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# Victoria GORDON, *et al*
# Blushwood vs Cancer

http://www.couriermail.com.au/news/queensland/possible-cancer-cure-found-in-blushwood-shrub/story-e6freoof-1225826874057
**The Courier-Mail**
**February 04, 2010**

## Possible cancer cure found in blushwood shrub

**Peter Michael**

**Cancer 'cure' in Qld rainforest**

CANCER patients are offering themselves as human guinea pigs as researchers investigate a possible cure for cancer that was found in north Queensland rainforests.

Scientists have identified a compound in the fruit of the native blushwood shrub that appears to "liquefy and destroy cancer with no side-effects", according to latest research.

Found deep in the remnants of a 130 million-year-old rainforest, the fruit extract may yet hold the secret antidote to Australia's No.1 killer disease.

Victoria Gordon, of EcoBiotics, an Atherton Tableland-based company, said they hoped to go to human clinical trials later this year.

Dr Gordon said a single dose injection of the extract, known as EBC-46, had been effective in

50 critically ill dogs and about a dozen cats and horses.

"This is proving to be something exceptional," she said. "The tumour literally liquefies. There is a rapid knock-down of the tumour, it disintegrates within 24 hours and we have a rapid healing response. The biggest tumour we treated was the size of a Coke can in a dog, and that animal is fully healed and healthy."

Dr Gordon said it had worked on skin cancers, such as carcinomas and melanomas, and bone cancer, and was a possible treatment for breast, colon and prostate cancer.

But she warned wannabe human guinea pigs against seeking under-the-table treatment.

She said it was "immoral, illegal, and unscientific" to seek to be administered the drug before approval, likely to take up to seven years, by the Therapeutic Goods Administration.

"We have been inundated with calls – it shows there is such a need for a breakthrough in anti-cancer treatment," she said. "Most people understand when we explain the situation."

Former breast cancer sufferer Mena Crew, 65, said many dying of cancer would "do anything for a miracle cure".

"We would all like a magic cure, that would be wonderful, and I hope in my lifetime we find it," the breast cancer support volunteer said.

She has worked with more than 200 sufferers and some victims in her role with the Cancer Council Queensland.

"I don't want to kill the enthusiasm of all the wonderful research, but until it is proven it will do the job, we recommend they go with proven and conventional treatments," she said.

"It is good, however, to think the secret antidote may be growing in the jungle above Cairns."

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**http://www.ecobiotics.com.au/**

**Overview**

We specialise in the discovery of small molecule drug candidates from nature

The products and services we offer range from licensing of individual chemicals from our internal discovery pipeline through to generating a customised small molecule portfolio in a specific therapeutic area.

We have significant flexibility in tailoring our products and discovery technology to the needs of individual clients by: devising new collection strategies for specific therapeutic areas and/or likely modes of action, and searching for particular classes of molecules in our collections and extract libraries.

Our products and services are underpinned by EcoLogic™, our powerful proprietary

technology which provides a rational basis for harnessing nature's chemistry in drug discovery.

**About QBiotics**

QBiotics Limited is an Australian lifesciences company in the business of human and veterinary pharmceutical product development for the oncology and wound healing markets. QBiotics was established in 2004 and is a subsidiary of EcoBiotics.

QBiotics current product portfolio consists of:

1. The anticancer drug EBC-46

EBC-46 is being developed for the intralesional treatment of solid tumours in both humans and companion animals. EBC-46 is a novel small molecule isolated from Blushwood, a native shrub found in the Australian tropical rainforest. To date, EBC-46 has been successful in the local treatment of a range of tumour types in horses, dogs and cats. Tumour destruction usually occurs within days with rapid healing of the site and no significant adverse effects when the drug is used at therapeutic doses. EBC-46 is currently at mid clinical development as a veterinary pharmaceutical and late preclinical development as a human pharmaceutical.

2. The wound healing product WH-1

WH-1 is proving to have promising potential as a wound healing agent. Anecdotal evidence gained from 'real world' animal clinical case studies treating dogs in the veterinary environment supports the ability of WH-1 to stimulate rapid wound closure, reduction or elimination of infection with good cosmetic outcomes in the form of low rate of scarring. In addition, in vitro studies have demonstrated the ability of WH-1 to selectively stimulate the migration of 'wound healing' cells to rapidly close the wound. WH-1 is currently in early preclinical development for both the human and veterianary markets.

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http://www.qbiotics.com

## EBC-46 & Animals

EBC-46 is a novel natural product small molecule with anticancer activity being developed as a local treatment for solid tumours in humans and companion animals (dogs, cats and horses). EBC-46 was discovered by applying the EcoLogic™ approach to drug discovery from the tropical rainforests of Far North Queensland. EcoLogic™ was developed by QBiotics parent entity EcoBiotics.

QBiotics plans to develop and register EBC-46 as a veterinary pharmaceutical for the treatment of solid tumours in dogs, cats and horses. To date, EBC-46 has been successful in the treatment of a diverse range of inoperable spontaneous solid tumours in dogs, cats and horses.

**Incidence of cancer in animals**

EBC-46 has the potential to revolutionise the treatment of many solid tumours in animals.

Accurate data on the rates of cancer (either incidence of new cases or overall prevalence in the population) in companion animals are difficult to obtain due to the lack of formal registries which gather population-based health statistics (e.g. population sizes and breed structure, longevity, neutering patterns, mortality rates and causes, etc).

Nonetheless, it is possible to make realistic estimates of the occurrence and epidemiology of different cancers in companion animals from survey data collected from university veterinary hospitals and large veterinary practices.

EBC-46 is a new experimental drug for treating cancers in dogs, cats and horses.

In treatment of over 100 dogs, cats and horses, intralesional injection of EBC-46 has successully ablated or significantly palliated a range of advanced, inoperable tumours (melanomas, sarcomas, carcinomas, mast cell tumours and sarcoids) while causing no significant long-term side effects. A topical formulation of EBC-46 has also been used to successfully treat ulcerative squamous cell carcinomas on cats and horses.

EBC-46 is delivered locally in a single treatment by direct injection into tumours, or by topical application onto the tumour surface.

EBC-46 is active against a wide range of tumour types and is potentially useful in treating any solid tumour that can be accessed for direct injection or topical application of the drug.

