, it is to be understood that the drawing and description in this disclosure are proffered to facilitate comprehension of the invention, and should not be construed to limit the scope thereof.

What is claimed is:

1. A 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of the general formula I:



wherein R is a saturated linear or branched lower alkyl, cycloalkyl, heterocycloalkyl, alcohol, alkoxy, aryloxy, or substituted or unsubstituted phenyl, phenyl(alkyl); and

x_1 and x_2 are selectably H or a halide

2. The 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of claim 1 wherein R is R is selected from the group consisting of -CH-(CH_2)n-CH3, -CH-(CH_2)n-$(CH_3)_2$, -CH-(CH_2)n-CH-OH, -CH_2-Ph, CH_3-$(CH_2)_n$-O-Ph, substituted -CH_3-$(CH_2)_n$-O-Ph, -$(CH_2)_n$-Pyr, where n=0-6

3. The 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of claim 1 wherein x_1 and x_2 are halides selected from the group consisting of chlorine and fluorine.

4. The 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of claim 1 which has an IC50 <10 μM.

5. The 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of claim 1 which is selected from the group consisting of 2,5-bis(benzylamino)cyclohexa-2,5-diene-1,4-dione; 2,5-bis[(pyridine-3-ylmethyl)amino]cyclohexa-2,5-diene-1,4-dione; and 2,5-dichloro-3,6-bis[3-methoxyphenyl)amino]cyclohexa-2,5,-diene-1,4-dione.

6. A formulation comprising a therapeutically effective amount of 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of claim 1, or a salt thereof, in a pharmaceutically-acceptable non-toxic carrier.

7. The formulation of claim 6 wherein the 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of claim 1 which is selected from the group consisting of 2,5-bis(benzylamino)cyclohexa-2,5-diene-1,4-dione; 2,5-bis[(pyridine-3-

ylmethyl)amino]cyclohexa-2,5-diene-1,4-dione; and 2,5-dichloro-3,6-bis[3-methoxyphenyl)amino]cyclohexa-2,5,-diene-1,4-dione.

8. The formulation of claims 7 or 8 further comprising at least one or more chemotherapeutic or cytotoxic pharmaceutical agents.

9. The formulation of claim 8 wherein the at least one or more chemotherapeutic or cytotoxic pharmaceutical agents is selected from the group consisting of gemcitabine and oxaliplatin.

10. A method of preventing and/or treating cancer comprising administering a therapeutically effective amount of the 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of claim 1, or a pharmaceutically acceptable salt thereof, to a subject either alone, or in combination with, one or more chemotherapeutic and/or cytotoxic pharmaceutical agents.

11. A method of sensitizing drug-resistant pancreatic cancer cells for the prevention of tumor progression and/or treatment of pancreatic tumors in a subject who has been diagnosed with pancreatic cancer comprising the step of administering to the subject a therapeutically effective amount of the 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of claim, either alone, or in combination with at least one other chemotherapeutic or cytotoxic pharmaceutical agent and/or mode of treatment.

12. A method of inhibiting the growth of chemo-resistant pancreatic cancer cells that are enriched in cancer stem-like cells in a subject having pancreatic cancer comprising administering to the subject a therapeutically effective amount of the 2,5-bis (alkyl/aryl amino) 1, 4-benzoquinone of claim 1, either alone, or in combination with at least one other chemotherapeutic or cytotoxic pharmaceutical agent and/or mode of treatment.

13. The method of claims 10, 11, and 12 wherein the at least one other chemotherapeutic or cytotoxic pharmaceutical agent is selected from the group consisting of gemcitabine and oxaliplatin.

