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(57) Abstract

The present invention discloses a composition suitable for pharmaceutical use which comprises at least one active ingredient from a brassica extract or an analogue of sulforaphane, and resveratrol or an analogue thereof.

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BRSSICA EXTRACTS OR SULFORAPHANE IN COMBINATION WITH RESVERATROL AS ANTITUMOR AGENTS

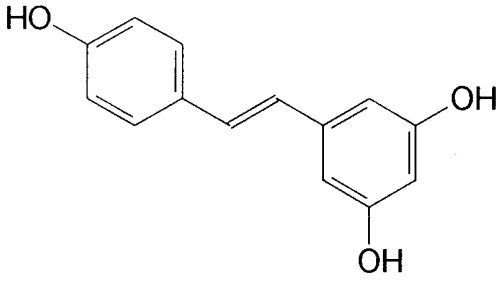
The present invention relates to compositions for retarding and/or preventing tumours.

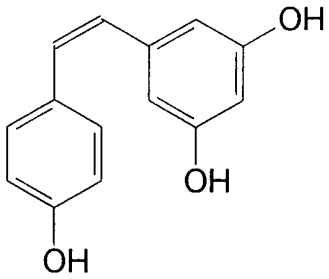
There have been numerous attempts at treating tumours. It is accepted that tumour development can be a multistage process, and that it can be inhibited by interfering with various discrete elements in the overall process. Inhibition of the earliest stages would generally be considered the most desirable protective effect.

Many compounds have been tested for their efficacy in preventing tumours. One such compound is resveratrol. *In vitro* experiments have been undertaken with resveratrol and there is evidence from *in vitro* cell culture and bacterial mutagenicity studies that resveratrol might retard tumour initiation when a mutagenic or carcinogenic challenge is given. The improved scavenging of free radicals, possibly by the induction of the phase II enzyme quinone reductase, has been proposed to account for the protection of the cells (Jang et al, *Science*, **275**, 218-20(1997)).

There is also evidence that resveratrol might inhibit cyclooxygenase, an enzyme considered to be involved in tumour promotion and various inflammatory conditions. Anti-proliferative effects of resveratrol have been noted *in vitro* and attributed to an inhibition of thymidine incorporation and the inhibition of esterase enzymes (Jang *et al.*, (1997)). In addition, in a leukaemia cell line, a marked inhibition of the enzyme ribonucleotide reductase has been reported (Fontcave *et al.*, *FEBS Letters*, **421**, 277-9 (1998)) and this should also have an anti-proliferative effect *in vivo*.

Resveratrol is a stilbene. It has two forms, the trans form and the cis form. A limited number of stilbene-containing plants have been consumed by man, and of these, the best known is the grape.

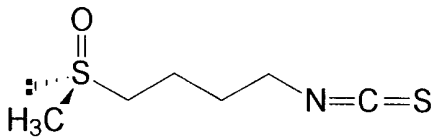




Several organic isothiocyanates have been tested for anti-cancer activity. One of these is sulforaphane which can be extracted from plants of the genus Brassica. It has been shown that sulforaphane does not induce phase I drug metabolising enzymes (Zhang et al(1992)). Later studies have shown that sulforaphane increased the activity of 2 isoforms of glutathione-S-transferase and decreased the major human cytochrome P450 CYP3A4 (Mahoe *et al.*, *Cancer Res.* **57**, 3649-3652 (1998)). Sulforaphane has been reported to reduce the activation of the Aflatoxin B_1 by human hepatocytes (Longuet *et al. Molecular Toxicology* **11**, 95-191, 1998) and to reduce the incidence and multiplicity of mammary tumours following administration of DMBA (Verhoeven *et al.*, 1997).