EBC-46 only works locally at the site of delivery and there is no current evidence to suggest that EBC-46 has any systemic efficacy against metastatic disease in affecting remote secondary tumours.
Veterinary Trials

QBiotics is currently conducting trials of EBC-46 for treating selected solid tumours in dogs and horses. Trials in cats will follow in the near future. The trials are focusing on treatment of tumours that are accessible for, and amenable to, direct injection of the drug including cutaneous and subcutaneous tumours. EBC-46 will become available through veterinarians following primary registration of the drug.

## EBC-46 & Humans

### An outstanding candidate for a human anti-cancer treatment

EBC-46 is a novel natural product small molecule with anticancer activity being developed as a local treatment for solid tumours in humans and companion animals (dogs, cats and horses). EBC-46 was discovered by applying the EcoLogic™ approach to drug discovery from the tropical rainforests of Far North Queensland. EcoLogic™ was developed by QBiotics parent entity EcoBiotics.

QBiotics plans to develop EBC-46 as a human pharmaceutical to Clinical Phase II and then seek a development partner for final development and marketing of the drug. The principles behind the development of cancer in animals and humans are similar. Consequently, it is likely that EBC-46 will have similar effects in human as in animals.

Potential indications for the drug include:

**What is ebc46**

Skin cancers including melanomas, squamous cell carcinomas (SCC) & basal cell carcinomas (BCC)
Head & neck cancers
Breast cancers
Prostate cancers
Other tumours where injection can be guided by imaging

Although only in late pre-clinical development for the human market, QBiotics has already demonstrated very compelling proof-of-concept of the drug's efficacy and safety both in cancer models in mice and in succussful treatment of advanced, spontaneous tumours in dogs, cats and horses that were considered untreatable with current standards of care.

**Clinical trials in humans**

EBC-46 is delivered locally in a single treatment by direct injection into tumours, or by topical application onto the tumour surface.

EBC-46 is active against a wide range of tumour types and is potentially useful in treating a range of tumours that can be accessed for direct injection or topical application of the drug.

EBC-46 only works locally at the site of delivery and there is no evidence to suggest that EBC-46 has any systemic efficacy against metastatic disease.

QBiotics is rapidly advancing the drug towards the necessary regulatory approvals for conducting human clinical trials.

An announcement as to the commencement of these trials will be made in due course

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# Tiglien-3-one derivatives
# US8598229

**Abstract**
Disclosed are bioactive natural products which may be obtainable from Fontainea australis, Fontainea borealis, Fontainea fugax, Fontainea oraria, Fontainea picrosperma, Fontainea rostrata, Fontainea subpapuana, Fontainea venosa or Hylandia dockrillii (Blushwood Tree). Isolated compounds from Fontainea picrosperma include: 12-tigloyl-13-(2-methylbutanoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliaen-3-one (EBI-46 / EBC-46), 12,13-di-(2-methylbutanoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-tigliaen-3-one (EBI-47), 12-(dodeca-2,4,6-trienoyl)-13-(2-methylbutanoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliaen-3-one (EBI-59), 12-(deca-2,4-dienoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliaen-3-one (EBI-61), 12,13-di-(2-methylbutanoyl)-1,2-2H-1,2,6,7-diepoxy-6-carboxy-4,5,9,12,13-pentahydroxy-tigliaen-3-one and 12,13-di-(2-methylbutanoyl)-5,20-di-acetoyl-4,5,9,12,13,20-hexahydroxy-tigliaen-3-one. Also disclosed is a composition which comprises a tiglien-3-one derivative such as those presented above, for the treatment of leukaemia, a

solid tumour cancer, including melanoma, prostate cancer, breast cancer, ovarian cancer, basal cell carcinoma, squamous cell carcinoma, fibrosarcoma, colon cancer or lung cancer or other solid tumours.

## FIELD OF THE INVENTION

This invention relates to bioactive molecules. More particularly, this invention relates to tiglien-3-one derivatives of potential therapeutic benefit and/or of use as a pharmaceutical and as an agrochemical.

## BACKGROUND OF THE INVENTION

Bio-discovery is a growing field, which investigates and screens for bioactive natural products from natural environments, including plants, microorganisms, coral and other marine life. In the search for bioactive natural products, biological material is screened for molecules having properties that may be of therapeutic benefit for potential use in a range of treatments, for example treatments for cancer, antiprotozoal treatments, antiparasitic treatments, antibiotic treatments and anti-inflammatory treatments, or for pesticidal activity.

## SUMMARY OF THE INVENTION

The present invention arises from the discovery of new tiglien-3-one derivatives which have potentially new therapeutic uses as cytotoxic agents, antiprotozoal agents, antiparasitic agents and antibiotic agents or potential as pesticidal agents for agricultural use.

One aspect of the invention provides compounds of the formula I wherein:
X is selected from -S-, -O-, -NH- or -N(C1-6 alkyl)-; ....

In one particular embodiment, the compound is a compound of formula II is 12-tigloyl-13-(2-methylbutanoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliaen-3-one (EBI-46):

In another embodiment the compound is 12,13-di-(2-methylbutanoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-tigliaen-3-one (EBI-47):

In yet another particular embodiment, the compound is 12-(dodeca-2,4,6-trienoyl)-13-(2-methylbutanoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliaen-3-one (EBI-59):

In still yet another particular embodiment, the compound is 12-(deca-2,4-dienoyl)-6,7-epoxy-4,5,9,12,13,20-hexahydroxy-1-tigliaen-3-one (EBI-61):

In yet another embodiment, the compounds is 12,13-di-(2-methylbutanoyl)-1,2-2H-1,2,6,7-diepoxy-6-carboxy-4,5,9,12,13-pentahydroxy-tigliaen-3-one:

In yet another embodiment, the compound is 12,13-di-(2-methylbutanoyl)-5,20-di-acetoyl-4,5,9,12,13,20-hexahydroxy-tigliaen-3-one:

The invention thus also relates to compounds in substantially pure isomeric form at one or more asymmetric centres e.g., greater than about 90% ee, such as about 95% or 97% ee or greater than 99% ee, as well as mixtures, including racemic mixtures, thereof. Such isomers may be obtained by isolation from natural sources, by asymmetric synthesis, for example

using chiral intermediates, or by chiral resolution. The compounds of the invention may exist as geometrical isomers. The invention also relates to compounds in substantially pure cis (Z) or trans E) forms or mixtures thereof.

The compounds of the present invention may be obtained by isolation from a plant or plant part, or by derivatisation of the isolated compound, or by derivatisation of a related compound.

