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(54) **Title:** THERAPEUTIC COMPOSITIONS AND METHODS FOR TREATMENT OF CANCER

(57) **Abstract:** The present invention relates to therapeutic compositions for the treatment of condition or disease in a subject. The present invention also relates to a method of treating cancer in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof.

WO 2024/214114 A1

THERAPEUTIC COMPOSITIONS AND METHODS FOR TREATMENT OF CANCER

FIELD OF THE INVENTION

The present invention relates to therapeutic compositions for the treatment of condition or disease in a subject. The present invention also relates to a method of treating a condition or disease in a subject.

BACKGROUND OF THE INVENTION

The modern healthcare industry is going through a major challenge in the form of various diseases such as cancer. According to global estimates, the number of new cases is expected to increase in the coming decades, reaching a staggering 30.2 million by 2040.

According to current views on the carcinogenesis of NSCLC, the transformation of a normal lung cell to malignant carcinoma requires the accumulation of multiple genetic alterations. It is observed that early alterations are caused from mutations or chromosomal deletions that inactivate TUMOUR Suppressor genes. Loss of TSG causes 85% of NSCLC Cases.

Further, P53 (Transcription factor) for various downstream targets that are involved cell progression DNA repair and regulation of APOPTOSIS. Mutations to P53 hinder its normal TUMOUR SUPRESSING capabilities. As a result of which damage of DNA continues, Faulty cells proceed through cell cycle. At this situation cell is more susceptible to further mutations.

One more critical alteration that occurs in the process of NSCLC is the activation of Oncogenes in 40% to 90% cases. The Epithelial Growth Factor Receptor, which is upregulated. In the majority of lung cancers, the activation of TELOMERASE, rise of which contributes to prolong cell survival in order the pre-malignant cells to progress towards malignancy.

To sustain growth and metastasize tumour cells adequate blood supply is required and consequently evolve angiogenesis. Tumour cells promote angiogenesis by producing vascular endothelial growth factor (VFGF). This sustains tumour growth. Genetic alterations may randomly occur in the genome leading to NSCLC. As NSCLC progresses the tumour may migrate to other areas like brain, liver, heart and other Organs.

To tackle these issues, researchers around the world are putting in tireless efforts to create more precise and speedy diagnostic methods and effective treatments. Despite these efforts, patients are still at a high risk of receiving a late diagnosis and facing post-treatment side effects after undergoing conventional treatments such as chemotherapy, surgery, and radiotherapy.

Although these traditional medical practices have been known to be moderately effective in treating certain types of cancer, they come with their own limitations that can negatively impact clinical outcomes.

Thus, there is a strong need to develop highly efficacious compositions and methods for treatment of diseases including cancer to overcome the long-standing problems associated with the existing traditional methods and provide a more accessible and affordable treatment.

OBJECTIVES OF THE PRESENT INVENTION

The main objective of the present invention is to provide a method of treating cancer in a subject.

Another objective of the present invention is to provide a composition comprising squaric acid, and/or croconic acid.

Yet another objective of the present invention is to provide a highly efficacious, more accessible, and affordable composition.

Yet another objective of the present invention is to provide a highly efficacious, more accessible, and affordable method of treating a condition or disease in a subject.

Yet another objective of the present invention is to provide the composition for use in the treatment of cancer.

Yet another objective of the present invention is to provide a compound for use in the treatment of cancer.

Yet another objective of the present invention is to provide a composition for use in the treatment of cancer.

SUMMARY OF THE PRESENT INVENTION

Main aspect of the present invention provides a method of treating cancer in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof.

Another aspect of the present invention provides a method of treating cancer in a subject, the method comprising administering to a subject in need of such treatment, a composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof.

Yet another aspect of the present invention provides a composition comprising:

- squaric acid, and/or croconic acid;
- squaric acid ester and/or croconic acid ester; and
- optionally at least one pharmaceutically acceptable carrier.

Yet another aspect of the present invention provides a highly efficacious, more accessible, and affordable composition.

Yet another aspect of the present invention provides a compound selected from squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide croconic acid diamide and a combination thereof, for use in the treatment of cancer.

Yet another aspect of the present invention provides a highly efficacious, more accessible, and affordable method of treating a condition or disease in a subject.

Yet another aspect of the present invention provides a composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof, for use in the treatment of cancer.

Yet another aspect of the present invention provides the use of a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide or a combination thereof, in the manufaturing of a medicament for treating cancer in a subject.

Yet another aspect of the present invention provides the use of a composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Illustrates A549 Cancer cell line (ACTREC), Tata Memorial Cancer, India.

Figure 2: Illustrates A549 Cancer cell line post Treatment with Adriamycin 9uM (ACTREC), Tata Memorial Cancer, India.

Figure 3: Illustrates A549 Cancer cell line post Treatment with AMSAA 9uM (ACTREC), Tata Memorial Cancer, India.

Figure 4: Illustrates Wi 38 Control and Treated with 0.01% AMSAA, Study Duration 24 hrs.

Figure 5: Illustrates Wi 38 Treated with 1% AMSAA, Study Duration 24 hrs.

Figure 6: Illustrates Wi 38 (Control) and Treated with 0.1% AMSAA, Study Duration 24 hrs.

Figure 7: Illustrates Wi 38 Treated with 1 % AMSAA, Study Duration 12 hrs.

Figure 8: Illustrates Control and Treated with 0.1 % AMSAA for 8 hrs.

Figure 9: Illustrates PC3 Control and Treated with 0.1% AMSAA for 24 hrs.

Figure 10: Illustrates PC3 Treated with 1% AMSAA Treated with Study of 24 hrs.

Figure 11: Illustrates Control RBC.

Figure 12: Illustrates AMSAA (5%) Treated RBC.

Figure 13: Illustrates AMSAA (15%) Treated RBC.

Figure 14: Illustrates AMSAA (30%) Treated RBC.

Figure 15: Illustrates ADR (9uM) Treated RBC.

Figure 16: Illustrates ADR (15uM) Treated RBC.

Figure 17: Illustrates the anti-tumor effect of AMSAA (Squaric Acid) on syngeneic breast cancer in BALB/c mice tumor model.

Figure 18: Illustrates MCF7 (Human Breast Cancer Cell Line) and 4T1 (Mice Breast Cancer Cell

Line)] killing efficacy.

DETAIL DESCRIPTION OF THE PRESENT INVENTION

At the very outset of the detailed description, it may be understood that the ensuing description only illustrates a particular form of this invention. However, such a particular form is an only exemplary embodiment, and without intending to imply any limitation on the scope of this invention. Accordingly, the description is understood as an exemplary embodiment and teaching of invention and is not intended to be taken restrictively.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skills in the art to which the methods belong.Although any formulation, composition and method similar or equivalent to those described hereincan also be used in the practice or testing of the methods and compositions, representative illustrative methods and compositions are now described.

Where a range of values is provided, it is understood that each intervening value between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within by the methods and compositions. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the methods and compositions, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding eitheror both of those included limits are also included in the methods and compositions.

It is appreciated that certain features of the methods, which are, for clarity, described in thecontext of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the methods and compositions, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination. It is noted that, as used herein and in the appended claims, the singular forms "a","an", and "the" include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as an antecedent

basis for use of such exclusive terminology as "solely," "only"and the like in connection with the recitation of claim elements or use of a "negative" limitation.

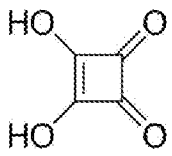
As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other embodiments without departing from the scope or spirit of the present methods. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

As those in the art will appreciate, the following description describes certain preferred embodiments of the invention in detail and is thus only representative and does not depict the actual scope of the invention. Before describing the present invention in detail, it is understood that the invention is not limited to the particular aspects and embodiments described, as these mayvary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the invention.