Some *in vivo* studies have also been performed on sulforaphane. Two studies showed a reduced binding of the aflatoxin B_1 following the administration of diets of cabbage and brussel sprouts. Reductions of DMBA or MNU-induced mammary tumours in rats have been found in 3 studies when cabbage, cauliflower, broccoli or brussel sprouts were included in the diet. Administration of cauliflower reduced the liver carcinogenesis induced by AFB1. A similar study, also with AFB1 showed a reduction in the number of tumours in the liver while a study on mice receiving cabbage along with DMH showed a reduction in the total number of tumours.

Sulforaphane is one of a number of organic thiocyanates released on hydrolysis of the aliphatic glucosinolates.



The present invention is based on the fact that a combination of resveratrol or its analogue and a brassica extract is surprisingly effective in treating tumours, especially testicular tumours. Accordingly, the present invention provides a composition suitable for pharmaceutical use which comprises at least one active ingredient obtainable from a brassica extract or an analogue of sulforaphane and resveratrol or an analogue thereof.

Resveratrol can also be obtained from plants. The composition can thus be obtained by mixing the plant extracts. The brassica extract may be obtained from any brassica vegetable which includes cabbage, kale, cauliflower, broccoli, mustard greens, kohlrabi, brussels sprouts and horseradish. The brassica extract is preferably a broccoli extract. Resveratrol may be extracted from grapes or other parts of grapevines or made via a synthetic preparation.

The analogues of resveratrol include stilbenes, hydroxylated stilbenes, for example trihydroxy-stilbenes and tetrahydroxy-stilbenes, which are typically phytoalexins, with or without one or more attached sugars or alkyl groups such as methyl; oligomers and/or polymers thereof, as well as oxidation or reduction products thereof. In particular, 3,4',5-trihydroxystilbene-3-beta-mono-D-glucoside or resveratrol which is preferred, may be used and the pharmacologically acceptable salts and esters thereof.

The sulforaphane analogue which can be used in the composition may be any compound having an isothiocyanate moiety and a polar functional group moiety,

wherein the two moieties are linked by a chain of one or more carbon atoms and the compound contains no pyridyl moieties, or a pharmacologically acceptable salt of such a compound.

The sulforaphane analogue is preferably not a heteroaromatic compound and is preferably not an arylalkyl compound. The analogue is preferably an olefin and is preferably aliphatic. The second moiety is preferably a polar functional group selected from a carboxylic ester, a carboxylic acid, a hydrocarbonoxy, a halogen, a hydroxyl, a ketone, a cyano, a nitro, a phosphine oxide, a sulfide, a sulfone, a sulfoxide, a thioether, and a thioester group, more preferably selected from a hydroxyl, a ketone, a phosphine oxide, a sulfone, and a sulfoxide group. The carbon chain of the sulforaphane component preferably comprises at least 3 carbon atoms, more preferably 3 to 5 carbon atoms. The carbon chain is preferably part of a non-aromatic ring.

The sulforaphane component is preferably selected from sulphoraphane itself, sulforaphene (4-isothiocyanato-(1R)-(methylsulfinyl)-1-(E)-butene), 6-isothiocyanato-2-hexanone, *exo*-2-acetyl-6-isothiocyanatonorbornane, *exo*-2-isothiocyanato-6-methylsulfonylnorbornane, 6-isothiocyanato-2-hexanol, 1-isothiocyanato-4-dimethylphosphonylbutane, *exo*-2-(1'-hydroxyethyl)-5-isothiocyanatonorbornane, *exo*-2-acetyl-5-isothiocyanatonorbornane, 1-isothiocyanato-5-methylsulfonylpentane and *cis*- or *trans*-3-(methylsulfonyl)cyclohexylmethylisothiocyanate and is preferably either form of sulforaphane, more preferably ((-) l-isothiocyanato-(4R)-(methylsulfinyl)butane). Bertoin, alyssin, erucin, erysolin, iberverin, iberin and cheirolin may also be used.

Although the brassica extract and resveratrol and its analogues both show potential for reducing the incidence of cancers, surprisingly, when used together, they show a synergistic effect. The brassica extract or analogue of sulforaphane appears to act principally on the initiation phase of carcinogenesis, whereas, resveratrol and its analogues may inhibit protein kinases *in vivo* and therefore affects the subsequent

proliferative phase of cancer.