Yet another aspect of the invention provides a method of isolating one or more compounds of formula (I) to formula (IV), which method includes the step of extracting said one or more compounds from a plant or plant part.

**Preferably, the plant is of the genus Fontainea or Hylandia.**

Preferably the species is Fontainea pancheri, Fontainea australis, Fontainea borealis, Fontainea fugax, Fontainea oraria, Fontainea picrosperma, Fontainea rostrata, Fontainea subpapuana, Fontainea venosa or Hylandia dockrillii, especially Fontainea picrosperma, Fontainea venosa or Hylandia dockrillii.

The parts of the plant may include fruit, seed, bark, leaf, flower, roots and wood.

Preferably the extract is obtained from the seed, bark and/or flowers.

For example, the biomass obtained from seeds, leaves, flowers and bark of the plant is subject to initial solvent extraction, for example with a polar solvent such as methanol. The initial extraction is then concentrated and diluted with water and subject to extraction with a second solvent, for example, ethyl acetate. The solvent samples from the second extraction are pooled and subject to separation by preparative HPLC fractionation. The fractions are analysed by analytical HPLC and pooled according to the retention time of compounds found in the samples. The pooled fractions are weighed, bioassayed and analysed by analytical HPLC. Further fractionation using one or more preparative HPLC is performed to isolate specific compounds. Each compound is bioassayed and its structure identified by UV, NMR and mass spectrometric techniques.

Other compounds of the invention may be obtained by derivatising compounds isolated from plants or parts of plants, especially from the genus Fontainea, especially from the species Fontainea picrosperma, especially the seeds, bark and/or flowers of Fontainea picrosperma...

A person skilled in the art would be able to determine suitable conditions for obtaining derivatives of isolated compounds, for example, by reference to texts relating to synthetic methodology, examples of which are Smith M. B. and March J., March's Advanced Organic Chemistry, Fifth Edition, John Wiley & Sons Inc., 2001 and Larock R. C., Comprehensive Organic Transformations, VCH Publishers Ltd., 1989. Furthermore, selective manipulations of functional groups may require protection of other functional groups. Suitable protecting groups to prevent unwanted side reactions are provided in Green and Wuts, Protective Groups in Organic Synthesis, John Wiley & Sons Inc., 3<rd >Edition, 1999...

An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular condition being treated. The amount varies depending upon the

health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the degree of protection desired, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. An effective amount in relation to a human patient, for example, may lie in the range of about 0.1 ng per kg of body weight to 1 g per kg of body weight per dosage. The dosage is preferably in the range of 1 [mu]g to 1 g per kg of body weight per dosage, such as is in the range of 1 mg to 1 g per kg of body weight per dosage. In one embodiment, the dosage is in the range of 1 mg to 500 mg per kg of body weight per dosage. In another embodiment, the dosage is in the range of 1 mg to 250 mg per kg of body weight per dosage. In yet another embodiment, the dosage is in the range of 1 mg to 100 mg per kg of body weight per dosage, such as up to 50 mg per kg of body weight per dosage. In yet another embodiment, the dosage is in the range of 1 [mu]g to 1 mg per kg of body weight per dosage. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals, or the dose may be proportionally reduced as indicated by the exigencies of the situation...

Reference herein to "treatment" and "prophylaxis" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, treatment and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition. The term "prophylaxis" may be considered as reducing the severity or onset of a particular condition. "Treatment" may also reduce the severity of an existing condition.

In another aspect of the invention, the compounds of the invention are suitable for use as a pesticide. The invention therefore further provides a pesticidal composition comprising a compound of formula (I) or formula (II) or an agriculturally or pesticidally acceptable salt thereof and a pesticidally acceptable carrier...

In yet another aspect of the invention, there is provided a method of controlling infestations of pests in a subject or an environment comprising applying a pesticidally effective amount of a compound of formula (I) or formula (II) to a subject or an environment infested with a pest.

The agricultural pest is preferably an insect, especially flies, beetles, grasshoppers, locusts, butterflies and moths and their larvae or nymphs, especially the flies (Diptera) such as true flies, fleas, ticks, lice, mosquitoes, gnats and midges.

In some embodiments, the pest infests plants. Examples of such pests include, but are not limited to, Acyrthosiphon kondoi (blue-green aphid), Acyrthosiphon pisum (pea aphid), Agrotis spp. (cutworm), Agrypnus variabilis (sugarcane wireworm), Anoplognathus spp. (christmas beetles), Aphodius tasmaniae (blackheaded pasture cockchafer), Austroasca alfalfae (lucerne leaf hopper), Bathytricha truncate (sugarcane and maize stemborer), Bemisia tabaci (whitefly), Brachycaudus helichiysi (leaf curl plum aphid), Brevicoryne brassicae (cabbage aphid), Bruchophagus roddi (lucerne seed wasp), Bruchus pisorum (pea weevil), Biyobia spp. (bryobia mite), Clampa arietaria (brown pasture looper), Chortoicetes terminifera (Australian plague locust), Chrysodeitis angentifena (tobacco looper), Chrysodeitis eriosoma (green looper), Contarinia sorghicola (sorghum midge), Deroceras spp. (slugs), Diachrysia oricalcea (soybean looper), Etiella behrii (lucerne seed-web moth),

Frankliniella schultzei (tomato thrips), Graphognathus leucoloma (white fringed weevil), Halotydeus destructor (redlegged earth mite), Hednota pedionoma (pasture webworm), Helicoverpa armigera (corn earworm), Helicoverpa punctigera (native budworm), Helix spp. (snails), Heteronychus arator (African black beetle), Leucania convecta (common armyworm), Lipaphis erysimi (turnip aphid), Listroderes difficilis (vegetable weevil), Melanacanthus scutellaris (brown bean bug), Merophyas divulsana (lucerne leaf roller), Myzus persicae (green peach aphid), Nala lividipes (black field earwig), Mythimna convector (common armyworm), Nezara viridula (green vegetable bug), Nysius vinitor (rutherglen bug), Nysius clevelandensis (grey cluster bug), Oncopera rufobrunnea (underground grass grub), Orondina spp. (false wireworm), Othnonius batesi (black soil scarabs), Penthaleus major (blue oat mite), Persectania ewingii (southern armyworm), Petrobia lateens (brown wheat mite), Pieris rapae (cabbage white butterfly), Piezodorus hybneri (redbanded shield bug), Plutella xylostella (cabbage moth/diamondback moth), Rhopalosiphum maidis (corn aphid), Sericesthis spp. (small brownish cockchafers), Sitona discoideus (sitona weevil), Sminthurus viridis (lucerne flea), Spodoptera exigua (lesser armyworm), Spodoptera letura (cluster caterpillar Spodoptera mauritia (lawn armyworm), Stomopteryx simplexella (soybean moth), Tetranychus ludeni (bean spider mite), Tetranychus urticae (two spotted mite), Therioaphis trifolii f. maculata (spotted alfalfa aphid), Thrips tabaci (onion thrips), Thrips imaginis (plague thrips), Zizina labradus (grass blue butterfly), Zygrita diva (lucerne crown borer).

