Title: GLYCOALKALOID AND TLR AGONIST COMBINATIONS AND VARIOUS USES THEREOF

Abstract: A method of treating a tumorous growth comprising the step of administering a therapeutically effective amount of: (a) a first composition comprising at least two glycoalkaloids, and (b) a second composition comprising a TLR agonist.
“Glycoalkaloid and TLR Agonist Combinations and Various Uses Thereof”

Field of the Invention

The present invention relates to the use of glycoalkaloids, such as solasonine and solamargine, in combination with TLR agonists to treat cancer and to compositions for use in such methods. More particularly the present invention relates to the use of glycoalkaloids in combination with TLR3, TLR7 or TLR9 agonists.

Background Art

Cytotoxic chemotherapy remains one of the premier treatment options to combat cancer. However, the efficacy of chemotherapy is limited by the fact that not all tumors respond optimally. Thus, single-modality chemotherapy is rarely curative. In addition, drug-resistant tumor cells often emerge when a single agent is used.

CORAMSINE™ is mixture of solasonine and solamargine with anti-cancer properties.

Toll-Like receptors (TLRs) play a critical role in early innate immunity to invading pathogens by sensing micro-organisms. These evolutionary conserved receptors, homologues of the Drosophila Toll gene, recognize highly conserved structural motifs only expressed by microbial pathogens, called pathogen-associated microbial patterns (PAMPs). PAMPs include various bacterial cell wall components such as lipopolysaccharides (LPS), peptidoglycans and lipopeptides, as well as flagellin, bacterial DNA and viral double-stranded RNA. Stimulation of TLRs by PAMPs initiates a signalling cascade that involves a number of proteins, such as MyD88 and IRAK. This signalling cascade leads to the activation of the transcription factor NF-κB which induces the secretion of pro-inflammatory cytokines and effector cytokines that direct the adaptive immune response.
A number of human and murine TLRs and their ligands have been characterized. TLR2 is essential for the recognition of a variety of PAMPs, including bacterial lipoproteins, peptidoglycan, and lipoteichoic acids. TLR3 is implicated in virus-derived double-stranded RNA. TLR4 is predominantly activated by lipopolysaccharide. TLR5 detects bacterial flagellin and TLR9 is required for response to unmethylated CpG DNA. TLR7 and TLR8 have been shown to recognize small synthetic antiviral molecules.

The present invention seeks to improve the effectiveness and/or patient outcomes obtained using CORAMSINE™ monotherapy through combination therapy with TLR agonists.

**Summary of the Invention**

The present invention also provides a method of treating a tumorous growth comprising the step of administering a therapeutically effective amount of: (a) a first composition comprising at least two glycoalkaloids of formula I:

![Chemical Structure](#)

wherein: either one or both of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

A: represents a radical selected from the following radicals of general formulae (II) to (V):
each of $R^1$ is a radical separately selected from the group consisting of hydrogen, amino, oxo and $OR^4$; each of $R^2$ is a radical separately selected from the group consisting of hydrogen, amino and $OR^4$; each of $R^3$ is a radical separately selected from the group consisting of hydrogen, alkyl and $R^4$-alkylene; each of $R^4$ is a radical separately selected from the group consisting of hydrogen, carbohydrate and a carbohydrate derivative; "X" is a radical selected from the group comprising $-CH_2^-, -O$ and $-NH_2^-$; and

wherein the compound includes at least one $R^4$ group in which $R^4$ is a carbohydrate or a derivative thereof selected from the group comprising glyceric aldehyde, glycerose, erythrose, threose, ribose, arabinose, xylose, lyxose, altrose, allose, gulose, mannose, glucose, idose, galactose, talose, rhamnose, dihydroxyacetone, erythulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, and other hexoses, heptoses, octoses, nanoses, decoses, deoxysugars with branched chains, (e.g. apiose, hamamelose, streptose, cordycepose, mycarose and cladinose), compounds wherein the aldehyde, ketone or hydroxyl groups have been substituted (e.g. N-acetyl, acetyl, methyl, replacement of $CH_2OH$), sugar alcohols, sugar acids, benzimidazoles, the enol salts of the carbohydrates, saccharinic acids, sugar phosphates; and

(b) and a second composition comprising a TLR agonist.

The present invention also provides a unit dosage form comprising a therapeutically effective amount of: (a) at least two glycoalkaloids of formula I:
wherein: either one or both of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

A: represents a radical selected from the following radicals of general formulae (II) to (V):

\[
\text{II): } \quad \begin{array}{c}
\text{III): } \\
\text{IV): } \quad \begin{array}{c}
\text{V): } \\
\end{array}
\end{array}
\]

each of \( R^1 \) is a radical separately selected from the group consisting of hydrogen, amino, oxo and \( OR^4 \);

each of \( R^2 \) is a radical separately selected from the group consisting of hydrogen, amino and \( OR^4 \);

each of \( R^3 \) is a radical separately selected from the group consisting of hydrogen, alkyl and \( R^4 \)-alkylene;

each of \( R^4 \) is a radical separately selected from the group consisting of hydrogen, carbohydrate and a carbohydrate derivative;
“X” is a radical selected from the group comprising –CH₂-, –O- and –NH₂-; and

wherein the compound includes at least one R⁴ group in which R⁴ is a carbohydrate or a derivative thereof selected from the group comprising glyceric aldehyde, glycerose, erythrose, threose, ribose, arabinose, xylose, lyxose, altrose, allose, gulose, mannose, glucose, idose, galactose, talose, rhamnose, dihydroxyacetone, erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, and other hexoses, heptoses, octoses, nanoses, decoses, deoxysugars with branched chains, (e.g. apirose, hamamelose, streptose, cordycepose, mycarose and cladinose), compounds wherein the aldehyde, ketone or hydroxyl groups have been substituted (e.g. N-acetyl, acetyl, methyl, replacement of CH₂OH), sugar alcohols, sugar acids, benzimidazoles, the enol salts of the carbohydrates, saccharinic acids, sugar phosphates; and

(b) a TLR agonist.

The present invention still further provides a pack comprising a unit dosage form of glycoalkaloids and a unit dosage form of a TLR agonist.

Brief Description of the Drawings

Figure 1a is a graph illustrating the effect of different doses of intra-peritoneal CORAMSINE™ on tumour size in mice;

Figure 1b is a graph plotting the survival of mice administered different doses of CORAMSINE™ intra-peritoneally;

Figure 2a is a graph illustrating the effect of different doses of intra-venous CORAMSINE™ on tumour size in mice;

Figure 2b is a graph plotting the survival of mice administered different doses of CORAMSINE™ intra-venously;

Figure 2c is a graph illustrating the effect of further (higher) doses of intra-venous CORAMSINE™ on tumour size in mice;

Figure 2d is a graph plotting the survival of mice administered further (higher) doses of CORAMSINE™ intra-venously;
Figure 3a is a graph illustrating the effect of CORAMSINE™ and FGK-45 monotherapy and combination therapy administered intra-peritoneally on tumour size in mice;

Figure 3b is a graph illustrating the survival of mice administered CORAMSINE™ and FGK-45 monotherapy and combination therapy intra-peritoneally;

Figure 4a is a graph illustrating the effect of CORAMSINE™ in combination with Poly I:C, loxoribine, CpG ODN or nonCpG ODN on tumour size in mice;

Figure 4b is a graph illustrating the survival of mice administered CORAMSINE™ in combination with Poly I:C, loxoribine, CpG ODN or nonCpG ODN;

Figure 4c is a graph illustrating the effect of CORAMSINE™ in combination with CpG ODN or nonCpG ODN on tumour size in mice;

Figure 4d is a graph illustrating the survival of mice administered CORAMSINE™ in combination with CpG ODN or nonCpG ODN;

Figure 5 is a plot illustrating the results of ApoStat staining in cells exposed to CORAMSINE™ or gemcitabine;

Figure 6a is a graph illustrating the IL-6 levels when cells are exposed to CORAMSINE™; and

Figure 6b is a graph illustrating the IL-6 levels when cells are exposed to gemcitabine.