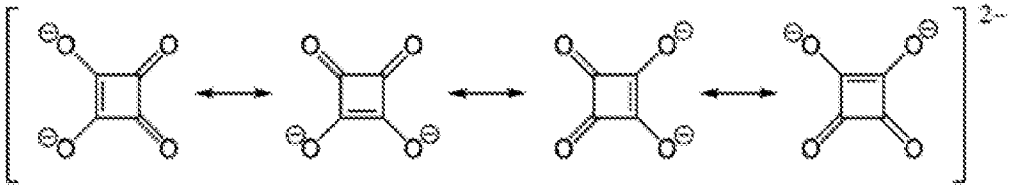
Before the formulations and methods of the present disclosure are described in greater detail, it is to be understood that the present invention generally relates to therapeutic compositionsfor the treatment of cancer, and prophylactic and therapeutic methods for cancer.

Squaric acid or salts thereof

Squaric acid, also known as 3,4-dihydroxy-3-cyclobutene-1,2 dione, has the following chemical structure.



Squaric acid has two acidic protons. The high acidity with pKa of 1.5 for the first proton and pKa of 3.4 for the second proton. It exists in dianion form at pH 7.2 with four-point hydrogenbonding sites and highly stable aromatic species.



Salt form of squaric or similar acids such as sodium salt, potassium salt or any other possible metallic salt which dissolve in water/polar protic/polar aprotic solvents may also be usedas an active agent.

Main embodiment of the present invention provides a method of treating cancer in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof.

Another embodiment of the present invention provides the method, wherein the method comprises administering (i) squaric acid, and/or croconic acid; and (ii) squaric acid ester and/or croconic acid ester.

Yet another embodiment of the present invention provides a method of treating cancer in a subject, the method comprising administering to a subject in need of such treatment, a composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof.

Yet another embodiment of the present invention provides the method, wherein the composition further comprises sodium lauryl sulfate, a base, water and a buffer.

Yet another embodiment of the present invention provides the method, wherein cancer is selected from prostate cancer, lung cancer, colon carcinoma, cervical cancer, kidney cancer, breast cancer, carcinoma such as adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, transitional cell carcinoma and the like, sarcoma such as chondrosarcoma, ewing sarcoma, leiomyosarcoma, liposarcoma, osteosarcoma and the like, lymphoma such as hodgkin lymphoma, Non-Hodgkin lymphoma, Leukemia, Acute lymphoblastic leukemia (ALL), Acute myeloid leukemia (AML),

Chronic lymphocytic leukemia(CLL), Chronic myeloid leukemia (CML) and the like, Myeloma, Melanoma, CNS tumors such as Astrocytoma, Glioblastoma, Medulloblastoma, Meningioma and the like, Gastrointestinal cancers such as Colorectal cancer, Gastric cancer, Liver cancer, Pancreatic cancerand the like, Gynecological cancers such as Cervical cancer, Ovarian cancer, Uterine cancer and the like, prostate cancer, Lung cancer, Breast cancer, Kidney cancer, Thyroid cancer, blood cancers such as Multiple myeloma, Hodgkin's lymphoma and Non-Hodgkin's lymphoma.

Yet another embodiment of the present invention provides the method, wherein the cancer is selected from breast cancer, colon carcinoma, kidney cancer, lung cancer, and prostate cancer.

Yet another embodiment of the present invention provides the method, wherein the subject is a human or animal.

Yet another embodiment of the present invention provides the method, wherein the concentration of squaric acid is in a range of 1 to 2 mg/mL.

Yet another embodiment of the present invention provides the method, wherein the concentration of squaric acid ester is in a range of 0.01 to 0.1 mg/mL.

Yet another embodiment of the present invention provides the method, wherein the concentration of sodium lauryl sulphate is in a range of 0.1 to 1 mg/ mL.

Yet another embodiment of the present invention provides a composition comprising:

- squaric acid, and/or croconic acid;
- squaric acid ester and/or croconic acid ester; and
- optionally at least one pharmaceutically acceptable carrier.

Yet another embodiment of the present invention provides the composition, wherein the composition further comprises sodium lauryl sulfate; a base; water and a buffer.

Yet another embodiment of the present invention provides the composition for use in the treatment of cancer.

Yet another embodiment of the present invention provides the composition for use as a medicament.

Yet another embodiment of the present invention provides a compound selected from squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide croconic acid diamide and a combination thereof, for use in the treatment of cancer.

Yet another embodiment of the present invention provides the compound, wherein the compound is a combination of (i) squaric acid and/or croconic acid; and (ii) squaric acid ester and/or croconic acid ester, for use in the treatment of cancer.

Yet another embodiment of the present invention provides a composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof, for use in the treatment of cancer.

Yet another embodiment of the present invention provides a composition comprising squaric acid, and/or croconic acid, squaric acid ester and/or croconic acid ester; water; and optionally at least one pharmaceutically acceptable carrier.

Yet another embodiment of the present invention provides the composition, wherein the composition further comprises sodium lauryl sulfate; a base; and a buffer.

Another embodiment of the present invention provides the composition, wherein the composition further comprises a combination of squaric acid and squaric acid monoamide or squaric acid diamide.

Yet another embodiment of the present invention provides the composition, wherein the composition further comprises a combination of croconic acid and croconic acid monoamide or croconic acid diamide.

Yet another embodiment of the present invention provides the composition, wherein squaric acid ester is squaric acid mono ester, squaric acid diester or a combination thereof. Further, diester of the squaric acid comprises two ester groups which may be the same or different.

Yet another embodiment of the present invention provides the composition, wherein croconic acid

ester is croconic acid mono ester, croconic acid diester or a combination thereof. Further, diester of the croconic acid comprises two ester groups which may be the same or different.

Yet another embodiment of the present invention provides the composition, wherein squaric acid monoester is selected from an alkyl squarate, an aryl squarate and a combination thereof. The squaric acid monoester is also known as squarate monoester. Further, the squaric acid monoester is selected from an alkyl squarate, an aryl squarate or a combination thereof.

Yet another embodiment of the present invention provides the composition, wherein alkyl squarate is selected from but not limited to a group comprising methyl squarate, propyl squarate, ethyl squarate, butyl squarate, pentyl squarate, hexyl squarate, heptyl squarate, octyl squarate, and a combination thereof.

Yet another embodiment of the present invention provides the squarate esters selected from not limited to mono/dimethyl squarate, mono/diethyl squarate, mono/diisopropyl squarate, mono/dibutyl squarate or any squaric acid mono/di esters, aryl esters and 2 or 2,3 or 2,3,5 or 2,3,5,6 mono/di/tri/tetra alkoxyor aryloxy cyclohexa-2,5-diene-1,4 dione.

Yet another embodiment of the present invention provides the composition, wherein croconic acid monoester is selected from an alkyl croconate, an aryl croconate and a combination thereof. The croconic acid monoester is also known as croconate monoester. Further, the croconic acid monoester is selected from an alkyl croconate, an aryl croconate or a combination thereof.

Yet another embodiment of the present invention provides the composition, wherein alkyl croconate is selected from but not limited to a group comprising methyl croconate, propyl croconate, ethyl croconate, butyl croconate, pentyl croconate, hexyl croconate, heptyl croconate, octyl croconate, and a combination thereof.

Yet another embodiment of the present invention provides the esters of croconic acid selected from but not limited to mono/di alkyl croconate such as mono/dimethyl croconate, mono/diethyl croconate, mono/diisopropyl croconate, mono/dibutyl croconate, aryl esters and 2 or 2,3 or 2,3,5 or 2,3,5,6 mono/di/tri/tetra alkoxyor aryloxy cyclohexa-2,5-diene-1,4 dione.

Yet another embodiment of the present invention provides the squaric acid diester also known as squarate diester. In certain embodiments, squaric acid diester is a dialkyl squarate, a diaryl squarate,

an alkyl aryl squarate or any combination thereof. Examples of dialkyl squarate include,but are not limited to, squaric acid dibutyl ester (SADBE), squaric acid diethyl ester (SADEE), squaric acid monobutyl ester (SAMBE), or squaric acid monoethyl ester (SAMEE) and/or any combination thereof.