Fig. 1

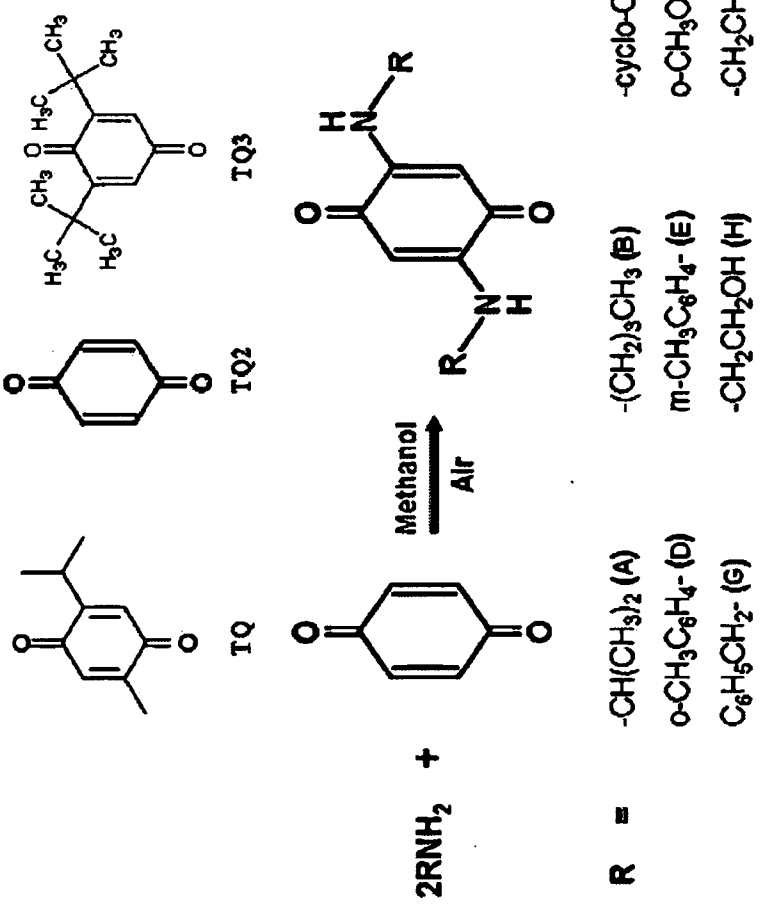

TQ
TQ2
TQ3
2RNH2 +
Methanol
Air
R =
-CH(CH3)2 (A)
o-CH3C6H4- (D)
C6H5CH2- (G)
-(CH2)3CH3 (B)
m-CH3C6H4- (E)
-CH2CH2OH (H)
-cyclo-C6H11 (C)
o-CH3OC6H4- (F)
-CH2CH3 (I)