Detailed Description of the Invention

Methods of Treating Tumours

The present invention also provides a method of treating a tumorous growth comprising the step of administering a therapeutically effective amount of: (a) a first composition comprising at least two glycoalkaloids of formula I:
wherein: either one or both of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

A: represents a radical selected from the following radicals of general formulae (II) to (V):

![Chemical Structures](image)

Each of $R^1$ is a radical separately selected from the group consisting of hydrogen, amino, oxo and $OR^4$; each of $R^2$ is a radical separately selected from the group consisting of hydrogen, amino and $OR^4$; each of $R^3$ is a radical separately selected from the group consisting of hydrogen, alkyl and $R^4$-alkylene; each of $R^4$ is a radical separately selected from the group consisting of hydrogen, carbohydrate and a carbohydrate derivative; "$X$" is a radical selected from the group comprising $–CH_2$, $–O$ and $–NH_2$; and

wherein the compound includes at least one $R^4$ group in which $R^4$ is a carbohydrate or a derivative thereof selected from the group comprising glycemic aldehyde, glycerose, erythrose, threose, ribose, arabinose, xylose, lyxose, altrose, allose, gulose, mannose, glucose, idose, galactose, talose, rhamnose, dihydroxyacetone, erythulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, and other hexoses, heptoses, octoses, nonoses, decoses, deoxy sugars with branched chains, (e.g. apiose, hamamelose, streptose, cordycepose, mycarose and cladinoise), compounds wherein the aldehyde, ketone or hydroxyl groups have been substituted (e.g. N-acetyl, acetyl, methyl, replacement of
CH$_2$OH), sugar alcohols, sugar acids, benzimidazoles, the enol salts of the carbohydrates, saccharinic acids, sugar phosphates; and

(b) and a second composition comprising a TLR agonist.

The present invention is based on the surprising discovery that glycoalkaloids can cause cell death without stimulating the immune system. Thus, there exists an opportunity to combine other immune system stimulating agents with the glycoalkaloids to develop tailored and improved therapies for treating a range of tumours.

Preferably, the glycoalkaloids and the TLR agonist are complimentary and/or synergistic in that they provide a better patient outcome relative to the respective monotherapies and relative to the additive effects of the respective monotherapies. Thus the present invention also provides a method for treating a tumorous growth comprising the steps of administering a therapeutically effective synergistic amount of the first and second compositions mentioned above.

The tumorous growth may be associated with a range of cancers including cancer selected from the group consisting of: melanomas and non-melanoma skin including lignin melanoma, solar keratosis, basal cell carcinoma, squamous cell carcinoma and actinic keratoses; mesothelioma; breast; prostate; liver; lung; colon; rectum; urinary bladder; non-Hodgkin lymphoma; kidney; pelvis; pancreas; pharynx; head & neck; ovarian; oral; thyroid; stomach; brain; multiple myeloma; oesophagus; liver and intrahepatic bile duct; cervix; larynx; acute myeloid leukemia; chronic lymphocytic leukemia; heart; Hodgkin lymphoma; testis; small intestine; chronic myeloid leukemia; acute lymphocytic leukemia; gallbladder; bones and joints; eye; nose; nasopharynx; peritoneum; omentum; and mesentery gastrointestinal.

The glycoalkaloids used in the method of the present invention may be varied. Preferably, the glycoalkaloids are triglycoside glycoalkaloids, solasodine glycosides or are selected from the group of glycoalkaloids consisting of:
solamargine, solasonine, solanine, tomatine, solanocapsine and 26-aminofurostane.

The glycoalkaloids may be chiral, stereoisomers and mixtures thereof including enantiomers and/or diastereoisomers. Furthermore, the glycoalkaloids may be obtained from natural sources, synthesized or produced by chemically modifying other glycoalkaloids.

The number of glycoalkaloids used may be varied, as may their relative ratios in the composition. However, when the composition comprises two glycoalkaloids they may be present in a ratio selected from the group of ratios consisting of approximately: 1:6 - 1:0.5; 1:5; 1:4; 1:3; 1:2, 1:1.5 and 1:1.

Preferably, the glycoalkaloids are solamargine and solasonine in a ratio between about 1:6 and 6:1 or more preferably in a ratio between about 1:4 and 4:1, 1:3 and 3:1 or 1:2 to 2:1. When the glycoalkaloids are solamargine and solasonine and they are present in a 1:1 ratio it is preferred that the glycoalkaloids are isolated. Alternatively, when the glycoalkaloids are solasonine and solamargine it is preferred that they do not constitute 66% of glycosides in the composition.

Preferably, the glycoalkaloids constitute greater than 70%-90% of the glycosides in the composition, more preferably 91-95% and even more preferably 96-100% of the glycosides in the composition.

The amount of glycoalkaloids in the compositions of the present invention may be varied depending on their intended end use. Preferably, the compositions comprise about 0.001% - 5% or 10% glycoalkaloids, more preferably 0.01% - 5% or 10% and even more preferably 0.1%- 5% or 10% glycoalkaloids.

The actual concentration of glycoalkaloids in the composition will vary and depend at least on the nature of the tumour being treated and the condition of the subject to be treated. Skilled practitioners can determine the most appropriate dose using their ordinary skill and taking into account various parameters that apply in such
situations. For example, the higher the cancer load in a particular patient the higher the dose of glycoalkaloids that can be administered and well tolerated by the patient. Preferably, the concentration of glycoalkaloids administered as part of the combination therapy is less than in a comparable situation in which it was to be administered as a monotherapy. The amount of glycoalkaloid may be about 0.1mg/kg - 100mg/kg, 1mg/kg-80mg/kg, 5mg/kg-60mg/kg or 10mg/kg-40mg/kg. Preferably the amount of glycoalkaloid is about 0.5-5mg/kg, 0.75-4mg/kg or 1-3mg/kg.

Preferably, the TLR agonist is a TLR3, TLR7, TLR8 or TLR9 agonist.

The TLR3 agonist may be double-stranded RNA (dsRNA) containing a molecular pattern associated with viral infection. More particularly the TLR3 agonist may be polyinosine-polyctidylic acid (poly(I:C)), a synthetic analog of dsRNA.

TLR7/TLR8 agonists include imidazoquinoline compounds such as Imiquimod™ (compound R387), guanine analogs such as loxoribine, single stranded PolyU, single stranded RNA40 and R484, a small anti-viral molecule.

When the agonist is a TLR9 agonist it may be a nucleotide comprising a sequence corresponding to a pathogen associated microbial pattern (PAMPs). More particularly, the TLR9 agonist is a CpG ODN such as a nucleotide comprising a sequence according to formula I:

\[ 5' X_1 X_2 CGX_3 X_4 3' \]

wherein C and G are unmethylated, \( X_1, X_2, X_3 \) and \( X_4 \) are nucleotides and a GCG trinucleotide sequence is not present at or near the 5' and 3' termini.