In other embodiments, the pests infest subjects and/or environments other than plants. Examples of such pests include, but are not limited to, lice, ants including Camponotus spp., Lasius alienus, Acanthomyops interjectus, Monoinorium pharaonis, Solenopsis molesta, Tetramorium caepitum, Monomorium minimum, Prenolepis impairs, Formica exsectoides, Iridomyrmex pruinosus, Cremastogaster lineolata, Tapinoma sessile, Paratrechina longicornis, cockroachs, mosquitos, bed bugs including Leptoglassus occidentalis, Acrosternum hiare, Chlorochroa sayi, Podius maculiventris, Murgantia histrionica, Oncopeltus fasciatus, Nabis alternatus, Leptopterna dolabrata, Lygus lineolaris, Adelpocoris rapidus, Poecilocapsus lineatus, Orius insidiosus, Corythucha ciliata, bees, wasps, black widow spider, booklice, boxelder bug, brown recluse spider, clothes moths including Tineola spp., Tinea spp., Trichophaga spp., carpet beetles, centipedes, clover mites, cluster and face flies, cigarette and drugstore beetles, crickets including Acheta spp., Gryllus spp., Gryllus spp., Nemobius spp., Oecanthus spp., Ceuthophilus spp., Neocurtilla spp., daddy-long-legs, domestic flies, drain flies, earwigs, European hornet, fleas including Ctenocephalides felis, Ctenocephalides canis, Ctenocephalides spp., Nosopsyllusfasciatus, Nosopsyllus spp., Xenopsylla cheopis, Xenopsylla spp., Cediopsylla simplex, Cediopsylla spp., fungus gnats, ground beetles, hide and larder beetles, horse/cattle/deer/pig flies, house dust mites including Dermatophagoides farinae, Dermatophagoides pteronyssinus, Dermatophagoides spp., mites including Ornithonyssus sylviarum, Dermanyssus gallinae, Ornithonyssus bacoti, Liponyssoides sanuineus, Demodexfolliculorum, Sarcoptes scabiei hominis, Pyemotes tritici, Acarus siro, Tyrophagus putrescentiae, Dermatophagoides sp., human lice, humbacked flies, Indian meal moth, millipedes, mud daubers, multicolored asian lady beetle, house borer, midges and crane flies, periodical and "dog-day" cicadas, powderpost beetles, roundheaded and flatheaded borers, pseudoscorpions, psyllids or jumping plant lice, spider beetles, sac spiders, sap beetles, termites, silverfish and firebrats, sowbugs and pillbugs, springtails, stinging hair caterpillars, tarantulas, vinegar flies, wasps and hornets, wharf borer, woods cockroach, yellowjacket wasps, fungus beetles, seed weevils, sawtoothed and merchant grain beetles, confused and red flour beetles, granery and rice weevils, indian meal moth, mealworms, drain flies, ticks including Dermacentar spp., Ixodes spp., Rhipicenphalus spp.,

carpenter bees, fleas, assassin bugs, human lice, chiggers, mystery bugs, european hornet, stinging hair caterpillars, black-legged tick, mayflies, black flies, horsehair worms, crickets, gypsy moths, grasshoppers, gnats, midges, locusts, mosquitoes including, Aedes albopictus, Aedes Canadensis Aedes triseriatus, Aedes tivittatus, Aedes vexans, Aedes spp., Anopheles quadrimaculatus, Anopheles spp., Coquillettidia perturbans, Coquillettidia spp., Culex pipiens, Culex spp.

An agriculturally effective amount may be determined by those skilled in the art using known methods and would typically range from 5 g to 500 g per hectare.

The compounds of the invention may be applied to any environment in which pests are present. For example, an environment in which agriculture is carried out, for example, the growing of crops, trees, and other plants of commercial importance. The agricultural environment includes not only the plant itself, but also the soil and area around the plants as they grow and also areas where parts of plants, for example, seeds, grains, leaves or fruit, may be stored. The environment may also be a household environment or industrial environment...

**BRIEF DESCRIPTION OF DRAWINGS**

**FIG. 1: Flowchart for initial solvent extraction of compounds of formula (I);**

**FIG. 2A: Flowchart showing the solvent partition for the aqueous concentrate obtained from the extraction shown in FIG. 1;**

**FIG. 2B: Flowchart showing the solvent partition for the ethyl acetate residue obtained from the extraction shown FIG. 1;**

**FIG. 3: Flowchart showing the steps in preparative HPLC chromatography;**

**FIG. 4: Graphically represents the selective inhibition of cell growth in culture by EBI-46;**

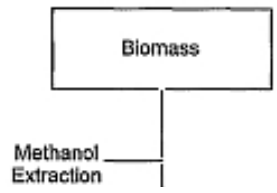
**FIG. 5: Graphically represents the results of topical treatment of B16 tumours in C57/B6 mice with EBI-46 (once a day application for three days starting from day 5);**