Fig. 2

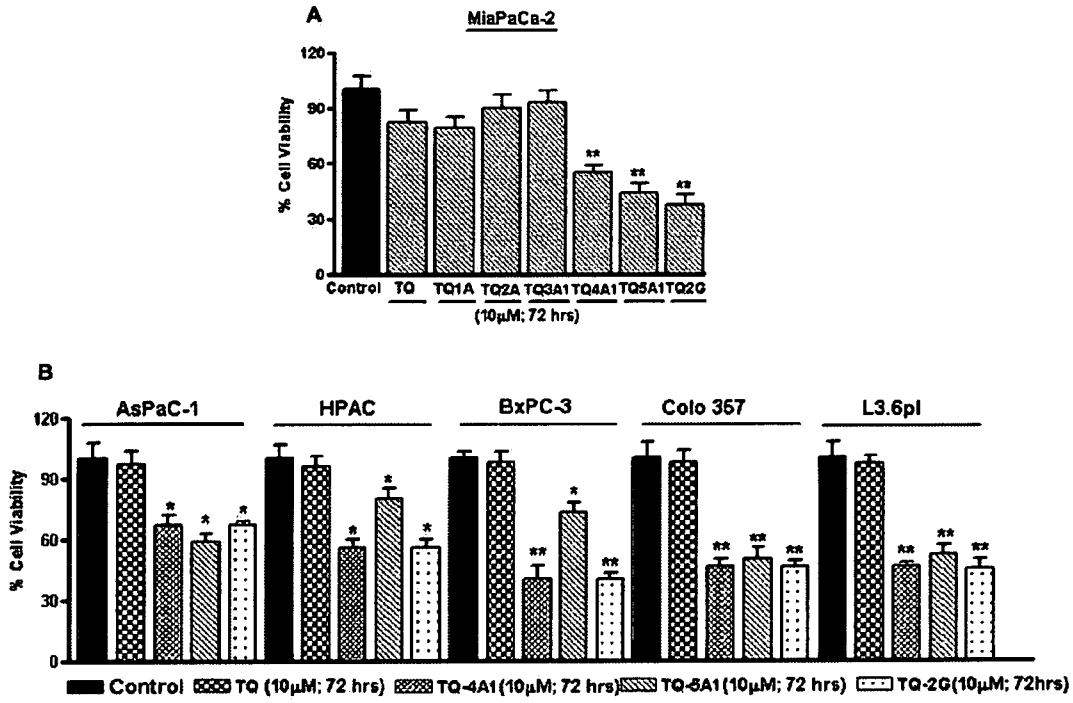

A
MiaPaCa-2
% Cell Viability
120
90
60
30
0
Control
TQ
TQ1A
TQ2A
TQ3A1
TQ4A1
TQ5A1
TQ2G
(10μM; 72 hrs)
B
AsPaC-1
HPAC
BxPC-3
Colo 357
L3.6pl
% Cell Viability
Control
TQ (10μM; 72 hrs)
TQ-4A1(10μM; 72 hrs)
TQ-5A1(10μM; 72 hrs)
TQ-2G(10μM; 72hrs)

Fig. 3

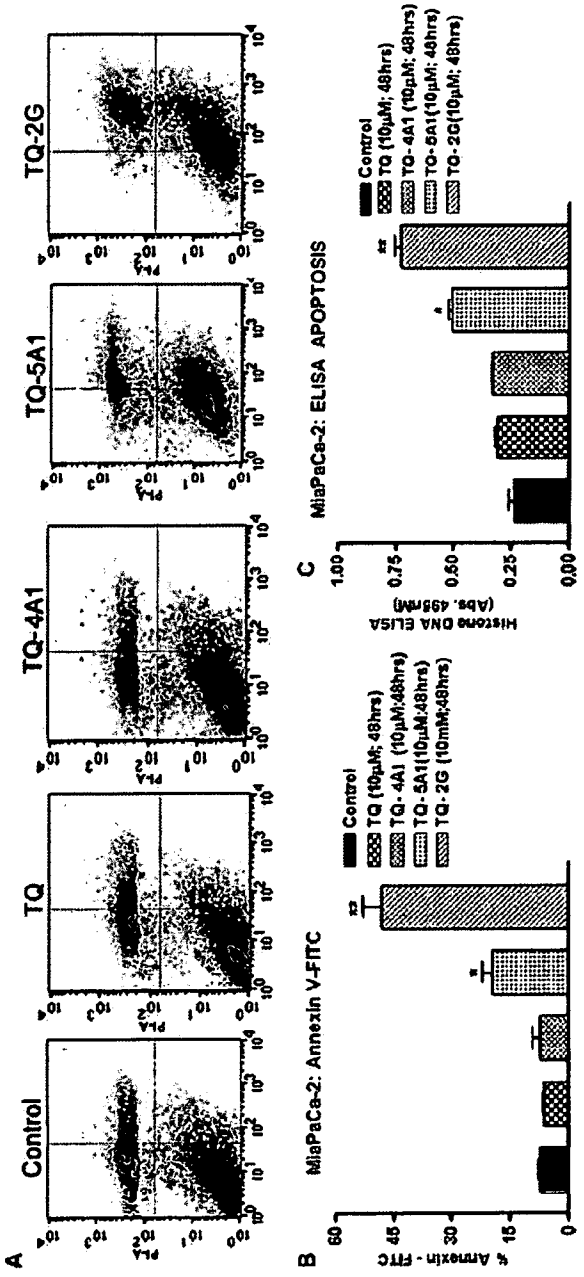

A
Control
TQ
TQ-4A1
TQ-5A1
TQ-2G
B
MiaPaCa-2: Annexin V-FITC
% Annexin - FITC
Control
TQ (10μM; 48hrs)
TQ - 4A1 (10μM;48hrs)
TQ - 5A1(10μM;48hrs)
TQ - 2G (10mM;48hrs)
C
MiaPaCa-2: ELISA APOPTOSIS
Histone DNA ELISA
(Abs. 495nM)
Control
TQ (10μM; 48hrs)
TQ - 4A1 (10μM; 48hrs)
TQ - 5A1(10μM; 48hrs)
TQ - 2G(10μM; 48hrs)