\( X_1, X_2 \) may be the dinucleotide GpA and/or \( X_3, X_4 \) may be the dinucleotide TpC or TpT. In a preferred form of the inversion \( X_1 X_2 CGX_3 X_4 \) is preceded on the 5' end by a T. Preferably, the nucleotide comprises the sequence TGACGTT or TGACGTC.
The nucleotide may be of various sizes but preferably the nucleotide is about 8 to 40 bases or about 16-20 bases. In one particular example the nucleotide is 5’ TCC ATG ACG TTC CTG ATG CT 3’ (CpG 1668). Other TLR9 agonists include oligonucleotides with human specific type B CpGs (e.g. ODN 2006), oligonucleotides with human specific type A CpGs (e.g. ODN 2216) and oligonucleotides with human specific type C CpGs (e.g. ODN M362).

When the agonist is a nucleotide it is preferably stabilized such that it is relatively resistant to in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilized oligonucleotides include those with a modified phosphate backbone e.g. a phosphorothioate modified phosphate backbone (i.e. at least one of the phosphate oxygen’s is replaced by sulfur). Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl- phosphonates, phosphodiester and alkylphosphotriesters. Oligonucleotides which contain a diol, such as tetraethylene glycol or hexaethylene glycol, at either or both termini have are also known to be resistant to nuclease degradation.

The actual concentration of TLR agonist in the composition will vary and depend at least on the nature of the tumour being treated and the condition of the subject to be treated. Skilled practitioners can determine the most appropriate dose using their ordinary skill and taking into account various parameters that apply in such situations. Preferably, the concentration of agonist administered as part of the combination therapy is less than in a comparable situation in which it was to be administered as a monotherapy. The amount of agonist may be about 0.1mg/kg - 100mg/kg, 1mg/kg-80mg/kg, 5mg/kg-60mg/kg or 10mg/kg-40mg/kg. Preferably, the amount of agonist is 0.005mg/kg-10mg/kg and more preferably 0.01-1mg/kg.

It is also possible to include other agents, in addition to the first and second compositions, in the combination therapy. Thus, the present invention also provides a method of treating a tumorous growth comprising the step of administering a therapeutically effective amount of: (a) a first composition comprising at least two glycoalkaloids of formula I:
wherein: either one or both of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

A: represents a radical selected from the following radicals of general formulae (II) to (V):

each of $R^1$ is a radical separately selected from the group consisting of hydrogen, amino, oxo and $OR^4$; each of $R^2$ is a radical separately selected from the group consisting of hydrogen, amino and $OR^4$; each of $R^3$ is a radical separately selected from the group consisting of hydrogen, alky1 and $R^4$-alkylene; each of $R^4$ is a radical separately selected from the group consisting of hydrogen, carbohydrate and a carbohydrate derivative; "X" is a radical selected from the group comprising $-\text{CH}_2$, $-\text{O}$ and $-\text{NH}_2$; and

wherein the compound includes at least one $R^4$ group in which $R^4$ is a carbohydrate or a derivative thereof selected from the group comprising glycemic aldehyde, glycose, erythrose, theose, ribose, arabinose, xylose, lyxose, altrose,
alloose, gulose, mannose, glucose, idose, galactose, talose, rhamnose, 
dihydroxyactone, erythulose, ribulose, xylulose, psicose, fructose, sorbose, 
tagatose, and other hexoses, heptoses, octoses, nanoses, decoses, deoxysugars 
with branched chains, (e.g. apiose, hamamelose, streptose, cordycepse, 
mycarose and cladinose), compounds wherein the aldehyde, ketone or hydroxyl 
groups have been substituted (e.g. N-acetyl, acetyl, methyl, replacement of 
CH₂OH), sugar alcohols, sugar acids, benzimidazoles, the enol salts of the 
carbohydrates, saccharinic acids, sugar phosphates;

(b) a second composition comprising a TLR agonist; and

c) a third composition comprising another anticancer agent.

Whilst the third composition may be varied, it is preferably an anti-CD40 antibody 
such as FGK-45. In this regard, a combination including FGK-45 was found to 
have very potent anti-tumour activity (results not shown).

The compositions can be formulated in various ways depending on the route and 
mode of administration and whether the compositions are combined in a single 
dosage unit or are administered as separate dosage units. Thus, the 
compositions used in the method of the invention may further comprise a 
pharmaceutically acceptable carrier.

Methods for the preparation of pharmaceutical compositions comprising one or 
more active ingredients are generally known in the art. Such pharmaceutical 
compositions will generally be formulated for the mode of delivery that is to be 
used and will usually include one or more pharmaceutically acceptable carriers. A 
"pharmaceutically acceptable carrier" is a material that is not biologically or 
otherwise undesirable, i.e., the material can be administered to an individual along 
with the glycoalkaloid and TLR9 agonist without causing unacceptable biological 
effects or interacting in a deleterious manner with any of the other components of 
the pharmaceutical composition in which it is contained.
In addition pharmaceutical compositions of the invention may further comprise suitable carriers, excipient and diluents that are pharmaceutically acceptable and compatible with the active ingredient. Some examples of suitable carriers, excipient and diluents include, without limitation, water, saline, ethanol, dextrose, cyclodextrins such as hydroxy propyl beta-cyclodextrin, glycerol, lactose, dextrose, sucrose sorbitol, mannitol, starches, gum acacia, calcium phosphates, alginate, tragacanth, gelatine, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water syrup, methyl cellulose, methyl and propylhydroxybenzoates, talc, magnesium stearate and mineral oil or combinations thereof.

The pharmaceutical compositions can additionally include lubricating agents, pH buffering agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavouring agents. The particular selection of constituent that can be included in the compositions described herein will generally depend on the particular mode of delivery used to bring the active agents into contact with their target cells or tissue.

Preferably, the first and second compositions are administered in separate dosage forms. When administered separately, the compositions may be administered simultaneously or sequentially. For the purposes of the present invention “simultaneously” means that the compositions are administered at the same time or within one hour of each other. When the compositions are administered greater than one hour apart they are deemed to be administered sequentially.

Compositions of the present invention should be administered in dosage unit form that is therapeutically effective. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated, each unit containing a quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on at least (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved and (b) the limitations inherent in the
art of compounding such an active material for the treatment sought. Thus the quantity of active compound to be administered will be largely dependent on the toxicity and specific activity of compound, the subject to be treated and the degree of treatment required. Precise amounts of compound required to be administered may depend on the judgement of the practitioner and may be peculiar to each subject.

It will be appreciated that the compositions may be given as a single dose schedule, or more preferably, in a multiple dose schedule. A multiple dose schedule is one in which a primary course of delivery may be with 1 to 100 separate doses, followed by other doses given at subsequent time intervals required to maintain or reinforce the treatment. The dosage regimen will also, at least in part, be determined by the need of the individual and the judgement of the practitioner.

The order in which the compositions are administered and their timing can be varied and will be determined by a practitioner of ordinary skill on a case by case basis. Furthermore, although it is preferred that the compositions be administered as separate dosage forms, the first and second compositions can be formulated into a single dosage form for more convenient administration. In this regard, when formulating the first and second compositions into a single dosage form unwanted interactions between the compositions must be managed to ensure the therapeutic activity of the compositions is not unduly compromised.