Yet another embodiment of the present invention provides the dialkyl squarate selected from but not limited to squaric acid diethyl ester (SADEE), and squaric acid dibutyl ester (SADBE).

Yet another embodiment of the present invention provides the croconic acid diester also known as croconate diester. In certain embodiments, croconic acid diester is a dialkyl croconate, a diaryl croconate, an alkyl aryl croconate or any combination thereof. Examples of dialkyl croconate include,but are not limited to, croconic acid dibutyl ester (CADBE), croconic acid diethyl ester (CADEE), croconic acid monobutyl ester (CAMBE), or croconic acid monoethyl ester (CAMEE) and/or any combination thereof.

Yet another embodiment of the present invention provides the dialkyl croconate selected from but not limited to croconic acid diethyl ester (CADEE), and croconic acid dibutyl ester (CADBE).

Yet another embodiment of the present invention provides the composition, wherein concentration of squaric acid is in a range of 1 to 2 mg/mL.

Yet another embodiment of the present invention provides the composition, wherein concentration of squaric acid dibutyl ester is in a range of 0.01 to 0.1 mg/mL.

Yet another embodiment of the present invention provides the composition, wherein the concentration of squaric acid dibutyl ester is 0.05 mg/mL.

Yet another embodiment of the present invention provides the composition, wherein concentration of croconic acid is in a range of 1 to 2 mg/mL.

Yet another embodiment of the present invention provides the composition, wherein concentration of croconic acid dibutyl ester is in a range of 0.01 to 0.1 mg/mL.

Yet another embodiment of the present invention provides the composition, wherein concentration of croconic acid dibutyl ester is 0.05 mg/mL

Sodium Lauryl Sulfate (SLS)

Yet another embodiment of the present invention provides the composition, wherein the composition also comprises sodium lauryl sulfate.

Yet another embodiment of the present invention provides the composition, wherein the concentration of sodium lauryl sulfate is in the range of 0.1-1mg per mL.

Yet another embodiment of the present invention provides the composition, wherein the concentration of sodium lauryl sulfate is 0.5 mg/mL. In another embodiment, the concentration of sodium lauryl sulphate is 0.65 mg/mL. In yet another embodiment, the concentration of sodium lauryl sulfate is 0.4 mg/mL.

Base and Buffer

Yet another embodiment of the present invention provides the composition, wherein the composition also comprises base to neutralize the squaric acid or croconic acid and to attain the pH of the composition to the desired range. Further, composition includes but not limited to any base capable of reacting with squaric acid or croconic acid to form squaric acid dianion or croconic acid dianion.

Yet another embodiment of the present invention provides the composition, wherein the base is selected from but not limited to a group comprising carbonate of alkali or alkaline earth metal, bicarbonate of alkali or alkaline earth metal, hydroxide of alkali or alkaline earth metal, and/or any combination thereof. In certain embodiments, the alkali and alkaline earth metals are selected from but not limited to the group comprising sodium, potassium, lithium, calcium, barium, and caesium.

Yet another embodiment of the present invention provides the composition, wherein the base is sodium hydroxide.

Yet another embodiment of the present invention provides the composition, wherein the concentration of base is in the range of 0.1 to 1 mg per mL.

Yet another embodiment of the present invention provides the composition, wherein, the concentration of sodium hydroxide is in the range from 0.1-1 mg per mL.

Yet another embodiment of the present invention provides the composition, wherein the composition also comprises a buffer to maintain the buffering action.

Yet another embodiment of the present invention provides the composition, wherein the buffer is selected from but not limited to a group comprising a carbonate of an alkali or alkaline earth metal, a bicarbonate of an alkali or alkaline earth metal, a hydroxide of alkali or alkaline earth metal, or any combination thereof.

Yet another embodiment of the present invention provides the composition, wherein the buffer is sodium bicarbonate.

Yet another embodiment of the present invention provides the composition, wherein the ratio of buffer is 4 to 10.

Yet another embodiment of the present invention provides the composition, wherein the concentration of sodium bicarbonate is in the range of 0.1-1 mg per mL.

Yet another embodiment of the present invention provides the composition, wherein the pH of the composition is from 5 to 10. In certain embodiments, base and/or buffer are present in the composition in an amount sufficient to maintain a pH of the composition from about 5 to about 10.

Yet another embodiment of the present invention provides the composition, wherein the pH of the composition is from 7 to 9. In certain embodiments, base and/or buffer in the composition are present in an amount sufficient to maintain a pH of the composition from about 7 to about 9.

Yet another embodiment of the present invention provides the composition, wherein the pH of the composition is about 7.5. In certain embodiments, base and/or buffer in the composition are present in an amount sufficient to maintain a pH of the composition is about 7.5.

Solvent

Yet another embodiment of the present invention provides the composition, wherein the composition also comprises solvent. Any suitable solvent can be used in the present invention such as any solvent which dissolves squaric acid and croconic acid and esters thereof.

Yet another embodiment of the present invention provides the composition, wherein the composition further comprises a solvent selected from a polar aprotic solvent, polar protic solvent, low polar aprotic solvent, a low polar protic solvent, and/or a mixture thereof. In certain embodiments, solvent is a polar aprotic solvent, polar protic solvent, low polar aprotic solvent, a low polar protic solvent, or a mixture thereof which can dissolve sodium salts of active agent.

Yet another embodiment of the present invention provides the composition, wherein the solvent is selected from dimethylformamide (DMF), acetone, alcohol, and/or combination thereof. Examples of alcohol include, but are not limited to, methanol, ethanol, propanol, isopropanol, butanol, iso butanol, and amyl alcohol. In a preferred embodiment, the solvent is deionized water.

Yet another embodiment of the present invention provides the composition, wherein the composition also comprises pharmaceutically acceptable carrier or excipient.

Yet another embodiment of the present invention provides the composition, wherein the pharmaceutically acceptable carrier is carrier selected from but not limited to a group comprising a sterile aqueous media, a solid diluent, a filler, an excipient, various non-toxic organic solvent, and/or any combination thereof.

Yet another embodiment of the present invention provides the composition as disclosed herein which can be used as a medicament or as a component in apharmaceutical composition.

Yet another embodiment of the present invention provides the pharmaceutical compositions which include but not limited to sprays, suppositories, pastes, ointments, jellies, lotions, injectable solutions, and parenteral solutions packaged in containers adapted for subdivision into individual doses.

Yet another embodiment of the present invention provides the parenteral compositions which include but not limited to pharmaceutically acceptable aqueous or non-aqueous solutions, dispersion, emulsions, suspensions for the preparation thereof. Non-limiting examples of carriers include water, ethanol, polyols (such as propylene glycol, polyethylene glycol),vegetable oils, and injectable organic esters such as ethyl oleate. Fluidity can be maintained by theuse of a coating such as lecithin, a surfactant, or maintaining appropriate particle size. Exemplary parenteral administration forms include solutions or suspensions of the compounds of the invention in sterile aqueous solutions, for example, aqueous propylene glycol or dextrose solutions. Such dosage forms can be suitably buffered, if desired.

Yet another embodiment of the present invention provides the composition, for use as a medicament, mouth wash, mouth freshener, sanitary napkins, adult diapers.

Yet another embodiment of the present invention provides the composition, for use as a perfume, deodorant and room freshener.

Yet another embodiment of the present invention provides the composition, for use in the treatment of a condition or disease in a subject, wherein the condition or disease is selected from a group comprising cancer, bacterial infection, viral infection, fungal infection, skin disease, wound related infections, or oral disease.

Yet another embodiment of the present invention provides a method of treating a condition or disease in a subject.