A further aspect of the invention provides for the use of the composition of the invention for treating tumours. The composition can be used in a method of treatment of tumours. The composition can be used to improve the condition of a patient suffering from a tumour, in particular a testicular tumour.

In a further aspect, the composition may additionally comprise pharmaceutically acceptable diluents or excipients. It may also comprise antioxidant compounds, vitamins and minerals, in particular, vitamin A, vitamin C, vitamin E, lycopene and selenium.

The composition preferably comprises the active ingredient obtainable from a brassica extract (or a sulforaphane analogue) and resveratrol or its analogue in a weight ratio of 1:1000 to 1:10, preferably 1:500 to 1:50, more preferably 1:150 to 1:75 and especially about 1:100, generally such that they are present in a quantity producing a synergistic antitumour effect. This last formulation typically contains sulforaphane and resveratrol in a ratio of 2:1.

The composition is preferably administered in doses containing 1 to 100mg of the active ingredient obtainable from a brassica extract or sulforaphane analogue and 0.5 to 100mg of resveratrol or an analogue, preferably in a dose of 10mcg of active ingredient obtainable from a brassica extract or sulforaphane analogue : 1mg resveratrol or its analogues. The composition may be administered with a frequency of several times a day to once every two days, preferably daily. Treatment should be ongoing.

The present composition can be administered in a variety of dosage forms, for example orally such as in the form of tablets, capsules, sugar- or film-coated tablets, liquid solutions or suspensions or parenterally, for example intramuscularly, intravenously or subcutaneously. The present composition may therefore be given

by injection or infusion.

The dosage depends on a variety of factors including the age, weight and condition of the patient and the route of administration. Typically, however, the dosage adopted for each route of administration when a compound of the invention is administered alone to adult humans is in the range of 0.01 to 100 mg/kg body weight. Such a dosage may be given, for example, from 1 to 5 times daily by bolus infusion, infusion over several hours and/or repeated administration.

The resveratrol or analogue thereof and sulphoraphane or analogue thereof or pharmaceutically acceptable salts thereof are formulated for use as a pharmaceutical or veterinary composition also comprising a pharmaceutically or veterinarily acceptable carrier or diluent. The compositions are typically prepared following conventional methods and are administered in a pharmaceutically or veterinarily suitable form.

The present composition may be administered in any conventional form, for instance as follows:

A) Orally, for example, as tablets, coated tablets, dragees, troches, lozenges, aqueous or oily suspensions, liquid solutions, dispersible powders or granules, emulsions, hard or soft capsules, or syrups or elixirs. Compositions intended for oral use may be prepared according to any method known in the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavouring agents, colouring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations.

Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for the manufacture of tablets. These excipients may be for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, dextrose, saccharose, cellulose, corn starch, potato starch, calcium

phosphate or sodium phosphate; granulating and disintegrating agents, for example, maize starch, alginic acid, alginates or sodium starch glycolate; binding agents, for example starch, gelatin or acacia; lubricating agents, for example silica, magnesium or calcium stearate, stearic acid or talc; effervescing mixtures; dyestuffs, sweeteners, wetting agents such as lecithin, polysorbates or lauryl sulphate. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and adsorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. Such preparations may be manufactured in a known manner, for example by means of mixing, granulating, tableting, sugar coating or film coating processes.

Formulations for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is present as such, or mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil.

Aqueous suspensions contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethyl-cellulose, sodium alginate, polyvinylpyrrolidone gum tragacanth and gum acacia; dispersing or wetting agents may be naturally-occurring phosphatides, for example lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides for example polyoxyethylene sorbitan monooleate.

The said aqueous suspensions may also contain one or more preservatives, for example ethyl or n-propyl p-hydroxybenzoate, one or more colouring agents, and/or one or more sweetening agents such as sucrose, saccharin, glucose, sorbitol and mannitol.