The compositions may be administered by any route deemed appropriate by the practitioner based on the particular circumstances of a given case. These routes include: intradermal, transdermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, topical, epidural, buccal, rectal, vaginal and intranasal.

Pharmaceutical compositions according to the invention may be administered to a patient using any technology or delivery route that permits contact between the active agents and their target site. Preferably, the composition is via the intraperitoneal or intravenous route. However, any technology that allows targeted delivery of the pharmaceutical composition via, subcutaneously, intramuscularly,
intraorbitally, ophthalmically, intraventricularly, intracranially, intracapsularly, intraspinally, intracisternally, buccal, rectally, vaginally, intranasally or by pulmonary administration may be used to deliver the composition.

The mode of administration must, however, be at least suitable for the form in which the composition has been prepared. The mode of administration for the most effective response may need to be determined empirically and the means of administration described below are given as examples and do not limit the method of delivery of the composition of the present invention in any way. All the formulations described below are commonly used in the pharmaceutical industry and are commonly known to suitably qualified practitioners.

Whilst not intending to be bound to any particular mode of delivery, further information on the characteristics of compositions that are used for three particular modes of delivery are provided hereunder.

(a) Parenteral delivery

Pharmaceutical compositions may be adapted for parenteral administration that facilitates delivery of a therapeutically effective amount of the glycoalkaloids and TLR agonist to the target cell or tissue.

Pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water-soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Alternatively, the glycoalkaloids may be encapsulated in liposomes and delivered in injectable solutions to assist their transport across cell membrane. The solution may be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol and the like), suitable mixtures thereof and vegetable oils or cyclodextrins such as hydroxy propyl beta-cyclodextrin. Proper fluidity may be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. Prolonged absorption of the injectable compositions can be brought about by the use in the
compositions of agents delaying absorption, for example, aluminium monostearate and gelatine.

Sterile injectable solutions may be prepared by incorporating the glycoalkaloids in the required amount in an appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle that contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying techniques that yield a powder of the active ingredient plus, optionally, any additional desired ingredient from previously sterile-filtered solution thereof.

(b) Topical delivery

Various topical delivery systems may be appropriate for administering the compositions of the present invention, particularly the glycoalkaloids, depending upon the preferred treatment regimen. Topical formulations may be produced by dissolving or combining the active agent in an aqueous or nonaqueous carrier. In general, any liquid, cream, or gel, or similar substance that does not appreciably react with the active or any other of the ingredients that may be introduced into the composition and which is non-irritating is suitable. Appropriate non-sprayable viscous, semi-solid or solid forms can also be employed that include a carrier compatible with topical application and have a dynamic viscosity preferably greater than water.

Suitable formulations are well known to those skilled in the art and include, but are not limited to, solutions, suspensions, emulsions, creams, gels, ointments, powders, liniments, salves, aerosols, transdermal patches, etc, which are, if desired, sterilized or mixed with auxiliary agents, e.g., preservatives, stabilizers, emulsifiers, wetting agents, fragrances, colouring agents, odour controllers, thickeners such as natural gums etc. Particularly preferred topical formulations include ointments, creams or gels.
Ointments generally are prepared using either (1) an oleaginous base, i.e., one consisting of fixed oils or hydrocarbons, such as white petroleum or mineral oil, or (2) an absorbent base, i.e., one consisting of an anhydrous substance or substances which can absorb water, for example anhydrous lanolin. Customarily, following formation of the base, whether oleaginous or absorbent, the glycoalkaloid is added to an amount affording the desired concentration.

Creams are oil/water emulsions. They consist of an oil phase (internal phase), comprising typically fixed oils, hydrocarbons and the like, waxes, petroleum, mineral oil and the like and an aqueous phase (continuous phase), comprising water and any water-soluble substances, such as added salts. The two phases are stabilised by use of an emulsifying agent, for example, a surface active agent, such as sodium lauryl sulfate; hydrophilic colloids, such as acacia colloidal clays, veegum and the like. Upon formation of the emulsion, the active is customarily added in an amount to achieve the desired concentration.

Gels comprise a base selected from an oleaginous base, water, or an emulsion-suspension base. To the base is added a gelling agent that forms a matrix in the base, increasing its viscosity. Examples of gelling agents are hydroxypropyl cellulose, acrylic acid polymers and the like. Customarily, the active is added to the formulation at the desired concentration at a point preceding addition of the gelling agent.

The amount of the glycoalkaloid compound incorporated into a topical formulation is not critical; the concentration should be within a range sufficient to permit ready application of the formulation to the affected tissue area in an amount that will deliver the desired amount of glycoalkaloid to the desired treatment site.

The customary amount of a topical formulation to be applied to an affected tissue will depend upon an affected tissue size and concentration of the glycoalkaloid in the formulation.
(c) Oral Delivery

Pharmaceutical compositions adapted for oral administration in such a manner that facilitates delivery of a therapeutically effective concentration of the active agent(s) to the target cell or tissue.

The effective dosages of the glycoalkaloid, when administered orally, must take into consideration the diluent, preferably water. The composition preferably contains about 1 to about 200mg active agent such as glycoalkaloids. When the compositions are ingested, desirably they are taken on an empty stomach.

Oral solid dosage forms are described generally in Martin, Remington's Pharmaceutical Sciences, 18th Ed. (1990 Mack Publishing Co. Easton PA 18042) at Chapter 89. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatised with various polymers (e.g., U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, in Modern Pharmaceutics, Chapter 10, Banker and Rhodes ed., (1979), herein incorporated by reference. In general, the composition will also include inert ingredients that allow for protection against the stomach environment and release of the active agents in inappropriate areas of the body.

In some instances it may be desirable to release the active agent(s) in the stomach, the small intestine (the duodenum, the jejunum, or the ileum), or the large intestine. One skilled in the art has available formulations that will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the composition or by release of the glycoalkaloids or TLR agonist beyond the stomach environment, such as in the intestine.

To ensure full gastric resistance, a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose
phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S and Shellac. These coatings may be used as mixed films.

A coating or mixture of coatings that are not intended for protection against the stomach can also be used on tablets. This can include sugar coatings, or coatings that make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatine) for delivery of dry therapeutic i.e. powder; for liquid forms, a soft gelatine shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, moulded tablets or tablet triturates, moist massing techniques can be used.

Colourants and flavoring agents may all be included. For example, compositions may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavouring agents.

One may also dilute or increase the volume of the composition with an inert material. These diluents could include carbohydrates, especially mannitol, alpha-lactose, anhydrous lactose, cellulose, sucrose, modified dextrins and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the pharmaceutical formulations of the present invention. Materials used as disintegrants include but are not limited to starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatine, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Other disintegrants include insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.
Binders may be used to hold the pharmaceutical composition together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatine. Others include methylcellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An antifrictional agent may be included in the glycoalkaloid composition to prevent sticking during the formulation process. Lubricants may be used as a layer between the glycoalkaloid and the die wall and these can include but are not limited to: stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights and Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the composition during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution into an aqueous environment, a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethonium chloride. The list of potential nonionic detergents that could be included in the pharmaceutical composition as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the composition either alone or as a mixture in different ratios.