Yet another embodiment of the present invention provides a method of treating a condition or disease in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester and/or croconic acid ester, wherein the condition or disease is selected from a group comprising cancer, bacterial infection, viral infection, fungal infection, skin disease, wound related infections, or oral disease. In reference to the treatment or prevention of disease, an effective amount refers to that amount which has the effect of reducing or inhibiting (that is, slowing to some extent, preferably stopping) one or more signs or symptoms characterizing one or more diseases.

Yet another embodiment of the present invention provides a method, wherein the condition or disease is cancer.

Yet another embodiment of the present invention provides a method of treating a bacterial infection in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester and/or croconic acid ester. In reference to the treatment or prevention of bacterial infection, an effective amount refers to that amount which has the effect of reducing or inhibiting (that is, slowing to some extent, preferably stopping) one or more signs or symptoms characterizing one or more bacterial infection.

Yet another embodiment of the present invention provides a method of treating a viral infection in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester and/or croconic acid ester. In reference to the treatment or prevention of viral infection, an effective amount refers to that amount which has the effect of reducing or inhibiting (that is, slowing to some extent, preferably stopping) one or more signs or symptoms characterizing one or more viral infection.

Yet another embodiment of the present invention provides a method of treating a fungal infection in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester and/or croconic acid ester. In reference to the treatment or prevention of fungal infection, an effective amount refers to that amount which has the effect of reducing or inhibiting (that is, slowing to some extent, preferably stopping) one or more signs or symptoms characterizing one or more fungal infection.

Yet another embodiment of the present invention provides a method of treating a skin disease in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester and/or croconic acid ester. In reference to the treatment or prevention of skin disease, an effective amount refers to that amount which has the effect of reducing or inhibiting (that is, slowing to some extent, preferably stopping) one or more signs or symptoms characterizing one or more skin diseases.

Yet another embodiment of the present invention provides a method of treating wound related infections in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester and/or croconic acid ester. In reference to the treatment or prevention of wound related infections, an effective amount refers to that amount which has the effect of reducing or inhibiting (that is, slowing to some extent, preferably stopping) one or more signs or symptoms characterizing one or more wound related infections.

Yet another embodiment of the present invention provides a method of treating a oral disease in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester and/or croconic acid ester. In reference to the treatment or prevention of oral disease, an effective amount refers to that amount which has the effect of reducing or inhibiting (that is, slowing to some extent, preferably stopping) one or more signs or symptoms characterizing one or more oral diseases.

Yet another embodiment of the present invention provides a method of treating a condition or disease in a subject, the method comprising administering to a subject in need of such treatment, a combination of squaric acid and squaric acid monoamide or squaric acid diamide, or of croconic acid with croconic acid monoamide or croconic acid diamide wherein the condition or disease is selected

from a group comprising cancer, bacterial infection, viral infection, fungal infection, skin disease, wound related infections, or oral disease.

Yet another embodiment of the present invention provides a method of treating bacterial infection in a subject, the method comprising administering to a subject in need of such treatment, a combination of squaric acid and squaric acid monoamide or squaric acid diamide, or of croconic acid with croconic acid monoamide or croconic acid diamide.

Yet another embodiment of the present invention provides a method of treating cancer in a subject, the method comprising administering to a subject in need of such treatment, a combination of squaric acid and squaric acid monoamide or squaric acid diamide, or of croconic acid with croconic acid monoamide or croconic acid diamide.

Yet another embodiment of the present invention provides a method of treating viral infection in a subject, the method comprising administering to a subject in need of such treatment, a combination of squaric acid and squaric acid monoamide or squaric acid diamide, or of croconic acid with croconic acid monoamide or croconic acid diamide.

Yet another embodiment of the present invention provides a method of treating fungal infection in a subject, the method comprising administering to a subject in need of such treatment, a combination of squaric acid and squaric acid monoamide or squaric acid diamide, or of croconic acid with croconic acid monoamide or croconic acid diamide.

Yet another embodiment of the present invention provides a method of treating skin disease in a subject, the method comprising administering to a subject in need of such treatment, a combination of squaric acid and squaric acid monoamide or squaric acid diamide, or of croconic acid with croconic acid monoamide or croconic acid diamide.

Yet another embodiment of the present invention provides a method of treating wound related infections in a subject, the method comprising administering to a subject in need of such treatment, a combination of squaric acid and squaric acid monoamide or squaric acid diamide, or of croconic acid with croconic acid monoamide or croconic acid diamide.

Yet another embodiment of the present invention provides a method of treating oral disease in a subject, the method comprising administering to a subject in need of such treatment, a combination

of squaric acid and squaric acid monoamide or squaric acid diamide, or of croconic acid with croconic acid monoamide or croconic acid diamide.

Yet another embodiment of the present invention provides a method of treating a condition or disease in a subject, the method comprising administering to a subject in need of such treatment, a composition, wherein the condition or disease is selected from a group comprising cancer, bacterial infection, viral infection, fungal infection, skin disease, wound related infections, or oral disease.

Yet another embodiment of the present invention provides a method of treating cancer in a subject, the method comprising administering to a subject in need of such treatment, a composition.

Yet another embodiment of the present invention provides a method of treating bacterial infection in a subject, the method comprising administering to a subject in need of such treatment, a composition.

Yet another embodiment of the present invention provides a method of treating viral infection in a subject, the method comprising administering to a subject in need of such treatment, a composition.

Yet another embodiment of the present invention provides a method of treating fungal infection in a subject, the method comprising administering to a subject in need of such treatment, a composition.

Yet another embodiment of the present invention provides a method of treating skin disease in a subject, the method comprising administering to a subject in need of such treatment, a composition.

Yet another embodiment of the present invention provides a method of treating oral disease in a subject, the method comprising administering to a subject in need of such treatment, a composition.

Yet another embodiment of the present invention provides a method of treating wound related infections in a subject, the method comprising administering to a subject in need of such treatment, a composition.

Yet another embodiment of the present invention provides a method, wherein cancer is selected from prostate cancer, lung cancer, colon carcinoma, cervical cancer, kidney cancer, breast cancer, carcinoma such as adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, transitional cell carcinoma and the like, sarcoma such as chondrosarcoma, ewing sarcoma, leiomyosarcoma, liposarcoma, osteosarcoma and the like, lymphoma such as hodgkin lymphoma, Non-Hodgkin lymphoma, Leukemia, Acute lymphoblastic leukemia (ALL), Acute myeloid leukemia (AML), Chronic lymphocytic leukemia(CLL), Chronic myeloid leukemia (CML) and the like, Myeloma,

Melanoma, CNS tumors such as Astrocytoma, Glioblastoma, Medulloblastoma, Meningioma and the like, Gastrointestinal cancers such as Colorectal cancer, Gastric cancer, Liver cancer, Pancreatic cancerand the like, Gynecological cancers such as Cervical cancer, Ovarian cancer, Uterine cancer and the like, prostate cancer, Lung cancer, Breast cancer, Kidney cancer, Thyroid cancer, blood cancers such as Multiple myeloma, Hodgkin's lymphoma and Non-Hodgkin's lymphoma.

Yet another embodiment of the present invention provides a method, wherein the cancer is selected from breast cancer, colon carcinoma, kidney cancer, lung cancer, and prostate cancer.

Yet another embodiment of the present invention provides a method, wherein the subject is a human or animal.

Yet another embodiment of the present invention provides a method for preventing or treating cancer byadministration of a therapeutically effective amount of composition as may be needed on a case to case basis.

In certain embodiments, composition may be administered in one or more dosage forms. Those skilled in the art will be able to determine, according to known methods, the appropriate amount, dose or dosage of the composition composition for administration to a subject taking intoaccount factors such as age, weight, general health, the compositions administered, the route of administration, the nature and advancement of malignancy requiring treatment, and the presence of other medications.

The composition may be administered together or independently of one another by any route known to a person skilled in the art, such as by oral, intravenous, topical, intraperitoneal or nasal route. A preferable mode for administration of the compositions of the present invention is topical and intravenous administration.