Oily suspension may be formulated by suspending the active ingredient in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol.

Sweetening agents, such as those set forth above, and flavouring agents may be added to provide a palatable oral preparation. These compositions may be preserved by this addition of an antioxidant such as ascorbic acid. Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, a suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavouring and colouring agents, may also be present.

The pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil, for example olive oil or arachis oils, or a mineral oil, for example liquid paraffin or mixtures of these. Suitable emulsifying agents may be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally occuring phosphatides, for example soy bean lecithin, and esters or partial esters derived from fatty acids an hexitol anhydrides, for example sorbitan mono-oleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsion may also contain sweetening and flavouring agents. Syrups and elixirs may be formulated with sweetening agents, for example glycerol, sorbitol or sucrose. In particular a syrup for diabetic patients can contain as carriers only products, for

example sorbitol, which do not metabolise to glucose or which only metabolise a very small amount to glucose.

Such formulations may also contain a demulcent, a preservative and flavouring and coloring agents;

B) Parenterally, either subcutaneously, or intravenously, or intramuscularly, or intrasternally, or by infusion techniques, in the form of sterile injectable aqueous or oleaginous suspensions. This suspension may be formulated according to the known art using those suitable dispersing of wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic paternally-acceptable diluent or solvent, for example as a solution in 1,3-butane diol.

Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition fatty acids such as oleic acid find use in the preparation of injectables;

C) By inhalation, in the form of aerosols or solutions for nebulizers;

D) Rectally, in the form of suppositories prepared by mixing the drug with a suitable non-irritating excipient which is solid at ordinary temperature but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials are cocoa butter and poly-ethylene glycols;

E) Topically, in the form of creams, ointments, jellies, collyriums, solutions or suspensions.

Daily dosages can vary within wide limits and will be adjusted to the individual requirements in each particular case. In general, for administration to adults, an appropriate daily dosage is in the range of about 5 mg to about 500 mg, although the upper limit may be exceeded if expedient. The daily dosage can be administered as a

single dosage or in divided dosages.

The following example further illustrates the present invention.

Example

Test materials

Resveratrol of defined purity (98.5-101.5%) and composition was provided. The material was a white powder and was stored at 4°C. Sulphoraphane of defined purity (99%) was used. The material was a brown oily substance with a slight pungent odour. It was stored at 4°C. NMR spectroscopy indicated a purity of 99% or better and that the material was stable stored at 4°C.

Animal Receipt

Male Wistar Albino rats weighing between 169-185g and of defined microbiological status were purchased from Charles River, Margate, Kent. All animals were examined upon receipt and were accepted for the study. The rats were randomly assigned to groups; tailmarked and weighed. The rats were allowed to acclimatise for 10 days to the conditions under which they were maintained throughout the study. During the acclimatisation period, they were allowed unrestricted access to Thames water tap water and to a standard rodent diet (B&K Rat and Mouse Diet, Hull - batch 17/04/00), certified to GLP standards.

Treatment groups were as follows:

Control	Unadulterated Diet
Treated group	2.0 mg/kg bodyweight/day of resveratrol and 10 mg/kg bodyweight/day of sulphoraphane.

The number of rats per group were 5 in the control group and 4 in the treated group. The dosing period was 14 days.

Throughout the study the rats were housed on sterilised sawdust in solid-bottom NKP polypropylene cages in the rodent facilities of the University of Surrey Experimental Biology Unit. The ambient temperature was maintained at 22°C ± 3°C and humidity controlled in the range of 30-70%. Lighting was artificial with a 12 h light and 12 h dark cycle. Tap water and diet was freely available at all times. Body weights were monitored during the study. Average food consumption was measured over the exposure period for both groups.

All animals were observed once daily during the treatment period for clinical or behavioural signs of toxicity, and for symptoms of ill health throughout the period of administration.