Additives, which potentially enhance uptake of the glycoalkaloids, are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release formulations may be desirable. The compositions could be incorporated into an inert matrix that permits release by either diffusion or leaching.
mechanisms i.e., gums. Slowly degenerating matrices may also be incorporated into the pharmaceutical composition. Another form of a controlled release is by a method based on the Oros therapeutic system (Alza Corp.), i.e. the composition is enclosed in a semipermeable membrane which allows water to enter and push the composition out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidised bed or by compression coating.

The active agents may be included in the pharmaceutical composition as fine multiparticulates in the form of granules or pellets of particle size about 1mm. The formulation for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The active agent(s) could be prepared by compression. Microparticles may be made by a variety of methods known to those in the art, for example, solvent evaporation, desolvation, complex coacervation, polymer/polymer incompatibility, interfacial polymerisation etc.

Hydrophilic polymers forming the microparticles may be attached to a targeting protein that acts to enable the microparticle to specifically bind selected target cells or tissues bearing the target molecule (e.g. characteristic marker). For example, the hydrophilic polymers may be conjugated to the Fab' fragment of an antibody. Smaller peptides from the hypervariable region or from another peptide interacting with a specific cell surface ligand may also be conjugated to the complexes. It is most preferred that the antibodies or antibody fragments are directed against target molecules associated with cancerous tissues or cells.

It will be appreciated that the targeting protein (e.g. an antibody or an antibody fragment) can be attached to the hydrophilic polymers either before or after formation of the microparticle. In a preferred embodiment, the targeting protein is coupled to the hydrophilic polymer, where the targeting protein/hydrophilic polymer is subsequently used to form the microparticle complex. This provides a convenient means for modifying the targeting specificity of an otherwise generic microparticle.
Targeted microparticles may be prepared by incorporating the Fab' fragment into the microparticles by a variety of techniques well known to those of skill in the art. For example, a biotin conjugated Fab' may be bound to a microparticle containing a streptavidin. Alternatively, the biotinylated Fab' may be conjugated to a biotin derivatised microparticle by an avidin or streptavidin linker. Typically about 30 to 125 and more typically about 50 to 100 Fab' fragments per microparticle complex are used.

Pharmaceutical compositions of the present invention may also be formed into powders or some other form that is suitable for delivery by inhalation. Whilst inhalation may be via the mouth it will be appreciated that the route of delivery may also by via the nose.

These compositions are particularly useful for treatment of diseases or disorders of the respiratory system such as lung cancer or cancer that may affect other parts of the respiratory system. When designing compositions for delivery to the lungs, they are preferably designed to reach the site of the alveoli. When the compositions are adapted for delivery by inhalation they may contain various doses of active agent and the particular dose will be determined by a skilled practitioner with due consideration to the recipient and the state of the disease to be treated. However, preferably, the compositions contain between about 100ug-100mg of glycolalkaloids, about 200ug-50mg of glycoalkaloids or 200ug – 10mg glycoalkaloids.

**Glycoalkaloid/TLR agonist Combinations**

As indicated above, the method of the present invention involves the administration of glycoalkaloids and TLR agonists. Particular dosage forms may be produced that are particularly useful and these form separate aspects of the present invention. Thus, the present invention also provides a unit dosage form comprising a therapeutically effective amount of: (a) at least two glycoalkaloids of formula I:
wherein: either one or both of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

A: represents a radical selected from the following radicals of general formulae (II) to (V):

![Chemical structures](image)

- each of $R^1$ is a radical separately selected from the group consisting of hydrogen, amino, oxo and $OR^4$;

- each of $R^2$ is a radical separately selected from the group consisting of hydrogen, amino and $OR^4$;

- each of $R^3$ is a radical separately selected from the group consisting of hydrogen, alkyl and $R^4$-alkylene;

- each of $R^4$ is a radical separately selected from the group consisting of hydrogen, carbohydrate and a carbohydrate derivative;
"X" is a radical selected from the group comprising –CH₂-, -O- and –NH₂-; and

wherein the compound includes at least one R⁴ group in which R⁴ is a carbohydrate or a derivative thereof selected from the group comprising glyceraldehyde, glycerose, erythrose, threose, ribose, arabinose, xylose, lyxose, altrose, allose, gulose, mannose, glucose, idose, galactose, talose, rhamnose, dihydroxyacetone, erythulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, and other hexoses, heptoses, octoses, nanoses, decoses, deoxysugars with branched chains, (e.g. apiose, hamamelose, streptose, cordyceplose, mycarose and cladinose), compounds wherein the aldehyde, ketone or hydroxyl groups have been substituted (e.g. N-acetyl, acetyl, methyl, replacement of CH₂OH), sugar alcohols, sugar acids, benzimidazoles, the enol salts of the carbohydrates, saccharinic acids, sugar phosphates; and

(b) a TLR agonist.

Alternatively the active components may be provided as separate unit dosage forms in a pack or kit arrangement. Thus, the present invention also provides a pack comprising a unit dosage form of glycoalkaloids and a unit dosage form of a TLR agonist.

General

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only.
Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

The entire disclosures of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference. No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working in the field to which this invention relates.

As used herein the term "derived" and "derived from" shall be taken to indicate that a specific integer may be obtained from a particular source albeit not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

For the purposes of the present invention the term "isolated" means essentially free of (i) mono and diglycosides and, preferably, essentially free of (ii) free sugars such as mono, di, tri, oligo or polysaccharides and (iii) aglycone. However, unless steps are taken to stabilise the glycoalkaloids, it will be appreciated that even in an isolated glycoalkaloid composition of the present invention there will be a small amount of free sugars and mono and diglycosides that result from degradation of the glycoalkaloids.

For the purposes of the present invention the phrase "consisting essentially of" means that the glycoalkaloids in the composition are the only glycosides therein.
Thus, a composition consisting essentially of solamargine and solasonine includes solamargine and solasonine and may include other non-glycoside constituents.

The present invention will now be described with reference to the following examples. The description of the examples in no way limits the generality of the preceding description.

Examples

General Materials/Methods

(a) Cell culture and reagents.

The murine AB1-HA (1, 2) and human JU77 and LO68 (3) mesothelioma cell lines were cultured in DMEM (Invitrogen) supplemented with 20 mM HEPES, 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin (CSL, Melbourne, Australia), 50µg/ml gentamicin (David Bull Labs, Warwick, UK) and 5% FCS (Invitrogen). SBP002 (CORAMSINE™) – a 1:1 solamargine and solasonine formulation in 3% acetic acid was dissolved to 200 µg/ml in 3% filter sterilised glacial acetic acid. Further dilutions were made in sterile sdH₂O. Gemcitabine (Eli Lilly, Indianapolis, IN, US) was diluted in sdH₂O to 1 µg/ml stock solution and diluted further with sdH₂O. All other reagents unless stated otherwise in text were purchased from Sigma (Castle Hill, NSW, Australia).

(b) Mice, in vivo tumor growth and chemotherapy treatments.