**FIG. 6: Graphically represents the results of topical treatment of LK2 mouse SCC in nude mice;**

**FIG. 7: Graphically represents the inhibition of growth of LKC SSC tumours by topical application of EBI-46; and**

**FIG. 8: Graphically represents the effect of injected EBI-46 on LK-2 SCC tumours.**

FIG. 1



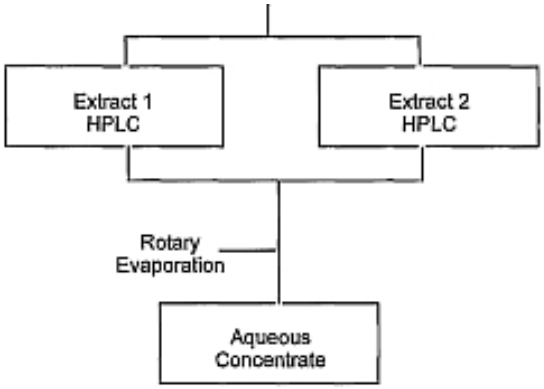



FIG. 2A

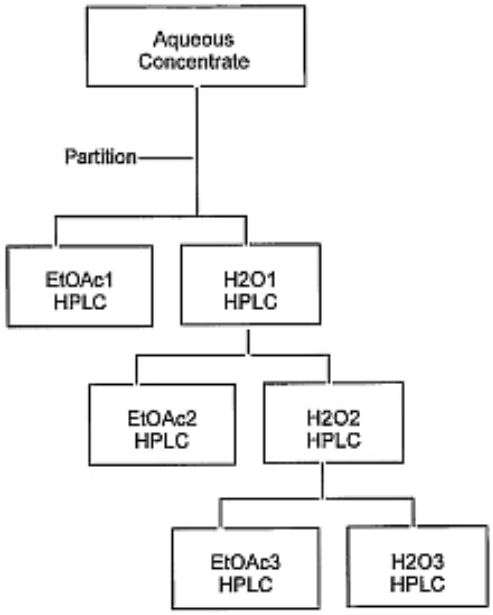



FIG. 2B

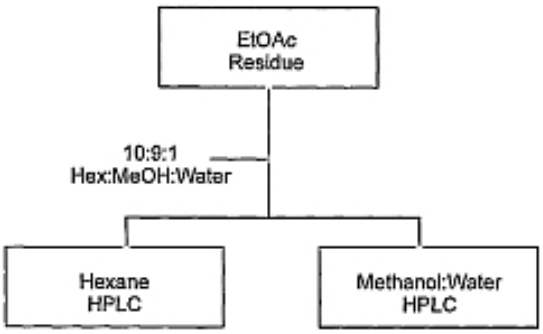
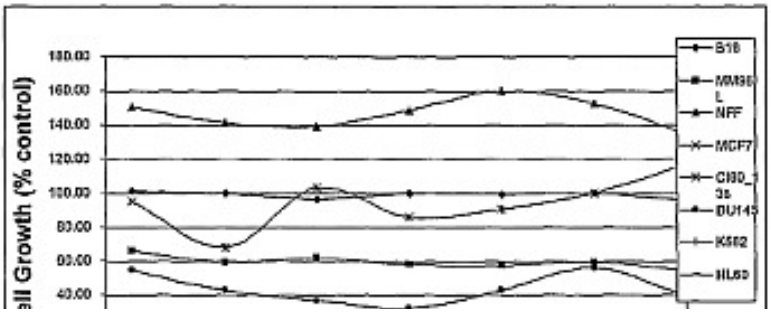



FIG. 4



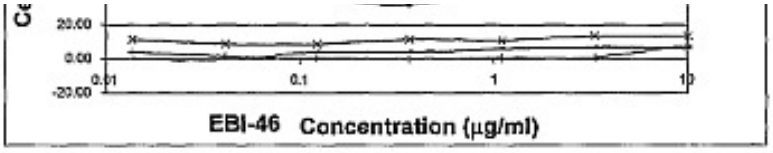



FIG. 5

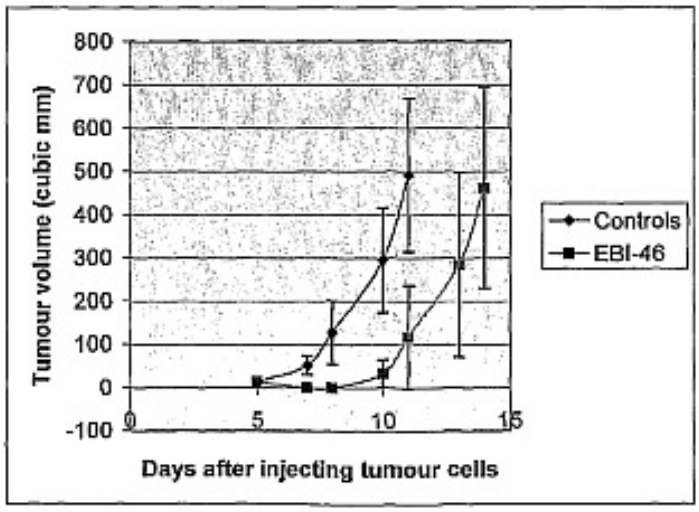



FIG. 6

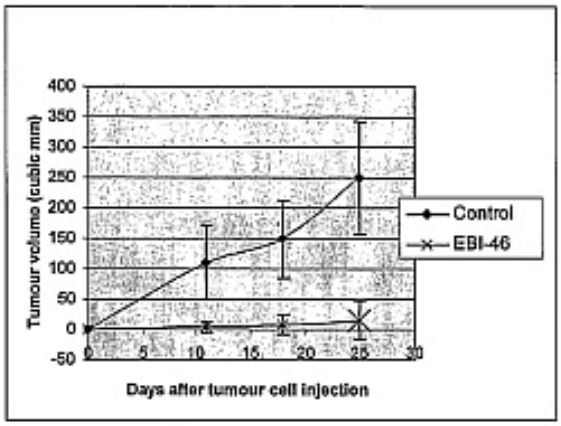



FIG. 7

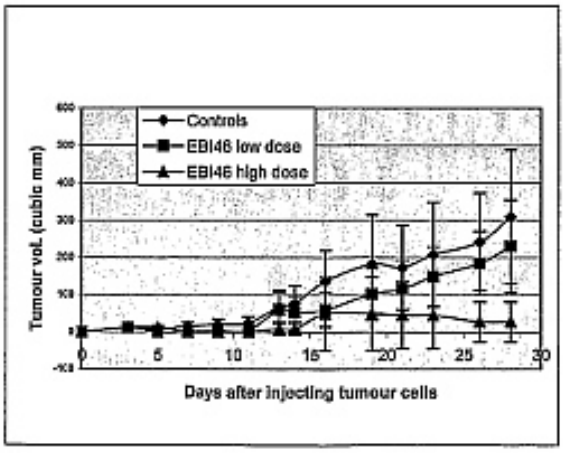
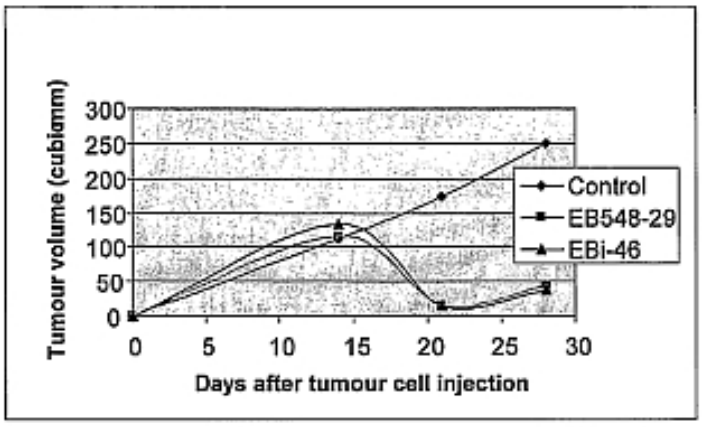



FIG. 8




**DETAILED DESCRIPTION**

**Activity Screening...**

**EXAMPLE 1**

**Methods**
**Extraction**

Biomass samples, including seeds, leaves and bark, from Fontainea picrosperma where collected and subject to the following extraction process. These samples and their subsequent fractions are referred in the below example as EB548.