Resveratrol was mixed directly into the diet at a nominal concentration of 2.0 mg/kg body weight/day. The oily nature of sulphoraphane would have made direct mixing difficult. Accordingly 250 mg was dissolved in 0.5 ml alcohol prior to mixing to ensure a thorough mix. It was administered at a concentration of 10.0 mg/kg body weight/day. The diet was stored at 4°C. No analysis of the diet for concentration or homogeneity of the test substances was carried out.

Alcohol (0.5ml) was added to the unadulterated diet to provide homogeneity throughout the administration period.

Termination of Experiment

On day 15 following administration of test materials, the surviving rats were injected intraperitonally with a lethal dose (0.15ml/100g body weight) of pentabarbitone (Sagatal, Rhone-Poulenc, Essex).

A 25% homogenate of the majority of the liver was prepared. The colon was scraped 5 times with a glass slide. The scrapings were put into a preweighed vial containing trypsin inhibitor (5 mg/ml) in KCl buffer (1.15%). The amount of material scraped was between 150-250 mg. The following assays were performed:-

Glutathione S-Transferase

NAD(P)H Quinone Reductase

The effect of the combination of resveratrol and sulphoraphane on the biological parameters glutathione S-Transferase and quinone reductase is shown in Table 1.

Table 1

	Control Group Nano mol reduced/min/mg protein	Treated Group Nano mol reduced/min/mg protein
Liver		
Glutathione S-Transferase (DCNB as substrate)	41.62	45.89
Glutathione S-Transferase (CDNB as substrate)	545.51	622.08
Quinone Reductase	10.68	11.83
Colon		
Glutathione S-Transferase	25.45	37.65
Quinone Reductase	13.19	19.11

CLAIMS

1. A composition suitable for pharmaceutical use which comprises at least one active ingredient from a brassica extract or an analogue of sulforaphane, and resveratrol or an analogue thereof.

2. A composition according to claim 1 in which the resveratrol or analogue thereof is an extract of a grape or a grapevine

3. A composition according to claim 1 or 2 which comprises resveratrol.

4. A composition according to any one of the preceding claims which comprises sulforaphane.

5. A composition according to any one of the preceding claims which comprises brassica extract and in which the weight ratio of brassica extract to resveratrol or its analogue is from 1:500 to 1:50.

6. A composition according to any one of the preceding claims which comprises 1 to 100mg of the brassica extract or sulforaphane analogue and 0.5 to 100mg of resveratrol or analogue thereof.

7. A composition according to any one of the preceding claims which further comprises one or more of vitamin A, vitamin C, vitamin E, lycopene and selenium.

8. A composition according to claim 1 substantially as hereinbefore described.

9. A process for producing a composition according to any one of the preceding claims which comprises mixing the active ingredients together.

10. A composition according to any one of claims 1 to 8 for use in a method of treatment of the human or animal body by therapy.

11. Use of a composition according to any one of claims 1 to 8 in the manufacture of a medicament for use in the treatment of tumours.

12. Use according to claim 11 of a composition according to any one of claims 1 to 8 in the treatment of testicular tumours.

13. A product containing at least one active ingredient from a brassica extract or an analogue of sulforaphane, and resveratrol or an analogue thereof, for s

simultaneous, separate or sequential use in the treatment of tumours.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00300

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K35/78 A61K31/26 A61K31/05

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)
IPC 7 A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WALLIG M A ET AL: "Induction of rat pancreatic glutathione S-transferase and quinone reductase activities by a mixture of glucosinolate breakdown derivatives found in Brussels sprouts." FOOD AND CHEMICAL TOXICOLOGY, (1998 MAY) 36 (5) 365-73. , XP000910743 see discussion abstract --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	HORAKOVA, K. ET AL: "Cytotoxic and cancerostatic activity of isothiocyanates and related compounds. I. Activity of some naturally occurring isothiocyanates and their synthetic analogs on HeLa cells" NEOPLASMA (1968), 15(2), 169-76 , XP000910737 see compound 5 table 1 page 173, paragraph 1 ---	1-13
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