In certain embodiments, the compositions are administered at a pre-determined daily dosage. This dosage regimen may be adjusted to provide the optimal therapeutic response. For example, the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation.

The practice of the method of this invention may be accomplished through various

administration or dosing regimens. The compositions of the present invention can be administered intermittently, concurrently or sequentially with other prescribed pharmaceutical compositions.

Repetition of the administration or dosing regimens may be conducted as necessary to achieve levels of treatment.

The compositions of the present invention may further comprise a pharmaceuticallyacceptable carrier, excipient or preservatives. The carriers include but are not limited to, solid diluents or fillers, excipients, sterile aqueous media and various non-toxic organic solvents. Dosage unit forms or pharmaceutical compositions include tablets, capsules, pills, powders, granules, aqueous and non-aqueous oral solutions and suspensions, creams, hard candies, lozenges, troches, sprays, salves, suppositories, gels, pastes, ointments, jellies, lotions, injectable solutions, elixirs, syrups, and parenteral solutions packaged in containers adapted for subdivisioninto individual doses.

Yet another embodiment of the present invention provides use of a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide or a combination thereof, in the manufaturing of a medicament for treating cancer in a subject.

Yet another embodiment of the present invention provides use of a composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof.

Yet another embodiment of the present invention provides the use, wherein the composition comprises (i) squaric acid and/or croconic acid; and (ii) squaric acid ester and/or croconic acid ester.

Yet another embodiment of the present invention provides the use, wherein the composition further comprises sodium lauryl sulfate, a base, water and a buffer.

Yet another embodiment of the present invention provides the use, wherein the cancer is selected from prostate cancer, lung cancer, colon carcinoma, cervical cancer, kidney cancer, breast cancer, carcinoma such as adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, transitional cell carcinoma and the like, sarcoma such as chondrosarcoma, ewing sarcoma, leiomyosarcoma, liposarcoma, osteosarcoma and the like, lymphoma such as hodgkin lymphoma, Non-Hodgkin

lymphoma, Leukemia, Acute lymphoblastic leukemia (ALL), Acute myeloid leukemia (AML), Chronic lymphocytic leukemia(CLL), Chronic myeloid leukemia (CML) and the like, Myeloma, Melanoma, CNS tumors such as Astrocytoma, Glioblastoma, Medulloblastoma, Meningioma and the like, Gastrointestinal cancers such as Colorectal cancer, Gastric cancer, Liver cancer, Pancreatic cancerand the like, Gynecological cancers such as Cervical cancer, Ovarian cancer, Uterine cancer and the like, prostate cancer, Lung cancer, Breast cancer, Kidney cancer, Thyroid cancer, blood cancers such as Multiple myeloma, Hodgkin's lymphoma and Non-Hodgkin's lymphoma.

Yet another embodiment of the present invention provides the use, wherein the cancer is selcted from breast cancer, colon carcinoma, kidney cancer, lung cancer, and prostate cancer.

Yet another embodiment of the present invention provides the composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof, for use as antibiotic and antiseptic.

EXAMPLES

The invention will now be further illustrated by the following non-limiting examples. Thespecific examples below are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The components and/or reagents of the present disclosure are commercially available and/or can be prepared according to methods readily available to a skilled person.

Example 1: Preparation of anti-cancer therapeutic compositions

For the preparation of anti-cancer therapeutic composition, a composition comprising squaric acid, squaric acid dibutyl ester, sodium hydroxide, sodium bicarbonate and sodium laurylsulphate was dissolved in deionized water. This composition was taken as a 100% composition.

Table-1: Composition of the present invention

Sr. No.	Components	Concentration (mg per mL)
1	Squaric Acid	1.745
2	Squaric Acid Dibutyl Ester	0.05
3	Sodium hydroxide	0.65
4	Sodium bicarbonate	0.4
5	Sodium lauryl sulphate	0.5
6	Deionized water	

The therapeutic composition was prepared at various concentrations such as 0.001%, 0.01%, 0.1%, 1%, 5% and 75%.

Example 2: The Efficacy of AMSAA at 9uM Comparing with ADR

Example 3: Cell Viability of WI38 while Treated with AMSAA and Efficacy of the Composition AMSAA against PC3 Prostate Cancer cell Line

Example 4: Efficacy of the Composition against other Cancer cell Line, Lymphocyte and Effect of AMSAA on RBC.

Other than prostate cancer cell lines, the cancer cell lines for other types of cancer were tested.

- The therapeutic composition was tested on the F549 human lung cancer cell line and compared with the WI38 human normal lung cell.
- The therapeutic composition was tested on the A-498 cell line for kidney cancer.
- The therapeutic composition was tested on the HEK-293 cell line for kidney cancer.
- The therapeutic composition was tested on the Caco-2 cell line for colon cancer.
- The therapeutic composition was tested on the MDA-MB-231 cell line for breast cancer.
- The therapeutic composition is suitable for the treatment of carcinoma such as adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, transitional cell carcinoma and the like.

- The therapeutic composition is suitable for the treatment of Sarcoma such as Chondrosarcoma, Ewing sarcoma, Leiomyosarcoma, Liposarcoma, Osteosarcoma and the like.
- The therapeutic composition is suitable for the treatment of prostate cancer, Lung cancer, Breast cancer, Kidney cancer, Thyroid cancer.
- Occupational carcinogens due to increased risk of exposure to Radon, radioactive inert gas and asbestos & Diet.
- The Anticancer drug screening formulation, containing a chemical entity and other excipients have been used in parallel with Adriamycin at various concentration levels. The normal Lung cancer cell line (Wi 38) and lung cancer cell line A-549 were treated.

Methodology

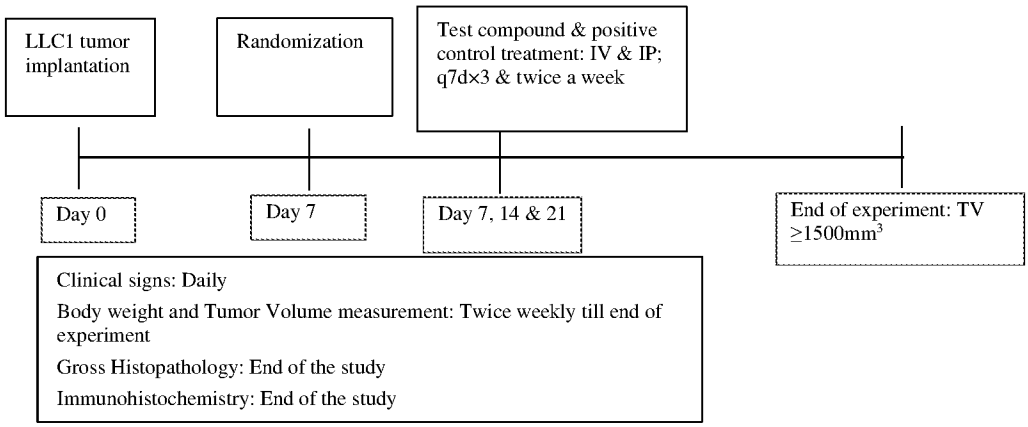
Study Title	**Determination of anti-tumor effect of AMSAA (Squaric Acid) on syngeneic lung carcinoma tumor model**		
Study No.	LB23-XX	**Functional Area**	Pre-Clinical
Version No.	00	**Effective Date**	XX Jan 2024

1. **Objective**: To determine the anti-tumor effect of AMSAA (Squaric Acid) on syngeneic lung carcinoma tumor mice model

1.1 Study Design

Tumor cell implantation (Day 0)	**Grouping at ~100mm^3 TV (Day ~7); n=8**	**Test Compound treatment dose, route and schedule (Day ~7)**	**Readout**
LLC1 cells @ 2×10^6	A: Vehicle	-	• Survivability • Body weights and clinical signs • Tumor volume measurement for TGI • Gross Histopathology • Immunohistochemistry for Ki-67
	B: Squaric Acid	5%; IV; q7d×3	
	C: Squaric Acid		
	D: Squaric Acid	15%; IV; q7d×3	
	E: Positive control (Cisplatin)	4mg/kg; IP; 6 doses (twice a week)	

Randomization: Mice will be randomized into 5 groups (n=8/group) at the tumor volume ~100mm^3 (Range 80-130 mm^3)



1.2 Study Schedule

- Study Initiation Date: XX Jan 2024
- Experiment Start Date: XX Jan 2024
- Experiment End Date: XX Feb 2024
- Study Completion Date: XX Feb 2024

Note: Actual dates shall be documented in raw data and report.