Fig. 4

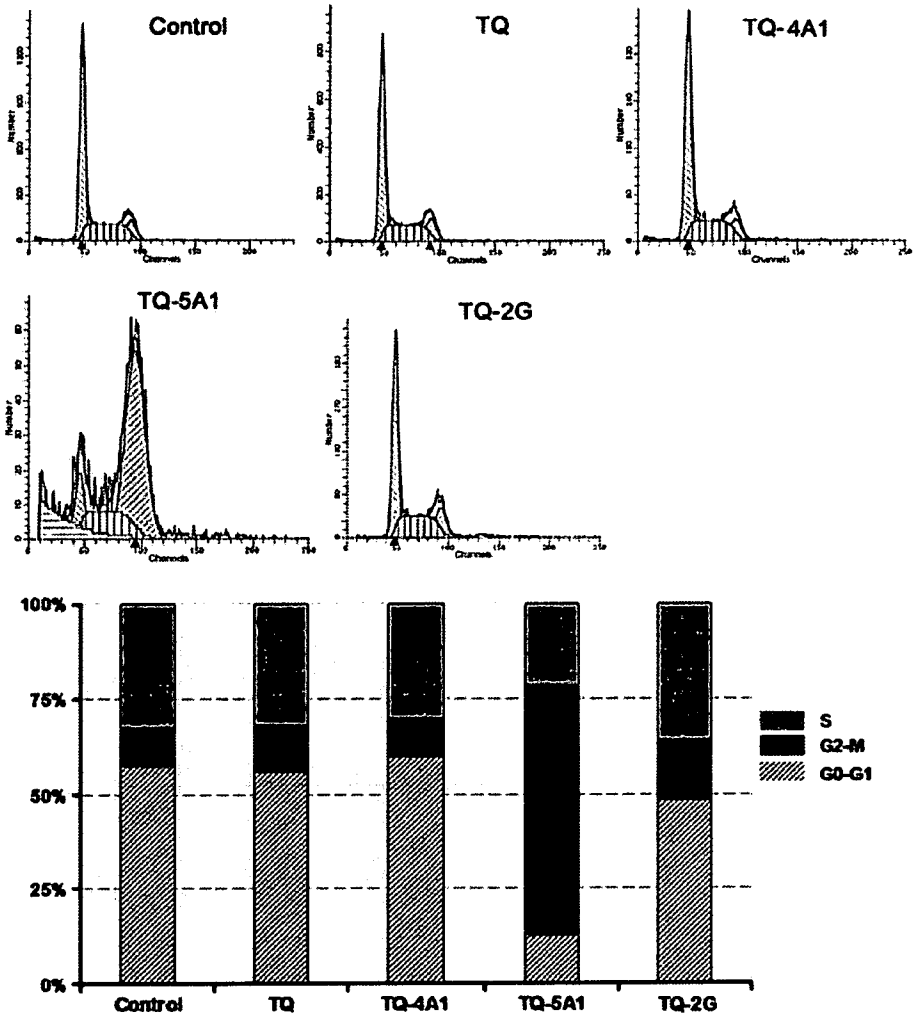

Control
TQ
TQ-4A1
TQ-5A1
TQ-2G
100%
75%
50%
25%
0%
Control
TQ
TQ-4A1
TQ-5A1
TQ-2G
S
G2-M
G0-G1