**Phase 1-Extraction**

The biomass is generously covered with methanol and shaken (~2 L, overnight) followed by filtration to give the first extract. This process is repeated a second time (~2 L, ~5 hours) to generate the second extract. Each extract is examined by analytical HPLC and bioassayed (FIG. 1). The sequential methanol extracts are combined and the solvent removed by rotary evaporation to afford an aqueous concentrate.

**Phase 2-Solvent Partition**

The aqueous concentrate from the extraction is diluted with water to 400 mL. The diluted sample (code 'Cr') is subsampled for HPLC and bioassay, then shaken with an equal volume of ethyl acetate (EtOAc) in a separatory funnel and the individual layers, EtOAc1 and H2O1, collected. Note, occasionally a precipitate would form that was insoluble in either layer. This precipitate was collected by filtration and dissolved in methanol (code 'Me'). The lower aqueous layer (H2O1) was twice more extracted with ethyl acetate to give EtOAc2 and EtOAc3 along with the remaining H2O3 layer. Subsamples of all layers are examined by analytical HPLC and bioassay (FIG. 2A).

The sequential ethyl acetate extracts are pooled and the solvent removed by rotary evaporation to afford a residue that is weighed. On occasions, analytical HPLC indicated the EtOAc extract contained considerable amounts of extremely lipophilic (RT>9 minutes) material. To remove this material a 10:9:1-hexane:methanol:water partition was performed (FIG. 2B).

**Phase 3-Preparative HPLC Fractionation**

The residue from the solvent partition is investigated by analytical HPLC to find optimum chromatographic conditions for separation of the metabolites present. Using these optimum conditions the residue (~2 g) is fractionated by preparative reverse phase HPLC (C18, single injection) into 100 fractions (FIG. 3). Subsamples of all 100 fractions are examined by analytical HPLC. After analysis of the HPLC traces, the 100 fractions are consolidated into 20 to 30 pooled fractions (pools), some of which may be >80% pure. These pooled fractions are weighed, bioassayed and examined by analytical HPLC.

**Solvent Partition Summary for EB548**

Biomass samples of Fontainea picrosperma under went extraction and solvent partitioning, using phase 1 and 2 described above. Table 1 summarises the amounts of extractable material obtained after solvent partitioning with ethyl acetate.

**TABLE 1**
**Weights after Ethyl Acetate Partition of Extracts**
Sample Weight<1> EtOAc<2> % Ext.<3> HPLC Comment
EB548 318 68.4 21.5% Excellent
<1>Weight: Total sample weight in grams of plant material supplied and used for the study.
<2>EtOAc: Ethyl acetate extractables.
<3>% Ext.: Ethyl acetate extractables expressed as a percentage of the total sample weight.

**Preparative HPLC**

The preparative HPLC was carried out on a system consisting of two Shimadzu LC-8A Preparative Liquid Chromatographs with static mixer, Shimadzu SPD-M10AVP Diode Array Detector and Shimadzu SCL-10AVP System Controller. The column used was 50*100 mm (diameter*length) packed with C18 Platinum EPS (Alltech).

Approximately 2 grams of ethyl acetate extracted material was dissolved in dimethyl sulphoxide (4 mL) and subjected to preparative HPLC with typically conditions being 60 mL/min with gradient elution of 30% to 100% acetonitrile/water over 20 minutes followed by acetonitrile for 10 minutes. One hundred fractions (20 mL) were collected, evaporated under nitrogen, and then combined on the basis of HPLC analysis...

**EXAMPLE 2**
**EB548: Extraction and Solvent Partition**

Extraction and solvent partitioning of EB548 afforded 318 g of material. Each of the extraction and solvent partition layers were tested for bioactivity using the above bioassays. It can be seen from Table 2 that the extracts and ethyl acetate layers of the solvent partition all contain high CyTOX and NemaTOX activity.

**TABLE 2**
**Activity of Extracts and Solvent Partitions.**
Ne Bs Tr Cy
Sample Titre LD99<4> Titre LD99<4> Titre LD99<4> Titre LD99<4><4>LD99 in [mu]g/mL calculated as weight of chemical in last well with activity, however the real value

may be lower as end point not attained.

The successive aqueous concentrated extracts were subjected to HPLC. The column used was 50*100 mm (diameter*length) packed with C18 Platinum EPS (Alltech). Approximately 2 grams of extracted material was dissolved in dimethyl sulfoxide (4 mL) and subjected to preparative HPLC with typical conditions being 60 mL/minute with gradient elution of 30% to 100% acetonitrile/water over 20 minutes followed by acetonitrile for 10 minutes.

For comparison purposes the first ethyl acetate partition and the third water layers were analysed by HPLC. There was little or no compounds of interest remaining in the third water layer of the third water/ethyl acetate solvent partition.

**First Preparative HPLC Fractionation**

In a manner similar to that described in Phase 3 above the EB548 ethyl acetate solvent partition samples where pooled and further worked up using preparative HPLC chromatograph.
The preparative HPLC was used to produce 100 fractions. These fractions were pooled depending on the relative concentration of compounds indicated in the preparative HPLC chromatograph.
The bioactivity of each fraction or pooled fraction resulting from the preparative HPLC was determined using the above bioassay method. The results are summarized below at Table 3.

<5>Weight in mg.
**Second Preparative HPLC Fractionation**

To prepare additional material a second preparative HPLC fraction was performed. The HPLC pools from the second preparative HPLC fraction did not require bioassay as the active bands were chosen based on the UV spectra from the first preparative HPLC.