1.3 Test System Details

Species	Mice
Strain	C57BL/6
Gender	Female
Age	8-10 Weeks
Weight	18-22g
No. of Animals	48

1.4 IAEC Details

Form B for study protocol approved by the IAEC committee.

2. Abbreviations

Abbreviation	Meaning
µg	Microgram
µl	Microliter
DMEM	Dulbecco's modified eagle medium

FBS	Fetal bovine serum
Gm	Gram
Hr	Hour
IV	Intravenous
IAEC	Institutional animal ethics committee
Kg	Kilogram
L	Length
LB	Lextro Bio Solutions
Ml	Milliliter
Mm	Millimeter
mm^3	Millimeter cube
PBS	Phosphate buffered saline
QD	Quaque die
SC	Subcutaneous
TV	Tumor Volume
W	Width

3. Reagents and Materials

Reagent/Material	Make	Catalogue#
LLC1 mouse lung cancer cells	ATCC	CRL-1642
DMEM medium	Gibco	31600-034
FBS	Gibco	10082-147
Trypsin EDTA	Gibco	25200-056
Trypan blue	MP Bio medicals	1691049
Phosphate buffered saline	Gibco	10010-031
10% neutral buffered formalin	Sigma Aldrich	HT501128-4L

4. Equipment

Equipment	Make	Model
Biosafety Cabinet	Thermo Scientific	1384

Water bath	Thermo Scientific	TSGP20
Centrifuge	Thermo Scientific	5430 R
Incubator	Thermo Scientific	381
Microscope	Olympus	CKX41
Automated cell counter	Invitrogen	Countess 3 FL
Weighing Balance	Mettler Toledo	ME403/A04
Biosafety Cabinet	NAV	NAV
Isoflurane Chamber	Allied Medical	ANA-VET
Digital vernier caliper	Mitutoyo	500-196-30

5. Procedure

5.1 Preparation of Animals

5.1.1 Five days prior to start of the experiment, animals are subjected to quarantine during which clinical signs are observed along with body weight measurements on the day of receipt

5.1.2 After completion of quarantine period, weigh the mice and consider the mice with weights 18-22g for the experiment.

5.2 Implantation of tumor cells

5.2.1 On the day of implantation, process the cells from cell culture and count the cells in suspension using automated cell counter.

5.2.2 Implant LLC1 lung cancer cells at 2x10^6 cells/100 µL DMEM medium per mice through subcutaneous route into shaved right flank of mice under isoflurane anesthesia on the experimental day 0.

5.2.3 Observe clinical signs and tumor development daily

5.3 Randomization

5.3.1 Measure the tumor volume and randomize animals into groups A, B, C, D and E once the tumor volume reaches to ~ 100 mm^3 (80-130 mm^3) tentatively on day 7.

5.3.2 Calculate tumor volume by using below formula:

$$TV = ½ (Length(L) \times Width^2 (W))$$

5.4 Treatment

5.4.1 Administer vehicle control and test compound intravenously or P.O at a strength of 5% (low dose) and 15% (high dose) at volume of 10 mL/Kg to the respective groups as per the study design table following schedule.

5.5 Clinical signs & Readouts

5.5.1 Observe clinical signs daily. If the animals are found to be under stress, judged to be in painful condition or found in moribund condition, sacrifice humanely during any stage of the experiment in consultation with the study director.

5.5.2 Measure tumor volume and body weights twice weekly.

5.5.3 End of experiment (more than 20% of mice tumor volume reaches >1500mm^3 from vehicle control or any treatment group), collect tumor tissue and fix it in 10% neutral buffered formalin for paraffin embedding and histological analysis.

Example 5: Efficacy of the composition against PC3 prostate cancer cell line

Prostate Cancer Cell line PC3 was treated with the therapeutic composition of Example 1. Microscopic images at 40X were captured after the treatment. The results are depicted in Figures1 to 9.

Example 6: Efficacy of the composition against other cancer cell line

Other than prostate cancer cell lines, the cancer cell lines for other types of cancer were tested.

The therapeutic composition was tested on the FI49 human lung cancer cell line and compared with the WI38 human normal lung cell.

The therapeutic composition was tested on the Hela Cell line for cervical cancer.

The therapeutic composition was tested on the A-498 cell line for kidney cancer.

The therapeutic composition was tested on the HEK-293 cell line for kidney cancer.

The therapeutic composition was tested on the Caco-2 cell line forcolon cancer.

The therapeutic composition was tested on the MDA-MB-231 cellline for breast cancer.

The therapeutic composition is suitable for the treatment of carcinoma such as adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, transitional cell carcinoma and the like.

The therapeutic composition is suitable for the treatment of Sarcoma such as Chondrosarcoma, Ewing sarcoma, Leiomyosarcoma, Liposarcoma, Osteosarcoma and the like.

The therapeutic composition is suitable for the treatment of Lymphoma such as Hodgkin lymphoma, Non-Hodgkin lymphoma, Leukemia, Acute lymphoblastic leukemia (ALL), Acute myeloid leukemia (AML), Chronic lymphocytic leukemia (CLL), Chronic myeloid leukemia (CML) and the like.

The therapeutic composition is suitable for the treatment of Myeloma.

The therapeutic composition is suitable for the treatment of Melanoma.

The therapeutic composition is suitable for the treatment of CNS tumors such as Astrocytoma, Glioblastoma, Medulloblastoma, Meningioma and the like.

The therapeutic composition is suitable for the treatment of Gastrointestinal cancers such as Colorectal cancer, Gastric cancer, Liver cancer, Pancreatic cancerand the like.

The therapeutic composition is suitable for the treatment of Gynecological cancers such as Cervical cancer, Ovarian cancer, Uterine cancer and the like.

The therapeutic composition is suitable for the treatment of prostate cancer, Lung cancer, Breast cancer, Kidney cancer, Thyroid cancer.

The therapeutic composition is suitable for the treatment of blood cancers such as Multiple myeloma, Hodgkin's lymphoma and Non-Hodgkin's lymphoma.

Occupational carcinogens due to increased risk of exposure to Radon, radioactive inert gas and asbestos & Diet.

Non-small cell lung cancer or NSCLC accounts for approx. 90% of all lung cancers and is subclassified into three sub types: Adenocarcinoma, Squamous cell carcinoma and large cell carcinoma. Different types of lung cancer originate in different locations of lungs. Adenocarcinoma develops in the peripheral portions of the lings in alveolar TYPE 2 cells or alveolar sacs (sometimes). Squamous cell carcinoma develops near the center of the lung in mucociliary epithelial cells. Large cell carcinoma may occur in any of the one location i.e., Squamous cells or glandular regions. As the risk factors associated with NSCLC which have been found across the globe as given below.

Smoking (85 %-90%) of lung cancer

Indirectly smoke due to Air pollution and all constitute 5%

Occupational carcinogens due to increased risk of exposure to Radon, radioactive inert gas and asbestos & Diet.

The Anticancer drug screening formulation, containing a chemical entity and other excipients have been used in parallel with Adriamycin at various concentration levels. The normal Lung cancer cell line (Wi 38) and lung cancer cell line A-549 were treated.