Fig. 5

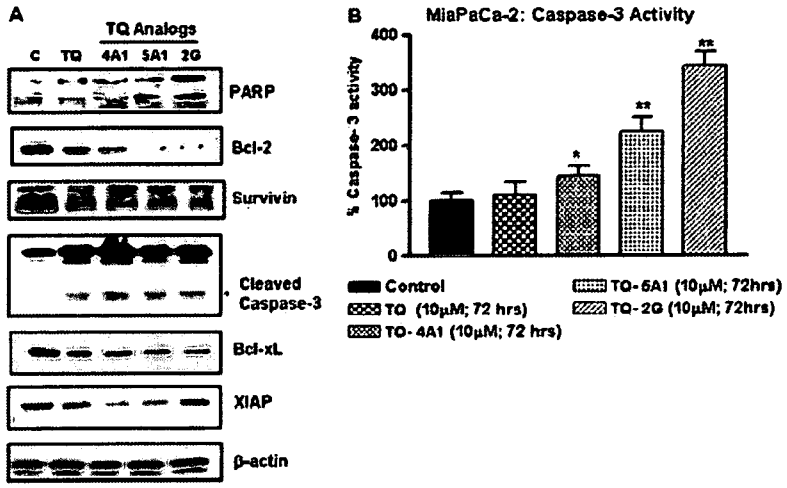

A
TQ Analogs
C
TQ
4A1
5A1
2G
PARP
Bcl-2
Survivin
Cleaved Caspase-3
Bcl-xL
XIAP
β-actin
B
MiaPaCa-2: Caspase-3 Activity
% Caspase-3 activity
400
300
200
100
0
*
**
**
Control
TQ (10μM; 72 hrs)
TQ- 4A1 (10μM; 72 hrs)
TQ- 5A1 (10μM; 72hrs)
TQ- 2G (10μM; 72hrs)