In performing the second preparative HPLC fractionation it was discovered that of the major active bands, fractions EB548.LA3.139-22/24, -35/38, -41/43 and -51/53, the latter three showed substantial instability. This instability was observed upon nitrogen evaporation but not while in acetonitrile/water solution at room temperature or on vacuum evaporation. To avoid decomposition the equivalent four bands of active metabolites from the second preparative HPLC were individually back extracted in the ethyl acetate and evaporated under vacuum. Analytical HPLC of these samples confirmed minimal decomposition...

**EXAMPLE 4**
**Mode of Activity**

While not wanting to be bound by any one theory the compound EBI-46 is considered to be a protein kinase C activator on the basis of it's similar in vitro growth inhibition profiles to phorbol ester (TPA), selective inhibition of the growth of K562 leukemia, MCF-7 breast cancer, Colo-205 colon cancer and D04 and SKMel-5 melanoma at doses approximately 100-fold lower than for other human tumour cell lines and for normal human fibroblasts. In addition, scattering of MCF-7 and HT-29 colon tumour cells was observed, typical of PKC activators. A particular human melanoma cell line MM96L undergoes a characteristic change to bipolar morphology when treated with TPA or with EBI-46. Additionally, given their similarity in structure, EBI-47, EBI-59 and EBI-61 are also considered to be protein kinase C

activators.

**EXAMPLE 5**

**Cytotoxicity and Reduction in Tumours with EBI-46**
**Materials and Methods**
**Isolation of EBI-46 and Related Compounds**

The raw plant material of Fontainea picrosperma (EB548 or EB610) was chopped, extracted with methanol, and partitioned between water and an organic solvent (diethyl ether or ethyl acetate). Pilot studies included HPLC and HPTLC activity-guided analysis, then optimization of the conditions for purification of bioactivity, and confirmation of stability. Fractionation of the bulk was conducted on silica gel 60 in petroleum spirit/acetone/methanol or in petroleum spirit/ethyl acetate/methanol, followed by HPLC. The latter involved reverse phase separation on a C18 Phenomenex Lunar 5 micron, 250*4.6 mm column in methanol-water.

**Results**

**1. Purification of EBI-46 and Related Compounds**

The organic extract was fractionated by chromatography on silica giving a fraction (548-35) containing a bioactive peak of high purity (RT: 25.131 minutes). Further purification by HPLC yielded >2 g of EBI-46 (RT: 25.262 minutes), from 2 kg of plant material.

**2. Purity, Stability and Solubility**

The bulk sample of EBI-46 was found to be >95% pure by UV and NMR, the limit of detection of the instruments.

Retention of bioactivity through extraction and chromatography steps implied that the structure was stable, and this has been confirmed to the extent that solutions of EBI-46 in ethanol retain bioactivity when held at 4[deg.] C. for 4 weeks. This was confirmed by an HPLC study of stability in the preferred delivery vehicle for intralesional injection (PEG 400 containing 10% ethanol) held at 37[deg.] C. The structure has no readily reactive groups which might otherwise confer instability.

Being a diterpene ester, EBI-46 is highly soluble in organic solvents including biocompatible solvents such as acetone, alcohols and PEG 400. It requires a small amount of such solvents to form aqueous solutions. Solubility tests have demonstrated 100% solubility at all 3 concentrations tested so far: 450 [mu]g/mL in 90% water, 50 [mu]g/mL in 99% water and 5 [mu]g/mL in 99.9% water. Higher solubilities may well be achievable.

Note that EBI-46 is a potent drug and only small amounts are required. Thus for intralesional injection of EBI-46, the concentration was 400 [mu]g/mL (and no water was required in this case).

3. Bioactivity Profile of EBI-46 and Related Compounds In Vitro: Arrest of Cell Growth

The ability of the EB548 crude extract (Table 13), and purified EBI-46 (FIG. 4) to block the growth of cultured human tumour cell lines and a normal strain (human diploid fibroblasts)

was tested in a clonogenic-type assay where many generations were allowed to elapse (5-6 days treatment) before measuring cell growth (Sulfurhodamine protein stain). Changes in morphology were also scored, and these were identical to those induced by the known protein kinase C (PKC) activator TPA (tetradecanoyl phorbol acetate), namely extreme bipolar morphology in the MM96L cell line and scattering of the MCF-7 cell clusters.

These compounds are therefore also considered to be PKC activators and of potential utility in the same indications as demonstrated for EBI-46 below.

4. Efficacy of EBI-46 in Treatment of Subcutaneous Tumours in Mice: Topical Application

Topical application of EBI-46 in an isopropanol gel was carried out on the aggressive B16 mouse melanoma in its natural (syngeneic) host, C57BL/6 mice (0.5 million tumour cells injected per site).

The frequency (1 daily dose for 3 days only) and dose level for topical application of PKC activators was selected on the basis of in vitro activity on cell lines. The materials were dissolved in acetone and diluted into an isopropanol gel for topical application.

The aggressive and rapidly growing B16 mouse melanoma is recognised as a very stringent tumour model in which to test anticancer agents. Stringency was further increased by injecting at least 10* more tumour cells than the minimum required to form a tumour in the animal. A confounding factor in determining the efficacy of topical treatments was that some tumour cells escaped from the subcutaneous site at a early stage and became established in the underlying muscle where it is assumed that the drug and its associated dermal host response did not reach. Such tumours could be distinguished from subcutaneous tumours by their immobility when the skin was pulled around the body of the animal.

It was therefore highly significant that approximately 150 [mu]g EBI-46/site gave a good response (4 mice and 4 controls, 2 sites/mouse), with one site apparently cured but the mouse had to be euthanased because the other site was growing. The inflammatory response was mild. There was no sign of systemic toxicity or lung metastases with any of the drugs.

The regrowth of tumour cells after 10 days was not surprising, given the stringency of the model (FIG. 5) and the somewhat arbitrary choice of dose and regimen.

The above was repeated using the UVB-induced mouse squamous cell carcinoma (SCC) grown on nude mice (FIG. 6) and treated with partially-purified EBI-46. This more realistic model for skin cancers showed an extremely high response rate which was maintained over a long period.

Again, an excellent result was obtained, with relapses occurring after 6 weeks due primarily to outgrowth of tumours from the underlying muscle, presumably due to being out of reach of the topical drug.

The most recent experiment with topical application used 2 different doses of EBI-46 (FIG. 7). The low dose was 100 [mu]g/site and the high dose was 350 [mu]g/site/treatment.