BALB/c (H-2d) mice were obtained from the Animal Resources Centre (Perth, WA, Australia) and maintained under standard conditions. AB1-HA cells (1 x 10⁶) were inoculated subcutaneously (s.c.) on one side of the ventral surface in the lower flank region. In general, treatment commenced 10 days later when a small palpable lump was evident, ranging from 1 to 2 mm in diameter (day 0). CORAMSINE™ was injected into mice either intraperitoneal (i.p.) or intravenous (i.v.) in a range of doses and volumes to optimize the treatment regimen: i.v.
doses were given in the tail vein of mice in 500 – 1000 μl volume (dose diluted in saline) and i.p. doses were given in a total of 200 μl. Acetic acid diluted in saline was used as the control treatment in the majority of experiments. This control was selected as CORAMSINE™ is initially resuspended in 3% glacial acetic acid before diluting to treatment concentrations. Tumor size was measured with callipers three times weekly during the course of chemotherapy and subsequently until tumor size reached 100 mm² at which point mice were euthanized.

(c) Immunotherapy and TLR-ligand treatments.

Mice receiving activating anti-CD40 antibody (FGK45; a gift from Dr. Antonius Rolink) received 100 μg in 100 μl PBS i.v. three times in 6 days (days 4, 6, and 9). Mice with palpable tumours were injected i.t. with 10 μg polynosinic:cytidylic acid (poly I:C; Invivogen, CA, USA), loxoribin (Invivogen), CpG 1668, or the non-CpG control CpG 1720 (TIB-MOLBIOL, Berlin, Germany), all in 50 μl saline, every 3 days until day 15. Mice with palpable tumors were also treated i.t. with 50 μg CpG 1668 or 1720.

(d) FACS and TUNEL analysis of apoptosis and necrosis.

Caspase induction was detected through the use of ApoStat-FITC (R&D, MN, US). 2 x 10⁵ AB1-HA cells (logarithmic growth) were inoculated into individual wells of 6-well plates and incubated overnight at 37°C with 5% CO₂. Individual wells were treated with dilutions of gemcitabine or CORAMSINE™ (in a total of 1 ml media). 30 minutes prior to completion of the incubation, 10μl of 50 μg/ml ApoStat was added. After 30 minute incubation with ApoStat, cells were harvested, washed in PBS and resuspended in 400 μl of PBS. 5 μl PI was added to the resuspended cells and incubated 20 minutes prior to flow cytometry. TUNEL (Roche, Castle Hill, NSW) was used to assess the presence of apoptotic cells after treatment with CORAMSINE™. 6-well plates were inoculated with 1 x 10⁶ AB1-HA cells/well and treated with lethal and sub-lethal doses of CORAMSINE™ for various time periods. 100 μl of cell suspension was spun onto microscope slides (cytospin) and the slides fixed in 95% ethanol for 10 minutes.
Slides were assessed for presence of nuclear DNA fragmentation using TUNEL, as per manufacturer's protocol. To perform in vivo TUNEL assays, BALB/c mice were injected with $1 \times 10^6$ AB1-HA cells subcutaneously in the flank and assessed every 2 days for tumour growth until the tumour became palpable (day 0). CORAMSINE™ (14 mg/kg) or saline/acetic acid solution equivalent to the 14 mg/kg dose (as control) was injected i.p. in a 300 µl volume at days 0, 1, 2, 7, 8, 9, 14, 15 and 16. Mice were sacrificed at Day 3, 10 and 17. Tumors were excised and made into histological blocks. Sections of the tumors were stained with TUNEL (Roche, Castle Hill, NSW, Australia) as per manufacturers protocol.

(e) Detection of IL-6.

The IL-6 assay was performed using a human IL-6 ELISA kit (eBioscience, USA) according to the manufacturers protocol. Briefly, 96-well plates were incubated at room temperature overnight with 100 µl of anti-human IL-6 monoclonal antibody. After washing and blocking steps, 100 µl of each experimental sample was added, followed by incubation with streptavidin-HRP conjugate and ABTS substrate. Plates were read at 405 nm on a SpectraMax 250 plate reader (Molecular Devices) and the concentration of IL-6 for each sample was calculated from the standard curve. Samples for analysis in the ELISA were prepared by seeding cells in 96-well plates with or without CORAMSINE™ or gemcitabine. Chemotherapeutic agents were added 24 hours after cell seeding. The concentration of IL-6 shown represents the mean value generated from three separate experiments.

(f) Statistical analyses.

Data comparing differences between groups were assessed using a Student $t$ test. Differences were considered significant when the $P$ was <0.05. Statistical analysis was conducted using Graph Pad Prism program (Graph Pad Software Inc. CA, USA).
Example 1: Efficacy of CORAMSINE™ in anti-cancer chemotherapy.

Results

AB1-HA mesothelioma cells (10^6) were inoculated subcutaneously and treatment was initiated when tumors became palpable (usually 10 days after inoculation). Chemotherapy treatment protocols were optimised by varying the route of administration, the dose, and the treatment schedule. We focused on systemic administration (intraperitoneal and intravenous) to stay close to the clinical reality. Whilst optimizing dosage and treatment schedules, we observed that systemic administration of CORAMSINE™ at higher doses (>16 mg/kg in daily intraperitoneal injections, or >32 mg/ml in a single intravenous injection) was associated with unacceptable morbidity and mortality, indicating that therapeutic efficacy and toxicity appeared to exist in a delicate balance in our mouse model.

To determine the anti-tumor activity of CORAMSINE™ after intraperitoneal (i.p.) injection, mice (5 per group) were injected with different doses of CORAMSINE™ (12 and 14 mg/kg) once AB1-HA tumors were palpable. Preliminary experiments had revealed that a treatment schedule of ‘three days on – four days off’ was optimal in the context of i.p. injection (i.e., mice did not tolerate daily injections for more than three days). Therefore, we used this protocol throughout this study. This dosing schedule significantly slowed tumor growth during the first two weeks of treatment with 12 or 14 mg/kg CORAMSINE™ (Fig.1a). In fact, tumor size even decreased at day 15 after treatment with 14 mg/ml CORAMSINE™. However, mice did not tolerate further treatment beyond day 15, and tumor growth rates increased after treatment stopped. Nevertheless, i.p. CORAMSINE™ treatments at these doses did prolong survival time, although all mice eventually succumbed to the tumor (Fig.1b).

Since intravenous (i.v.) administration more closely mimics the clinical situation, we evaluated the therapeutic efficacy of a single i.v. injection of CORAMSINE™ (8 and 16 mg/kg) once AB1-HA tumors were palpable. We found that treatment with either dose significantly slowed tumor growth (Fig.2a) and prolonged survival
time (Fig.2b). Since i.v. injection of CORAMSINE™ was associated with less morbidity than i.p. treatment, we then asked how a large tumor would respond to a single high-dose i.v. injection of CORAMSINE™.

Mice (5/group) were inoculated s.c. with AB1-HA and treated with a single high dose of CORAMSINE™ (30 mg/kg) when their tumors had reached 77 ± 23 mm². We found that i.v. injection of CORAMSINE™ resulted in reductions of tumor volume in 3/5 mice (Fig.2c). Moreover, the mice that did not show reduced tumor volume, did exhibit a significant reduction of the tumor growth rate compared to the controls (data not shown). Although i.v. injection of CORAMSINE™ at this dose was lethal in 20% of mice, we found that this treatment significantly prolonged survival in 2/5 of mice tested (Fig.2d).

Example 2 - CORAMSINE™ is potentiated by immunotherapy with CpG-containing oligodeoxynucleotides.