Cell line and culture condition

PC3, HCT-116, MCF7, WI-38, Caco-2, A549 cells were purchased from the National Centre for Cell Science (Pune, India) and maintained at 37 °C, 5% CO_2 and 95% relative humidity (RH) in DMEM (Dulbecco's Modified Eagle Medium), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 U/ml). Cells were seeded for overnight and subsequently treated with AMSA and doxorubicin at different concentration after withdrawal of etoposide, incubated in fresh medium for indicated time period.

Survivability (MTT) assay

After AMSA and doxorubicin treatment cells were washed with 1 × PBS and incubated in medium with 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (0.5 mg/ml) for 3 h at 37 °C. The resulting formazan crystals were dissolved in MTT solubilization buffer (40 ml

Isopropanol, 10 ml TritonX 100, 170 µl HCl) and the absorbance were taken with a UV vis spectrophotometer (Biotek USA) at a wavelength of 570 nm.

Lymphocyte culture

10 ml of blood is collected from the vein. The collected blood is transferred to four 7ml heparinized green top tubes (Total: 20ml). Pool all the blood from the heparinized tubes were pooled together into one 50ml blue cap tube. Made 1:1 dilution of whole blood with 1X PBS. Carefully underlay 10ml of histopaque-1077. 20ml of diluted blood is transferred to a 50ml tube. Immediately centrifuge at 1800rpm [700g] for 30 min in a cooling centrifuge (4°C). Carefully remove the tube from the centrifuge, observing the three layers: top is clear supernatant, middle is opaque fluid containing the PBMC, and bottom is RBC. Carefully remove and discard the supernatant within 15 ml of the opaque layer of the PBMC. Quickly transfer the buffy coat layer of PBMC (around 5 ml) into a new 50ml tube. Add 1x PBS to the PBMC suspension to obtain a final volume of 45 ml. Mix well and centrifuge at 1800rpm for 10 min in a cooling centrifuge (4°C). Pour off and discard the supernatant and combine 1 x PBS to obtain the final volume of 30 ml. Mix well and centrifuge at 1500rpm (500g) for 10 min in a cooling centrifuge (4°C) to remove platelets. Pour off and discard the supernatant. Resuspend the pellet in 5ml of the freezing medium (90% FBS+ 10% DMSO filter sterilized).

Microscopy images

Cells were seeded on 18 mm sterile glass cover slips. After treatment cells were mounted with 50% glycerol and examined under a microscope (Leica, Germany).

MTT ASSAY & RESULTS

WI 38 Cell Line Treated with AMSAA and ADR

Table 2: Time point: 24 Hours after Treatment with AMSAA & ADR

	Ctrl	0.1% AMSAA	1% AMSAA	5 uM ADR	15 uM ADR	50 uM ADR
1		0.288 0.253	0.239	0.150	0.152	0.11
2		0.282 0.242	0.222	0.150	0.149	0.103
3		0.290 0.244	0.240	0.152	0.151	0.11
4		0.282 0.254	0.219	0.152	0.150	0.102
5		0.285 0.248	0.230	0.151	0.150	0.106
% Average Cell viability	100%	87%	81%	53%	52%	37.1%

Table 3 A-549 Cell Line and Treated with AMSAA & ADR:

Time Point: 24 hours after Treatment with AMSAA & ADR MTT Assay

	Ctrl	0.1% AMSAA	1% AMSAA	5 uM ADR	15 uM ADR	50uM ADR
1	0.197	0.193	0.162	0.063	0.063	0.057
2	0.179	0.178	0.153	0.150	0.063	0.053
3	0.195	0.190	0.163	0.064	0.065	0.058
4	0.180	0.182	0.154	0.055	0.065	0.054
5	0.196	0.180	0.153	0.065	0.055	0.054
% Average Cancer cell viability	100%	91%	78%	33%	28%	27%

Table 4: Time Point: 24 hrs. after the Treatment of Cancer cell Viability with AMSAA and ADR MTT Assay

	Ctrl	5%	15%	30%	9 uM ADR	15 uM ADR
A498 (Kidney cancer)	100	51.79	48.20	32.37	48.2	43.1
MCF 7 (Breast cancer)	100	59.77	45.80	38.54	31.84	32.90
Caco 2 (colon cancer)	100	71.11	67.77	55.35	75.55	66.66
PC3 (Prostate cancer)	100	89.82	52.96	41.11	43.60	34.10

Table 5: Lymphocyte Viability by MTT Assay with AMSAA & ADR

Ctrl	5% AMSAA	10% AMSAA	15% AMSAA	20% AMSAA	30% AMSAA	9uM ADR	15uM ADR
0.230	0.204	0.168	0.161	0.058	0.041	0.183	0.166
100%	88.69%	73.04%	70%	25.2%	17.8%	79.6%	72.17%

Table 6 WI 38 (Lung Normal Cell) MTT Data Post Treatment with AMSAA & ADR

Ctrl	5% AMSAA	10% AMSAA	15% AMSAA	20% AMSAA	9uM ADR
0.140	0.111	0.089	0.080	0.058	0.049
100%	79.28%	63.57%	57.14%	41.4%	35%

Calculation: 10% AMSAA = 1530.7uM

Discussion: The cell membrane rupture, degradation of even nucleus of the cancer cells were found. At 5% AMSAA treatment on PC3 entirely breaks down to powdered form and highly cidal. The complete profile study on Lung, Liver, Kidney, Colon, Prostate, Breast, Lymphocyte have been done at various concentration of AMSAA. 1% to 15% concentration of AMSAA shows excellent results as cidal to cancer cells with much higher viability of normal cells compared to normal therapeutic drug used in 21 days treatment cycle.

Conclusion: Safety data shows The Acute, Subacute oral & inhalation GLP OECD studies on Animals show LD 50 Oral > 5000 mg/kg, Non-toxic, Non-irritant to eyes, human skin, inhalation, on Wister rats and no mortality and morbidity in the duration as per OECD guidelines in all 2x, 5x and 10 x repeat dose study (Conclusively NOAEL)as per the Regulatory guidelines. Alternative therapeutic treatment could be very much effective at affordable price with the formulation AMSAA. <u>The anti-tumor effect of AMSAA (Squaric Acid) on syngeneic breast cancer in BALB/c mice tumor model:</u>

A total of 24 female BALB/c mice were distributed into three groups, each containing 8 individuals.

Group G1 was designated as the control and received Water for Injection (WFI) at a dose volume of 10mL/Kg. Group G2 was treated with AMSAA (Squaric acid) at a dose corresponding to 15% of the human equivalent (18.33 mg/kg). Group G3 received Doxorubicin at a dosage of 1.63 mg/kg. Intravenous (I.V) administration was employed for all test and standard drugs. Results are provided in Figure 17.

Significant increase in tumor growth inhibition was observed in the treatment group of G2(AMSAA) when compared to control treated group on 7^{th}, 11^{th}, 14^{th}, 18^{th} post treatment day.

Significant increase in tumor growth inhibition was observed in the treatment group of G3(AMSAA) when compared to control treated group on 4^{th}, 7^{th}, 11^{th}, 14^{th}, 18^{th} post treatment day.

[*MCF7* (Human Breast Cancer Cell Line) and *4T1* (Mice Breast Cancer Cell Line)] killing efficacy:

- MCF7 (Human Breast Cancer Cell Line) and 4T1 (Mice Breast Cancer Cell Line) when treated with 15% AMSAA, the **early apoptotic cell population is very high** compared to cells which are untreated irrespective of time. AMSAA treatment showed higher percentage of early apoptotic population compared to doxorubicin.

- MCF7 cell line shows a **sub G0 phase arrest** when they are exposed to AMSAA. In case of 4T1, it was noticed that AMSAA treatment induces a cell cycle arrest at G2-M (6hrs treatment) and Sub G0 stage (24 hrs treatment). AMSAA treatment showed higher percentage of Sub G0 cell population compared to doxorubicin. Results are indicated in Figure 18.