Fig. 6

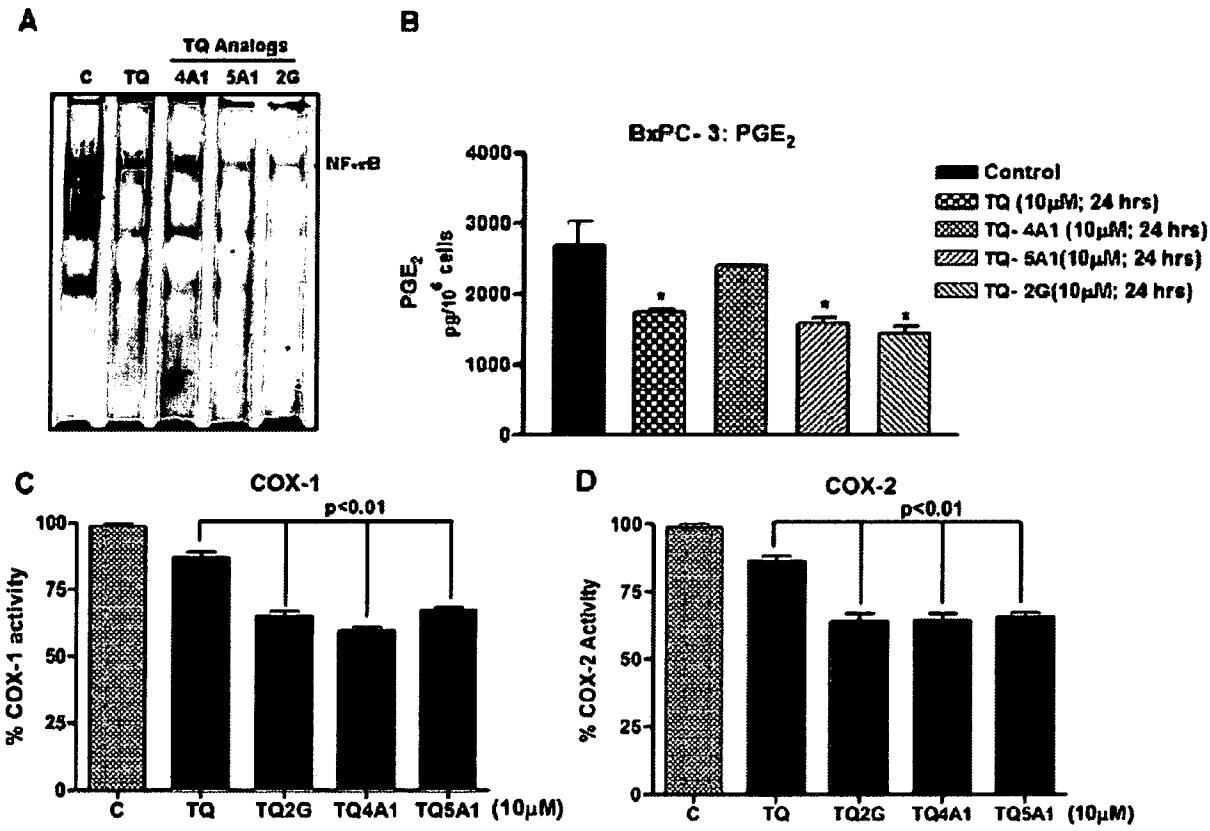

A
TQ Analogs
C
TQ
4A1
5A1
2G
NF-κB
B
BxPC-3: PGE2
PGE2 pg/10^6 cells
4000
3000
2000
1000
0
Control
TQ (10μM; 24 hrs)
TQ- 4A1 (10μM; 24 hrs)
TQ- 5A1(10μM; 24 hrs)
TQ- 2G(10μM; 24 hrs)
C
COX-1
p<0.01
% COX-1 activity
100
75
50
25
0
C
TQ
TQ2G
TQ4A1
TQ5A1
(10μM)
D
COX-2
p<0.01
% COX-2 Activity
100
75
50
25
0
C
TQ
TQ2G
TQ4A1
TQ5A1
(10μM)

Fig. 7

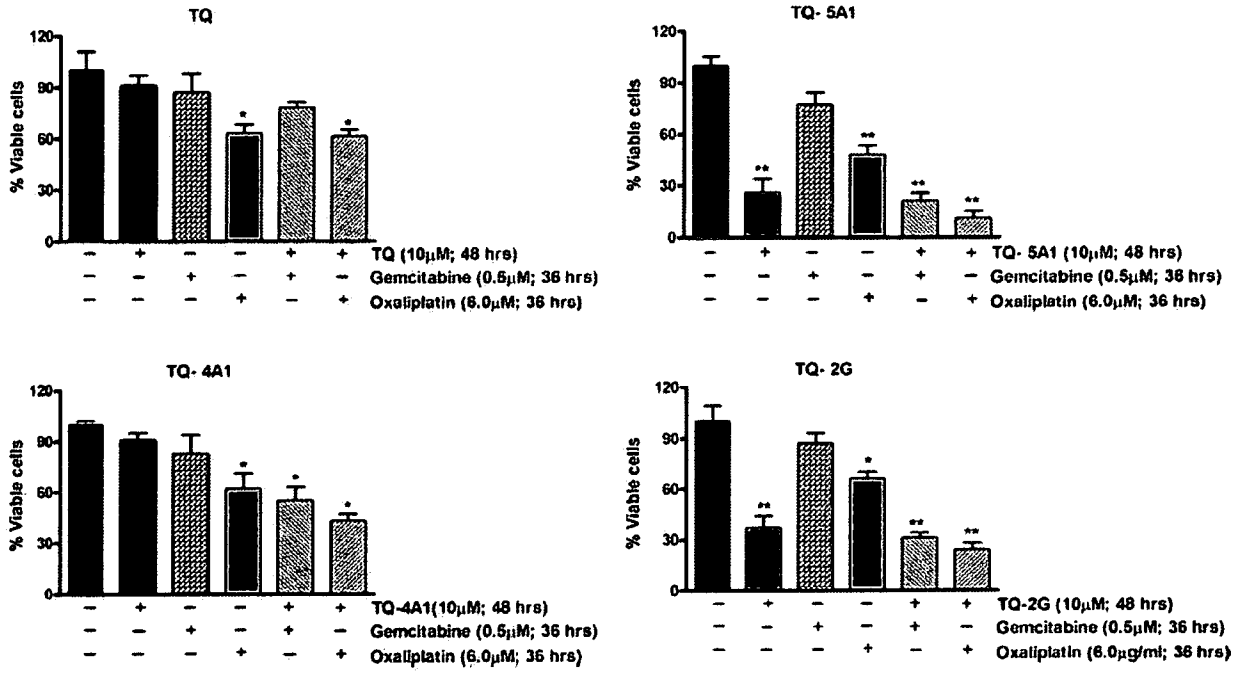
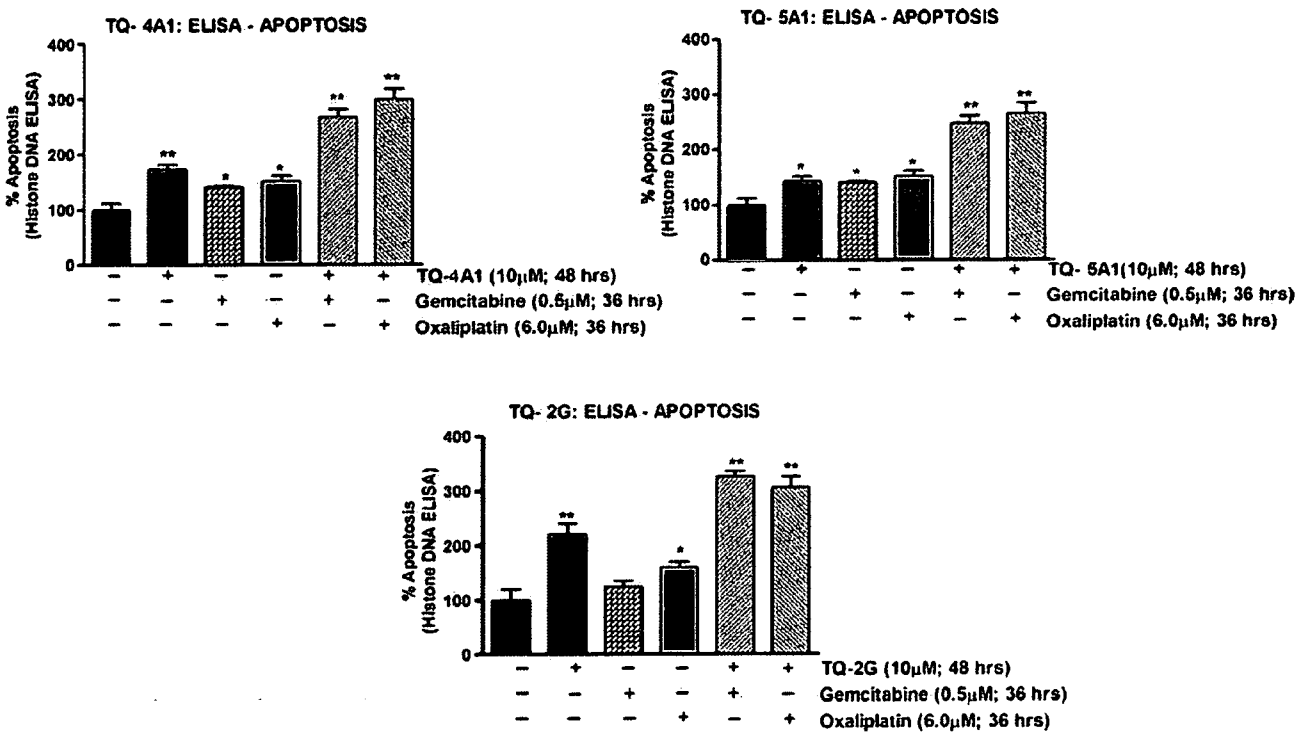