This experiment gave an excellent result at 350 [mu]g EBI-46/site, and showed that it was important to achieve a certain dose level to achieve efficacy.

5. Efficacy of EBI-46 in Treatment of Subcutaneous Tumours in Mice: Intralesional Injection

A pilot study was conducted on 6 mm*6 mm LK-2 tumours established in nude mice. Approximately 50 [mu]L of a solution of EB548 fractions (approximately 20 [mu]g EBI-46 in the EB548-35 fraction) in saline containing 20% acetone were injected in 3 sites around the periphery of each lesion. This was only done once.

The result showed rapid ablation of visible tumours, (FIG. 8) and an inflammatory response at the site of injection. Tumour growth eventually recovered, presumably due to non-optimal delivery.

The above procedure was then modified by using PEG 400 containing 10% ethanol as the vehicle. EBI-46 is freely soluble in this mixture and the increased viscosity served to restrict delivery of drug to the tumour site.

With PEG 400 delivery, 10 [mu]g EBI-46 in 25 [mu]L solution was injected (29 gauge) with a 0.5 mL insulin needle into a 7 mm*7 mm tumour, highly visible on the left flank of a nude mouse.

By 16 hours, a marked inflamed area had developed and the tumour lump had largely gone. A small area of normal skin on the ridge of the back was accidentally treated topically with the preparation, and developed a mild inflammatory response.

Seven days later, the tumour site was still flat and a scab had formed. The normal treated skin on the ridge of the back also formed a scab. This mouse remained tumour-free for over 9 months and was finally euthanased due to an unrelated condition (swollen penis).

In addition to the advantage of viscosity for localization of drug, PEG 400 gave fewer problems with leaking out after withdrawal of the needle. PEG 400 alone had no effect when injected into an LK2 tumour on another mouse.

A second mouse model was tested in a pilot study, involving an 8 mm diameter human nasopharyngeal tumour implanted and growing subcutaneously on the neck of a SCID-NOD mouse. Up to 3 injections of EBI-46 (total of 25 [mu]g in 75 [mu]L 25% propylene glycol-saline) were made into the NPC tumours of 2 mice. The scab sloughed off the treated site in one mouse, with no sign of residual tumour. Growth of the tumour in the second mouse was delayed but not ablated.

**Systemic Administration of EBI-46**

A variety of reports using cultured cells suggest that PKC activators may have potential for the treatment of lymphoid neoplasms. The murine B-cell lymphoma line A20 was used as an experimental model because it has been reported to grow well in mice and closely models the human situation.

SCID-NOD mice (BALB/c background) were shaved and 10E7 A20 cells injected subcutaneously (2 sites per mouse). The tumours tended to grow in a flat, diffuse manner and became raised and measurable at the 10E7 sites only after about 15 days. One mouse with 10E7 tumours was then injected intraperitoneally from day 18 with a total of 5 doses of 20-25 [mu]g EBI-46 in 25% propylene glycol-saline. The solution was stable for weeks at 4[deg.]

C., and there was no sign of insolubility at this concentration (250 [mu]g/mL).

The results suggest that tumour growth in the injected mouse was strongly inhibited by EBI-46, compared with an untreated 10E7 mouse. Growth increased when the treatment stopped and the mouse was euthanased at 27 days.

**Delivery Vehicles for EBI-46**

Consideration of the structure and stability of EBI-46 leads to the use of protic solvents that are biocompatible. Benzyl alcohol and Cremaphor would be possibilities but have not been tested. PEG 400 was chosen because of its common usage, but similar solvents could well be suitable; and the use of larger needles would obviate the need to dilute slightly with ethanol. None of the above materials were deliberately sterilized, despite being used in the immunocompromised nude mice.

**Safety Issues**

The operator, as with any potent drug, should wear personal protection (gloves, coat/gown, eye protection). EBI-46 can cause inflammation of skin. It can be deactivated with sodium carbonate solution.

The animals have shown no weight loss, signs of distress or side effects. Internal organs appeared to be normal on dissection but no histology or formal toxicology has been done.

**Discussion**

EBI-46 is solvent extractable from EB548 material and although other compounds with similar activity are present, EBI-46 travels in an uncluttered region of the chromatogram and therefore is relatively easy to purify. The same bioactivities were obtained from the crude extract through to the purified structure. Properties relevant to its potential use as a pharmaceutical have so far been favourable: availability, purity, stability and solubility in delivery vehicle.

The cell growth inhibition profile revealed EBI-46 to be a PKC activator, showing very high selectivity for a subset of solid tumour and leukemia cell lines, compared with normal cells and some other tumour cell lines. Local application such as topical cream or intralesional injection into lesions is likely to clear these sites because of a combination of direct killing (high local dose) and elimination of peripheral tumour cells by the host's innate immune response as evidenced by the early inflammatory reaction at the site of application.

Intralesional injection required less drug than topical application, and only one treatment, to obtain a significant response. If relapse occurs, for example on one side of the original lesion, repeated injections would be possible. Injection also provides a more positive delivery than relying on topical application on sites of different skin thickness.

It is important to note that efficacy of local treatment does not require the target tumour to be intrinsically sensitive to EBI-46. The aggressive B16 mouse melanoma cells for example are quite resistant in culture but respond to the drug topically in the mouse. Presumably, the vigorous host response is a major factor. This C57BL/6 strain is very different from the BALB/c background of the nude mouse, indicating that neither strain differences nor lack of

adequate T-cell immunity inhibit the efficacy of EBI-46.

The ability of local treatment with EBI-46 to work in species other than mice remains to be evaluated. Mouse skin is very thin (half the thickness of human skin), thus making intralesional injection more attractive. PEG 400 was chosen as a vehicle because it is used extensively for drug delivery and because its viscosity (lowered slightly with 10% ethanol to achieve injectability) may limit spreading away to far from the injection site.

Note also that EBI-46 causes an inflammatory response in normal skin. There are anecdotal reports of various plant saps being used to treat warts. This raises the possibility of using EBI-46 to ablate keloid scars, psoriasis, warts, proud flesh and other non-malignant conditions of the skin.

Local treatment may find significant application beyond skin lesions. It may be feasible to locally treat, by injection or suitably-formulated topical preparations, life-threatening tumours such as those of the oral cavity, esophagus and bowel. This could be carried out in conjunction with physical or pharmacological means of limiting escape of the drug into the circulation.

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

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.