Since i.p. administration of CORAMSINE™ at the optimized dose of 14 mg/kg caused a significant delay in AB1-HA tumor growth, we asked whether this effect could be enhanced by immunotherapy. We first tested the efficacy of anti-CD40 immunotherapy (using the agonistic FGK-45 antibody, administered i.v.) in combination with i.p. CORAMSINE™ treatment. Although both CORAMSINE™ and FGK-45 treatment delayed tumor growth as single agents, we found no enhanced anti-tumor efficacy in the combination therapy (Figs.3a and 3b).

Because treatment with FGK-45 stimulates immune responses after the priming event in the draining lymph nodes, the lack of FGK-45/CORAMSINE™ synergy could suggest that CORAMSINE™-induced tumor cell death differs in its immune stimulating potential when compared to gemcitabine. To test this, we used the capacity of inflammatory stimuli associated with pathogens (LPS, double-stranded RNA, CpG–containing ODN) to stimulate dendritic cell maturation and stimulate an immune response. In this study, ligands that stimulate responses through TLR-3 (poly I:C), TLR-7 (loxoribine) and TLR-9 (CpG-containing ODN) were assessed for their capacity to synergize with CORAMSINE™ treatment.
To evaluate possible synergy, mice (n = 4) were inoculated with AB1-HA tumor cells. When tumors became palpable, they were injected either intra- or peritumorally with either poly I:C, loxoribine, CpG ODN, non-CpG ODN (a non-stimulatory control), or PBS. Mice were also injected i.p. with 14 mg/ml CORAMSINE™ (3 days on – 4 days off). We found that mice did not tolerate i.p. CORAMSINE™ treatment in combination with i.t. PBS or poly I:C treatment (50% mortality) (Fig.4a). Nevertheless, combination of CORAMSINE™ and poly I:C did slow tumor growth (Fig.4a). There was no observed anti-tumor effect of combining loxoribine with CORAMSINE™. Strikingly however, all mice that received the CpG/CORAMSINE™ combination treatment had significant delays in tumor growth and experienced prolonged survival (Fig.4a/b), demonstrating synergy between the TLR9 ligand and CORAMSINE™. This combination treatment was lethal for 1/4 mice, but also cured 1/4 mice (Fig.4b). In comparison, there was no enhanced anti-tumor effect for the non-CpG/CORAMSINE™ (control) combination therapy (Fig.4). The one surviving mouse was rechallenged with AB1-HA cells and did not develop a tumor, suggesting that successful CORAMSINE™/CpG treatment resulted in the generation of anti-tumor memory (data not shown).

The synergy between CORAMSINE™ and CpG was reproduced in an additional experiment (n = 5), in which we also tested the effect of a higher dose of CpG (50 µg instead of 10 µg) in combination with CORAMSINE™ (Fig.4c and d).

**Example 3 - CORAMSINE™ does not induce cell death through apoptosis.**

The lack of synergy between CORAMSINE™ and FGK-45 suggests that tumor cell destruction by CORAMSINE™, in contrast to gemcitabine-induced tumor cell death, may not be immunologically stimulating. This prompted us to investigate the mechanism of CORAMSINE™-induced cell death in more detail. To address this question, we have investigated the mechanism of CORAMSINE™-induced cell death both *in vitro* and *in vivo.*
Initial experiments revealed that AB1 mesothelioma cells treated with lethal doses of CORAMSINE™ (20 μg/ml) caused cell lysis within minutes (data not shown). This suggests that the mechanism of cell death is via primary necrosis or direct cell lysis, as apoptosis usually requires several hours to cause cell death. To confirm this, we sought to exclude involvement of apoptosis in CORAMSINE™ induced cell death. We used two different assays to detect apoptosis, i.e., visualization of caspase activation (ApoStat staining), and TUNEL staining. Neither of these assays provided any evidence for CORAMSINE™-induced apoptosis. First, when gemcitabine- and CORAMSINE™-exposed cells were stained with the ApoStat reagent, which binds activated caspases, we detected no signs of apoptosis in CORAMSINE™-treated AB1 cells (4-10 μg/ml, 16 h) (Fig.5). Note that CORAMSINE™-exposed cells do take up PI (see the increased number of PI+/ApoStat- cells in CORAMSINE™-treated cells), which is indicative of primary necrosis when observed in the absence of indications for apoptosis (32,33). In contrast, gemcitabine treatment (1-10 μg/ml) did induce apoptosis using ApoStat staining (Fig.5). Second, TUNEL staining revealed similar numbers of apoptotic cells in CORAMSINE™- (10 μg/ml, 16 hours) and PBS-treated AB1 cells (data not shown). Finally, to determine the mechanism of CORAMSINE™-induced cell death induction in vivo, we quantified apoptotic cells in CORAMSINE™- or PBS-treated tumors, using the TUNEL staining assay. We found no difference in the number of apoptotic cells present in tumors isolated from CORAMSINE™ and PBS-treated mice (data not shown), suggesting that CORAMSINE™-induced tumor growth retardation was not due to tumor cell apoptosis.

Example 4- Linking chemotherapy to immune priming capacity: the role of IL-6.

Since (i) the pro-inflammatory cytokine IL-6 plays a key role of activating immune responses in the presence of immuno-suppressive CD25+CD4+ T cells and (ii) TLR-ligation results in enhanced IL-6 production, we investigated whether differential production of IL-6 could, in part, explain our results, i.e., the
immunological silence of CORAMSINE™-induced cell death and the rescue by CpG ODN treatment.

To test this, we measured IL-6 production by mesothelioma cells in response to CORAMSINE™ and gemcitabine. As shown in Fig.6, we found that treatment of LO68 mesothelioma cells with lethal or near-lethal levels of gemcitabine induced a surge of IL-6 production (Fig.6a). In contrast, treatment of these cells with CORAMSINE™ led to a decrease of IL-6 production as cell viability decreased (Fig.6b). Similar data were obtained with JU77 mesothelioma cells (data not shown). Thus, these results indicate that the different immune stimulatory capacities of gemcitabine and CORAMSINE™ are associated with differential kinetics of IL-6 production.
REFERENCES


The Claims Defining the Invention are as Follows:

Methods of Treating Tumours

1. A method of treating a tumorous growth comprising the step of administering a therapeutically effective amount of: (a) a first composition comprising at least two glycoalkaloids of formula I:

![Chemical Structure Image]

wherein: either one or both of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

A: represents a radical selected from the following radicals of general formulae (II) to (V):

![Chemical Structure Images]

each of R\(^1\) is a radical separately selected from the group consisting of hydrogen, amino, oxo and OR\(^4\); each of R\(^2\) is a radical separately selected from the group consisting of hydrogen, amino and OR\(^4\); each of R\(^3\) is a radical separately
selected from the group consisting of hydrogen, alkyl and \( R^4 \)-alkylene; each of \( R^4 \) is a radical separately selected from the group consisting of hydrogen, carbohydrate and a carbohydrate derivative; "X" is a radical selected from the group comprising \(-\text{CH}_2-, -\text{O-} \) and \(-\text{NH}_2-\); and

wherein the compound includes at least one \( R^4 \) group in which \( R^4 \) is a carbohydrate or a derivative thereof selected from the group comprising glyceric aldehyde, glycerose, erythrose, threose, ribose, arabinose, xylose, lyxose, altrose, allose, gulose, mannose, glucose, idose, galactose, talose, rhamnose, dihydroxyacetone, erythulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, and other hexoses, heptoses, octoses, nanoses, decoses, deoxysugars with branched chains, (e.g. apiose, hamamelose, streptose, cordycepose, mycarose and cladinose), compounds wherein the aldehyde, ketone or hydroxyl groups have been substituted (e.g. N-acetyl, acetyl, methyl, replacement of \( \text{CH}_2\text{OH} \)), sugar alcohols, sugar acids, benzimidazoles, the enol salts of the carbohydrates, saccharinic acids, sugar phosphates; and

(b) a second composition comprising a TLR agonist.