TQ
% Viable cells
120
90
60
30
0
TQ (10μM; 48 hrs)
Gemcitabine (0.5μM; 36 hrs)
Oxaliplatin (6.0μM; 36 hrs)
TQ- 5A1
TQ- 5A1 (10μM; 48 hrs)
TQ- 4A1
TQ-4A1(10μM; 48 hrs)
TQ- 2G
TQ-2G (10μM; 48 hrs)
Oxaliplatin (6.0μg/ml; 36 hrs)

Fig. 8


TQ- 4A1: ELISA - APOPTOSIS
% Apoptosis (Histone DNA ELISA)
400
300
200
100
0
TQ-4A1 (10μM; 48 hrs)
Gemcitabine (0.5μM; 36 hrs)
Oxaliplatin (6.0μM; 36 hrs)
TQ- 5A1: ELISA - APOPTOSIS
% Apoptosis (Histone DNA ELISA)
TQ- 5A1(10μM; 48 hrs)
Gemcitabine (0.5μM; 36 hrs)
Oxaliplatin (6.0μM; 36 hrs)
TQ- 2G: ELISA - APOPTOSIS
% Apoptosis (Histone DNA ELISA)
TQ-2G (10μM; 48 hrs)
Gemcitabine (0.5μM; 36 hrs)
Oxaliplatin (6.0μM; 36 hrs)