Reversal of Immune suppression:

When hPBMCs treated with AMSAA,

- The M1/M2 ratio does not vary much between control and treated group.
- CD206+ (TAM) declines irrespective of time
- **Suppressive TAM (PDL1+) population is increased**.

When hPBMCs were co cultured with MCF7 cancer cell and treated with AMSAA,

- **CD206+ TAM (Tumor associated macrophage) population increases.**

- also their (TAM) **suppressive population PDL1+ TAM is increased which** indicates its involvement in the induction of suppressive ability of tumor associated macrophages that causes severe immunosuppression.

Immune stimulatory role of AMSAA:

- Human Blood Mono-nuclear Cells when treated with AMSAA, CD3+ cell population is increased whereas their surface CD3 expression declines indicating possible **T lymphocyte activation.**
- **No such significant change in CD4+/CD8+ cell population** has been noticed when hPBMCs are treated with AMSAA.
- It has been noticed that the **immune cell proportion across different immune population is increased** upon AMSAA treatment.

WE CLAIM

1. A method of treating cancer in a subject, the method comprising administering to a subject in need of such treatment, a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof.

2. The method as claimed in claim 1, wherein the method comprises administering (i) squaric acid, and/or croconic acid; and (ii) squaric acid ester and/or croconic acid ester.

3. A method of treating cancer in a subject, the method comprising administering to a subject in need of such treatment, a composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof.

4. The method as claimed in claim 3, wherein the composition further comprises sodium lauryl sulfate, a base, water and a buffer.

5. The method as claimed in any of claims 1 to 4, wherein cancer is selected from prostate cancer, lung cancer, colon carcinoma, cervical cancer, kidney cancer, breast cancer, carcinoma such as adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, transitional cell carcinoma and the like, sarcoma such as chondrosarcoma, ewing sarcoma, leiomyosarcoma, liposarcoma, osteosarcoma and the like, lymphoma such as hodgkin lymphoma, Non-Hodgkin lymphoma, Leukemia, Acute lymphoblastic leukemia (ALL), Acute myeloid leukemia (AML), Chronic lymphocytic leukemia(CLL), Chronic myeloid leukemia (CML) and the like, Myeloma, Melanoma, CNS tumors such as Astrocytoma, Glioblastoma, Medulloblastoma, Meningioma and the like, Gastrointestinal cancers such as Colorectal cancer, Gastric cancer, Liver cancer, Pancreatic cancerand the like, Gynecological cancers such as Cervical cancer, Ovarian cancer, Uterine cancer and the like, prostate cancer, Lung cancer, Breast cancer, Kidney cancer, Thyroid cancer, blood cancers such as Multiple myeloma, Hodgkin's lymphoma and Non-Hodgkin's lymphoma.

6. The method as claimed in claim 5, wherein the cancer is selected from breast cancer, colon carcinoma, kidney cancer, lung cancer, and prostate cancer.

7. The method as claimed in any of claims 1 to 6, wherein the subject is a human or animal.

8. The method as claimed in any of claims 1 to 3, wherein concentration of squaric acid is in a range of 1 to 2 mg/mL.

9. The method as claimed in any of claims 1 to 3, wherein concentration of squaric acid ester is in a range of 0.01 to 0.1 mg/mL.

10. The method as claimed in claim 4, wherein concentration of sodium lauryl sulphate is in a range of 0.1 to 1 mg/ mL.

11. A composition comprising:

- squaric acid, and/or croconic acid;
- squaric acid ester and/or croconic acid ester; and
- optionally at least one pharmaceutically acceptable carrier.

12. The composition as claimed in claim 11, wherein the composition further comprises sodium lauryl sulfate; a base; water and a buffer.

13. The composition as claimed in claim 11 or 12, for use in the treatment of cancer.

14. The composition as claimed in any of claim 11 or 12, for use as a medicament.

15. A compound selected from squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide croconic acid diamide and a combination thereof, for use in the treatment of cancer.

16. The compound as claimed in claim 15, wherein the compound is a combination of (i) squaric acid and/or croconic acid; and (ii) squaric acid ester and/or croconic acid ester, for use in the treatment of cancer.

17. A composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof, for use in the treatment of cancer.

18. Use of a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid

monoamide, croconic acid diamide or a combination thereof, in the manufaturing of a medicament for treating cancer in a subject.

19. Use of a composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof.

20. The use as claimed in claim 19, wherein the composition comprises (i) squaric acid and/or croconic acid; and (ii) squaric acid ester and/or croconic acid ester.

21. The use as claimed claim 18 or 19, wherein the composition further comprises sodium lauryl sulfate, a base, water and a buffer.

22. The use as claimed in any of claims 17 to 21, wherein the cancer is selected from prostate cancer, lung cancer, colon carcinoma, cervical cancer, kidney cancer, breast cancer, carcinoma such as adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, transitional cell carcinoma and the like, sarcoma such as chondrosarcoma, ewing sarcoma, leiomyosarcoma, liposarcoma, osteosarcoma and the like, lymphoma such as hodgkin lymphoma, Non-Hodgkin lymphoma, Leukemia, Acute lymphoblastic leukemia (ALL), Acute myeloid leukemia (AML), Chronic lymphocytic leukemia(CLL), Chronic myeloid leukemia (CML) and the like, Myeloma, Melanoma, CNS tumors such as Astrocytoma, Glioblastoma, Medulloblastoma, Meningioma and the like, Gastrointestinal cancers such as Colorectal cancer, Gastric cancer, Liver cancer, Pancreatic cancerand the like, Gynecological cancers such as Cervical cancer, Ovarian cancer, Uterine cancer and the like, prostate cancer, Lung cancer, Breast cancer, Kidney cancer, Thyroid cancer, blood cancers such as Multiple myeloma, Hodgkin's lymphoma and Non-Hodgkin's lymphoma.

23. The use as claimed in claim 22, wherein the cancer is selcted from breast cancer, colon carcinoma, kidney cancer, lung cancer, and prostate cancer

24. A composition comprising squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof, for use as antibiotic and antiseptic.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2024/050372

A. CLASSIFICATION OF SUBJECT MATTER
A61K31/185,C07C35/02,C07C49/527,C07C49/593,C07C49/597,A61P31/00
Version=2024.01
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)

A61K, C07C, A61P

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

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

PatSeer, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2022074667A1 (MAZUMDER AMITAVA); 14 April 2022 (14.01.2022). abstract, claim 1, 8-9, 11, 18-19, page no. 20, 22-23, page no. 28 para. 2.	11-24
Y	US20080200523A1 (MURTHI KRISHNA K; KOESTLER ROLAND; SMITH CHASE; BRANDSTETTER TILMAN; KLUGE ARTHUR F; FORMA TM LLC); 21 August 2008 (21.08.2008). abstract, claims 1-5, para. [0193].	11-24
Y	WO2022185326A1 (MAZUMDER AMITAVA); 09 September 2022 (09.09.2022) [FAMILY: NONE] abstract, claims 1-6, 23.	11-24

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23-07-2024	23-07-2024
Name and mailing address of the ISA/ Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Authorized officer Himanshu Singh Telephone No. +91-1125300200

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2024/050372

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-10
because they relate to subject matter not required to be searched by this Authority, namely:
The subject matter of claims 1-10 relates to a method of treating cancer using a therapeutically effective amount of squaric acid, croconic acid, squaric acid ester, croconic acid ester, squaric acid monoamide, squaric acid diamide, croconic acid monoamide, croconic acid diamide, or a combination thereof, which does not require an

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

International application No.
PCT/IN2024/050372

Continuation of Claims found unsearchable(Box II)

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international search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and [Rule 39.1(iv)].

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IN2024/050372

Citation	Pub.Date	Family	Pub.Date
WO 2022074667 A1	14-04-2022	US 2022287302 A1	15-09-2022
US 2008200523 A1	21-08-2008	US 8450348 B2	28-05-2013