2. A method according to claim 1 wherein in the first and second compositions act in a complimentary fashion.

3. A method according to claim 2 wherein in the first and second compositions act synergistically.

4. A method according to any one of claims 1 to 3 wherein the tumorous growth is associated with a cancer selected from the group consisting of: melanomas and non-melanoma skin including lignin melanoma, solar keratosis, basal cell carcinoma, squamous cell carcinoma and actinic keratoses; mesothelioma; breast; prostate; liver; lung; colon; rectum; urinary bladder; non-Hodgkin lymphoma; kidney; pelvis; pancreas; pharynx; head & neck; ovarian; oral; thyroid; stomach; brain; multiple myeloma; oesophagus; liver and intrahepatic bile duct; cervix; larynx; acute myeloid leukemia; chronic lymphocytic leukemia;
heart; Hodgkin lymphoma; testis; small intestine; chronic myeloid leukemia; acute lymphocytic leukemia; gallbladder; bones and joints; eye; nose; nasopharynx; peritoneum; omentum; and mesentery-gastrointestinal.

5. A method according to any one the preceding claims wherein the glycoalkaloids are triglycoside glycoalkaloids.

6. A method according to claim 5 wherein the triglycoside glycoalkaloids are solasodine glycosides.

7. A method according to claim 6 wherein the solasodine glycosides are selected from the group of glycoalkaloids consisting of: solamargine, solasonine, solanine, tomatine, solanocapsine and 26-aminofurostane.

8. A method according to claim 1 wherein the first composition comprises two glycoalkaloids present in a ratio selected from the group of ratios consisting of approximately: 1:6 - 1:0.5; 1:5; 1:4; 1:3; 1:2; 1:1.5 and 1:1.

9. A method according to claim 8 wherein the glycoalkaloids are solamargine and solasonine.

10. A method according to claim 9 wherein the solamargine and solasonine are present in a ratio of 1:1.

11. A method according to any one of claims 1 to 10 wherein the TLR agonist is a TLR3, TLR7, TLR8 or TLR9 agonist.

12. A method according to claim 11 wherein the TLR3 agonist is a double-stranded RNA (dsRNA) containing a molecular pattern associated with viral infection.

13. A method according to claim 12 wherein the TLR3 agonist is polyinosine-polycytidylic acid (poly(I:C)).
14. A method according to claim 11 wherein the TLR7/TLR8 agonist is selected from the group consisting of: imidazoquinoline compounds such as Imiquimod™ (compound R387), guanine analogs such as loxoribine, single stranded PolyU, single stranded RNA40 and R484, a small anti-viral molecule.

15. A method according to claim 11 wherein the TLR9 agonist is a nucleotide comprising a sequence corresponding to a pathogen associated microbial pattern (PAMPs).

16. A method according to claim 15 wherein the TLR9 agonist is a CpG ODN.

17. A method according to claim 16 wherein the CpG ODN is a nucleotide comprising a sequence according to formula I:

\[ 5' X_1 X_2 CGX_3 X_4 3' \]

wherein C and G are unmethylated, \( X_1, X_2, X_3 \) and \( X_4 \) are nucleotides and a GCG trinucleotide sequence is not present at or near the 5' and 3' termini.

18. A method according to claim 17 wherein \( X_1, X_2 \) are the dinucleotide GpA.

19. A method according to claim 17 or 18 wherein \( X_3, X_4 \) are the dinucleotide TpC or TpT.

20. A method according to claim 17 wherein \( X_1, X_2 \) CGX_3 X_4 is preceded on the 5' end by a T.

21. A method according to claim 17 wherein the nucleotide comprises the sequence TGACGTT or TGACGTC.

22. A method according to any one of claims 17 to 21 wherein the nucleotide is about 8 to 40 bases.
23. A method according to any one of claims 17 to 22 wherein the nucleotide is 5’ TCC ATG ACG TTC CTG ATG CT 3’ (CpG 1668).

24. A method according to any one of the preceding claims wherein at least one of the compositions further comprise a pharmaceutically acceptable carrier, excipient or diluents.

25. A method according to any one of the preceding claims wherein the first and second compositions are administered in separate dosage forms.

26. A method according to claim 25 wherein the compositions are administered simultaneously.

27. A method according to claim 25 wherein the compositions are administered sequentially.

28. A unit dosage form comprising a therapeutically effective amount of: (a) at least two glycoalkaloids of formula I:

![Chemical Structure](image)

wherein: either one or both of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

A: represents a radical selected from the following radicals of general formulae (II) to (V):
each of \( R^1 \) is a radical separately selected from the group consisting of hydrogen, amino, oxo and \( OR^4 \); 

each of \( R^2 \) is a radical separately selected from the group consisting of hydrogen, amino and \( OR^4 \); 

each of \( R^3 \) is a radical separately selected from the group consisting of hydrogen, alkyl and \( R^4 \)-alkylene; 

each of \( R^4 \) is a radical separately selected from the group consisting of hydrogen, carbohydrate and a carbohydrate derivative; 

"\( X \)" is a radical selected from the group comprising \(-CH_2-, -O- and -NH_2-\); and 

wherein the compound includes at least one \( R^4 \) group in which \( R^4 \) is a carbohydrate or a derivative thereof selected from the group comprising glyceraldehyde, glycerose, erythrose, threose, ribose, arabinose, xylose, lyxose, altrose, allose, gulose, mannose, glucose, idose, galactose, talose, rhamnose, dihydroxyacetone, erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, and other hexoses, heptoses, octoses, nanoses, decoses, deoxysugars with branched chains, (e.g. apiose, hamamelose, streptose, cordycepose, mycarose and cladinose), compounds wherein the aldehyde, ketone or hydroxyl groups have been substituted (e.g. N-acetyl, acetyl, methyl, replacement of
CH₂OH), sugar alcohols, sugar acids, benzimidazoles, the enol salts of the carbohydrates, saccharinic acids, sugar phosphates; and

(b) a TLR agonist.
Figure 1a
Figure 1b
Figure 2a

- Vehicle
- △ 8 mg/ml
- ▽ 16 mg/ml

Days since tumor palpable

Tumor size
Figure 2b
Figure 2c
Figure 2d
Figure 3a
Figure 3b
Figure 4a
Figure 4b
Figure 4c
Figure 6a
Figure 6b
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.


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)

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


C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>Y</td>
<td>St. C. Barnetson, R et al., &quot;Imiquimod induced regression of clinically diagnosed superficial basal cell carcinoma is associated with early infiltration by CD4 T cells and dendritic cells&quot; Clinical and Experimental Dermatology, (2004) 29, 639-643.</td>
<td>1 to 28</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C

See patent family annex

Date of the actual completion of the international search

21 April 2006

Date of mailing of the international search report

1 May 2006

Authorized officer

K.G. ENGLAND

Telephone No: (02) 6283 2292

Form PCT/ISA/210 (second sheet) (April 2005)
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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<td>AU 2003238544  CA 2489675  EP 1539191</td>
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX