

ZINC-PGA COMPOSITIONS AND METHODS FOR TREATING CANCER

FIELD OF THE INVENTION

The invention relates to compositions comprising gamma-polyglutamic acid (γ -PGA) carrier and a zinc salt, and, optionally, an NF- κ B inhibitor, pharmaceutical formulations thereof, and methods using any of the compositions and formulations as anti-tumor agents to treat cancer in a patient.

BACKGROUND OF THE INVENTION

Inherent and acquired drug resistance to cancer drugs is a major cause of cancer treatment failures. Common mechanisms for resistance include dysfunctions in p53 apoptosis protein and/or overexpression of energy-dependent drug ejection pumps encoded by *MDR1* or *MRP1* genes. One tumoricidal strategy for overcoming the drug resistance problem is to individually correct the dysfunctional p53 apoptosis function or to inhibit the drug ejection pumps. An alternate approach is to utilize PARP1-mediated energy depletion-induced necrotic cell death mechanism ("PARP1-mediated necrosis") that bypasses the p53-mediated apoptosis mechanism altogether.

PARP1-mediated necrosis, initially observed in post-ischemic necrosis of heart or brain tissues, is caused by depletion of cellular energy (NAD⁺ and ATP) from excessive DNA-repair activity by PARP1 enzyme. Hyperactivation of PARP1/PARG in response to genetic damage triggers depletion of NAD⁺ and ATP cellular energy commodities, which subsequently triggers mitochondria-initiated necrosis from MPTP activation. This set of events is illustrated in Fig. 1. Because the necrosis mechanism bypasses p53-

mediated apoptosis, it was proposed that this mechanism could be used to target cancer (NPL3). However, no one succeeded in translating this idea into a clinically useful therapeutic treatment because the methods tried proved to be too toxic. PARP1-mediated necrosis could only be induced in experimental tumors by excessive radiation exposure and/or administration of highly toxic chemotherapeutic agents such as doxorubicin. Another problem with using toxic agents for activating PARP1-mediated necrosis was that the agents also activated p53 proteins at sub-critical levels, effectively disabling PARP1-mediated necrosis via p53-induced fragmentation of PARP1 enzymes. Given that drug distribution within a tumor is heterogeneous due to physical and structural constraints, it was inferred that toxic agents would simultaneously render a large portion of the cancer tumor mass devoid of PARP1 and therefore insensitive to PARP1-mediated necrosis.

The problem to be solved in a great number of clinical cancer cases is that some cancers are innately resistant to conventional anticancer drugs and others develop multidrug resistance over the course of systemic treatment, resulting in treatment failure. Although there was a theoretical suggestion that harnessing PARP1-mediated tumor necrosis through excessive dosing with radiation and chemotherapeutic agents could be used to treat cancer, realizing this potential result was difficult due to the inherent toxicity of the treatment and the inherent self-contradictory nature of the mechanism mentioned above. Thus, there remains an unmet need to find a composition and/or treatment method based on actively inducing PARP1-mediated tumor necrosis. Furthermore, a composition and/or treatment method comprising a carrier and targeting system that can specifically deliver such an inducer to

tumor tissues without interfering with the tumor necrosis process or being excessively toxic is highly desired. There is also a continued desire to reduce the tumoricidal dose needed against a wide variety of cancer types and/or drug resistance traits, and to reduce unwanted side effects in healthy tissue.

A report assessing neurotoxicity of zinc salts describes that high concentrations of zinc ion from simple zinc salts (400 μ M or 26 μ g/mL) induces PARP/PARG-mediated NAD⁺ and ATP depletion and subsequent necrosis in cultured cortical cells (NPL6). The report, however, did not study tumoricidal activity of zinc salts or their therapeutic use against cancer.

A report assessing the toxicity of zinc pyrithione against immune cells showed that nanomolar concentrations of zinc pyrithione induced zinc-specific apoptosis in various leukocyte-originating cells, including murine thymocytes, murine splenic lymphocytes, human Ramos B, and human Jurkat T cells (NPL7). The report disclosed that zinc pyrithione induced apoptosis via activation of caspase 9, which has the effect of blocking necrotic cell death (NPL11). Collectively, these reports indicate that nanomolar doses of zinc pyrithione induce apoptotic cell death and not necrotic cell death in the immune cells studied.

Later, it was demonstrated that micromolar concentrations of zinc pyrithione (1-10 μ M) elicited ATP-depletion and, eventually, ERK and PKC-dependent necrosis in androgen-dependent LNCaP and androgen-independent PC3, DU145 prostate cancer cell lines (NPL2). However, the dose of zinc pyrithione used in NPL2 to elicit necrosis was previously shown to cause acute neurological toxicity in rats after 9~14 days of dietary administration (240 ppm) with clinical symptoms of progressive hind-limb

weakness, motor incoordination, spinal kyphosis with muscle atrophy and penile prolapse (NPL10).

Thus, although there was a report that zinc pyrithione was capable of causing selective necrotic cell death against prostate cancer cell lines, it required high (μM) concentrations of the agent (NPL2), but zinc pyrithione had been shown to cause severe and permanent neurotoxicity at such concentrations (NPL12), which would have dissuaded attempting to develop it into an antitumor therapeutic agent. Furthermore, NPL2 did not demonstrate broad-spectrum anticancer activity against multiple cancer cell types, show efficacy towards reversing drug resistance arising from MDR1 or MRP1 multidrug resistance gene overexpression, or demonstrate necrotic efficacy in any animal cancer models.

NPL5 developed insulin-mimetic zinc (2+) complexes and investigated the *in vitro* insulin-mimetic activity as well as the *in vivo* antidiabetic effects in type-2 diabetic KKA^y mice of zinc(gamma-polyglutamic acid) complexes. Specifically, the study showed that oral administration of 10-20 mg Zn per kg body mass for 30 days with gamma-polyglutamic acid-complexed zinc normalized the hyperglycemia in KKA^y mice, and improved the impaired glucose tolerance, elevated HbA(1c) levels, and metabolic syndromes relative to treatment with ZnSO₄ (NPL5). In NPL5, the authors concluded that the zinc(gamma-polyglutamic acid) complexes have antidiabetic potency through their high blood glucose-lowering effect and their ability to attenuate the derangement in β cell secretion of insulin and the insulin resistance in type-2 diabetic KKA^y mice, however they did not understand the mechanism of action responsible for the insulin-mimetic activity of the complex, and they did

not suggest in any way antitumor activity by zinc(gamma-polyglutamic acid) complexes nor its effectiveness for treating drug-refractory cancer types.

In summary, the art has not even suggested, much less succeeded in using zinc compounds to achieve the above-mentioned goal of effectively treating broad-spectrum cancers *in vivo*, including those with important drug-resistance traits, and moreover to do so without risking severe toxicity. Hence, there is an unmet need for a clinically active and safe zinc composition for treating cancer that works across many cancer types and even those having drug-resistance phenotypes (e.g., dysfunctional p53, MDR1 overexpression, MRP1 overexpression), without toxicity issues. To solve the problem we performed systematic research in the field and developed formulations of zinc complexes meeting these needs, and accordingly completed our invention as described herein.

CITATIONS

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SUMMARY OF THE INVENTION

Compositions, pharmaceutical formulations, and methods disclosed herein are based on the surprising observation that complexes of zinc and γ -polyglutamic acid (γ -PGA) can induce necrotic cell death in various human and mouse cancer cell lines.

The present invention relates to a zinc-containing γ -polyglutamic acid composition that triggers a PARP1-mediated necrotic cell death mechanism.

Without being limited by theory, zinc apparently over-activates PARP1, which in turn leads to depletion of ATP and NAD⁺ in cells. As a result, the cells are depleted of energy sources, and then enter a necrotic cell death pathway.

This mechanism to induce necrosis is expected to be similarly available for most cancer cell types and thus zinc-containing γ -polyglutamic acid compositions demonstrate broad-spectrum tumoricidal activity. Furthermore, this mechanism suggests that tumors having a drug-resistant phenotype with respect to different tumoricidal mechanisms may also respond to this PARP1-mediated mechanism.

Compositions according to the invention comprise (i) zinc(II) species (equivalently, Zn²⁺) as an active ingredient and (ii) γ -PGA as a carrier in a unmodified form and/or modified form, wherein folic acid and/or RGD tumor targeting peptides are covalently joined to γ -PGA. Compositions may further comprise NF- κ B inhibitors or NF- κ B signaling cascade inhibitors to sensitize the tumor cells (make them more susceptible to) the tumoricidal effect of Zn(II) and γ -PGA.

The compositions may be formulated for oral administration. In some embodiments, oral formulations that comprise gastro-resistant materials, such as enteric bindings and coatings, to prevent, delay, or attenuate dissociation of zinc ions from the complex in the strongly acidic environment of the stomach are provided.

The invention also relates to methods for preparing the above-mentioned compositions and pharmaceutical formulations, and the therapeutic uses thereof.

It is one object of the invention to provide a composition that can actively induce PARP1-mediated tumor necrosis, and it is a further object to do so using compositions and formulations that are not toxic to the patient.

It is another object of the invention to provide pharmaceutical formulations for use in treating a wide variety of tumors and cancer cells having drug-resistant phenotypes in a patient.

It is another object of the invention to provide a composition comprising a γ -PGA carrier that can target delivery of Zn(II) to tumor cells. Also, it is an object of the invention to provide a strong tumoricidal agent having a reduced dose requirement, or with reduced profile of unwanted side effects in healthy tissue.

One embodiment of a method of inducing PARP1-mediated tumor necrosis in a tumor in a patient comprises administering a therapeutically effective amount of a Zn(II) salt and a γ -polyglutamic acid carrier to the patient with the tumor wherein said γ -polyglutamic acid carrier comprises γ -polyglutamic acid and/or a tumor-targeting γ -polyglutamic acid derivative and/or a charge-modified γ -polyglutamic acid derivative and/or a tumor-targeting charge-modified γ -polyglutamic acid derivative. In another embodiment of a method, a therapeutically effective amount of a Zn(II) salt and a γ -polyglutamic acid carrier are administered to a patient with a tumor that has a drug-resistant phenotype.

In another embodiment of a method, a therapeutically effective amount of a Zn(II) salt and a γ -polyglutamic acid carrier are administered in combination with a therapeutic amount of an NF- κ B inhibitor.

In one embodiment, a therapeutic amount of Zn(II) salt and γ -polyglutamic acid carrier are administered together in a solid dosage form or in a liquid dosage form. In several embodiments, a solid dosage form is selected from a tablet, a minitab, a hard capsule, a soft capsule, a caplet, a gelcap, an oral disintegrating films, granules, pellets, a paste, and a powder sachet. In several embodiments, a liquid dosage form is selected from a liquid solution, a liquid suspension, a syrup, and an oral spray.

In several embodiments, a therapeutic amount of Zn(II) salt and γ -polyglutamic acid carrier are administered together by oral administration, topical administration, or an injection administration.

One embodiment of the invention is a pharmaceutical composition comprising (i) a pharmaceutically acceptable Zn(II) salt, (ii) γ -polyglutamic acid containing a tumor-targeting moiety and/or a charge-modifying moiety, and (iii) optionally further comprising γ -polyglutamic acid.

In several embodiments said tumor-targeting moiety is selected from folic acid and RGD peptide, and either or both of said moieties are covalently joined to γ -polyglutamic acid. In several embodiments said charge-modifying moiety is selected from citric acid, ethylenediamine tetraacetic acid, 1,4,7,10-tetracyclododecane-N,N',N'',N'''-tetraacetic acid, and diethylenetriamine pentaacetic acid, and any combination of said moieties are covalently joined to γ -polyglutamic acid.

In another embodiment, the pharmaceutical compositions further comprise γ -polyglutamic acid. In another embodiment, in the pharmaceutical compositions a substantial portion of said Zn(II) salt is a bound complex of the Zn(II) ion with γ -polyglutamic acid and/or said tumor-targeting moiety and/or

said charge-modifying moiety. In another embodiment, in the pharmaceutical compositions a Zn(II) salt and (ii) said γ -polyglutamic acid polymers are mixed together in a solid mixture.

In another embodiment, the pharmaceutical compositions further comprise an NF- κ B inhibitor.

In another embodiment, the pharmaceutical compositions are formulated as a solid dosage form. In several further embodiments, the solid dosage forms further comprise a gastro-resistant binder and/or a gastro-resistant outer coating. In another embodiment, the pharmaceutical compositions are formulated as a liquid dosage form. In one further embodiment, the liquid dosage form is a suspension of a pharmaceutical composition that further comprises a gastro-resistant material.

One embodiment of a method for treating a tumor in a patient comprises administering a therapeutically effective amount of the pharmaceutical composition according to any one of the foregoing embodiments of a pharmaceutical composition to the patient with the tumor. In a further embodiment of the method, a therapeutically effective amount of the foregoing pharmaceutical compositions are administered to a patient with a tumor that has a drug-resistant phenotype.

These and other objects and features of the invention will become apparent to one of ordinary skill in the art from the following detailed description of the invention and the claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic summary of PARP1-mediated necrosis.

Figure 2 shows the results of an in vitro flow cytometric analysis of human cancer lines treated with one embodiment of a ZnPGA composition.

Figure 3 shows the results of treating LL2 murine lung cancer allografts with embodiments of ZnPGA compositions.

Figure 4 shows the results of treating subcutaneous xenografts of H460 human lung cancer in mice with an embodiment of a ZnPGA composition.

DETAILED DESCRIPTION OF THE INVENTION

The components used in the compositions, formulations, and methods described herein are of a grade accepted by regulatory authorities for pharmaceutical use, or for use in foods, or for use in products for human consumption. In some instances the components are pharmaceutical grade or medical grade compounds or substances.

The meaning of abbreviations used herein is as follows: “kDa” means kiloDalton; “wt%” means percent by weight.

Active Ingredients

Zinc is provided as a zinc(II) salt (equivalently, a Zn^{2+} salt), wherein the counterion (anion) may be any suitable inorganic or organic anion. Suitable anions are those that are tolerated by the human body, including those that are not toxic. Generally, the zinc salt can be represented by the formulas $\text{Zn}^{2+}\text{X}^{2-}$ or $\text{Zn}^{2+}(\text{X}^-)_2$ or even $\text{Zn}^{2+}(\text{X}^-)(\text{Y}^-)$, where X and Y are suitable anions. The anion may be selected from the group of anions that are a component of an approved food additive or nutritional supplement. In some embodiments, the zinc(II) salt is a nutritionally acceptable zinc salt, wherein said zinc(II) salt is selected from the group of zinc(II) salts that have been approved for use in

foods or nutritional supplements. The anion may be selected from the group of anions that are a component of an FDA-approved pharmaceutical product. In some embodiments, the zinc(II) salt is a pharmaceutically acceptable zinc salt. Examples of zinc salts include zinc chloride, zinc sulfate, zinc citrate, zinc acetate, zinc picolinate, zinc gluconate, amino acid-zinc chelates, such as zinc glycinate, or other amino acids known and used in the art. Two or more different zinc salts may be used together as the active zinc ingredient in any proportion.

In some embodiments, Zn(II) is provided complexed to γ -polyglutamic acid compounds in the composition or formulation. Typically, when complexed forms of Zn(II) and γ -PGA ("ZnPGA") are provided, the ZnPGA is purified and free Zn(II) ions as well as the original counterions to the Zn cation are removed in the process.

The amount of zinc included in a single solid dosage form is generally in the range of about 1 to about 100 mg of zinc (zinc(II) ion). Thus, the particular amount of zinc salt(s) used in a formulated composition will be higher because amount of the salt must account for the weight of the counterion. Considering only zinc(II), the amount provided in a dosage form may be up to about 100 mg, up to about 75 mg, up to about 50 mg, up to about 25 mg, up to about 10 mg of zinc, or up to about 5 mg. The amount of zinc(II) provided in a solid dosage form is generally at least about 1 mg. By way of comparison, commonly available supplements provide, for example, 20, 25, 30, 50, 75, and even 100 mg of zinc. Any amount of zinc in this range, or even higher, is acceptable so long as the amount provided does not cause physiologically excessive levels of zinc to be absorbed. What might be

considered an excessive level and the risk therefrom, however, is to be balanced against the therapeutic benefit gained by treating a tumor. Although a tolerable upper intake level of zinc in most adults is about 40 mg/day (and for children it is lower), it should be recognized that all of the zinc in the solid dosage form taken orally is unlikely to be absorbed; some of it will pass through the body without being adsorbed. Because the amount of zinc absorbed will also vary with the formulation, the upper limit for zinc content in a particular formulation can be tested by methods known in the art to ascertain the level of uptake provided by the formulation, and then in view of any therapeutic benefit in the treatment gained by administering the formulation, one may adjust the amount administered for a given dosage form or formulation accordingly.

The concentration of zinc provided in a composition or formulation in a liquid dosage form is generally in the range of about 1 mg/L to about 100 g/L of zinc (zinc(II) ion). This corresponds to a range of about 0.0001 wt% to about 10 wt% of zinc. The concentration of Zn(II) may be at least about 10 mg/L, or at least about 100 mg/L, or at least about 1 g/L, or at least about 10 g/L, or the range for the concentration of Zn(II) may fall within any two of these exemplary concentrations. In one embodiment, the concentration may be in the range of about 100 mg/L about 500 mg/L. The amount of the liquid provided in the dosage form will determine the total dosage amount. For example, 100 mL amount of liquid would provide about 10 mg to about 50 mg of Zn(II) for the exemplary range. The disclosure regarding dosage amounts of zinc in solid dosage forms may be used as guidance as to the amount of Zn(II) solutions to provide as a liquid dosage amount. As disclosed in

Example 4, mice were treated with 160 µg/mL solutions of Zn(II) (equal to 160 mg/L) and received a physiologically relevant dose of 16 mg/day/kg body weight of Zn(II).

Zinc may also be provided as part of a solid suspended in liquid. The amount of zinc(II) and the volume of the suspension provided follows the guidance set out above for solid and liquid dosage forms.

Gamma-polyglutamic acid (alternatively γ -polyglutamic acid or γ -PGA) is a polymer of glutamic acid, an amino acid, where the polymer backbone is formed by a peptide bond joining the amino group and carboxyl group in the amino acid side chain (at the γ -carbon). γ -PGA can be formed from the L isomer, the D isomer, or the DL racemate of glutamic acid. Any of these forms may be used, and two or more different forms may be used together in any proportion. The various isomeric forms of γ -PGA may be synthetic or derived from natural sources. γ -PGA is found, for example, in Japanese natto and in sea kelp. Whereas organisms usually only produce poly(amino acids) from the L isomer, certain bacterial enzymes that produce γ -PGA can produce polymers from either isomer or both isomers.

γ -PGA of various sizes and various polymer dispersities may be used. The polymer molecular weight of γ -PGA is generally at least about 5 kDa and at most about 1000 kDa. In some embodiments, the polymer molecular weight of γ -PGA is at least about 30 kDa, or at least about 35 kDa, or at least about 40 kDa. In some embodiments, the polymer molecular weight of γ -PGA is at most about 700 kDa, or at most about 500 kDa, or at most about 300 kDa, or at most about 200 kDa, or at most about 100 kDa. An acceptable polymer molecular weight range may be selected from any of the above

indicated polymer molecular weight values. In an embodiment, the polymer molecule weight is in the range of about 5 kDa to about 500 kDa. In an embodiment, the polymer molecule weight is in the range of about 50 kDa to about 100 kDa. In one embodiment, the polymer molecular weight is about 50 kDa. Polymer molecular weights are typically given as a number average molecular weight (M_n) based on a measurement by gel permeation chromatography (GPC). The above polymer masses are cited as M_n ; other measurement techniques can be used to determine, e.g., a mass (weight) average molecular weight (M_w), and the specification for any given polymer can be converted among the various polymer mass representations.

The amount of γ -PGA included in a solid dosage form is generally in the range of about 10 wt% to about 40 wt%. In some embodiments the amount is about 20 wt% or about 30 wt%. The amount used is generally based upon the desired molar ratio between zinc and polyglutamic acid monomer units, the mass of the zinc salt (accounting for the weight of the counterion), and the amount of excipients needed to provide an acceptable formulated dosage form. For example, the greater the amount of γ -PGA and zinc salt used, the lesser the amount of excipients that can be added for a given overall dosage form size. Those of skill in the art can readily balance the amount of active ingredients versus the amount and type of excipients needed to obtain stable dosage forms. The desired ratio between zinc and γ -PGA can also be expressed as a ratio of milligrams of zinc to wt% of γ -PGA per dosage form. Exemplary ratios include 5 mg:10 wt%; 5 mg: 20 wt%; 5 mg: 40 wt%; 30 mg:10 wt%; 30 mg: 20 wt%; 30 mg: 40 wt%; or even 100

mg:10 wt%; 100 mg: 20 wt%; 100 mg: 40 wt%; or any other sets of values apparent from the values cited for each ingredient.

The amount of γ -PGA included in a liquid dosage form is generally in the range of about 0.01 wt% to about 10 wt%. In some embodiments the amount is about 0.1 wt% or about 1 wt%.

The amount used is generally based upon the desired molar ratio between zinc and polyglutamic acid monomer units, the nature of the carrier γ -PGA (that is whether it is unmodified, or modified with a tumor-targeting moieties and/or a charge-modifying moieties), and the degree of formation of Zn(II) complexes with the carrier γ -PGA. For example, as illustrated in Examples 1 and 2, ZnPGA complexes were obtained as solution comprising approximately 1 wt% PGA with approximately 400 $\mu\text{g/mL}$ (mg/L) of complexed zinc.

To arrive at suitable solid or liquid compositions and formulations having an effective amount of a Zn(II) salt and a γ -polyglutamic acid carrier, the relative amounts and the respective concentrations of γ -PGA and zinc can be adjusted readily by those of skill in the art in accordance with the disclosure.

γ -PGA may comprise tumor-targeting moieties. Such moieties may be selected from folic acid and RGD peptide. Either or both of said moieties may be covalently joined to γ -polyglutamic acid to form a folate conjugate and/or an RGD peptide conjugate of γ -PGA. Folate receptor protein is often expressed in many human tumors. Folates naturally have a high affinity for the folate receptors, and further, upon binding, the folate and the attached conjugate may be transported into the cell by endocytosis. In this way, a

ZnPGA modified with folic acid can target and accumulate at tumor cells and deliver zinc(II) to the inside of the tumor cells. Similarly, RGD peptides are known to bind strongly to $\alpha(V)\beta(3)$ integrins, which are expressed on tumoral endothelial cells as well as on some tumor cells. Thus RGD conjugates are a strategy for targeting and delivering antitumor agents to the site. As contemplated in this invention, γ -PGA may be conjugated (i.e., modified) with one or both of these tumor targeting agents. Other similar tumor targeting moieties are also within the scope of the invention.

γ -PGA has a free carboxylic acid group at the α -carbon of each glutamic acid unit that can be used to form a conjugate with folic acid and with RGD peptide. Folic acid has an exocyclic amine group that may be coupled with the α -carbon carboxylic acid group of glutamic acid to form an amide bond joining the two. RGD conjugates are also well-known in the art, and can also be similarly covalently joined to the α -carbon carboxylic acid group via, for example, the free α -amino group in RGD. Alternatively, either moiety may be conjugated to γ -PGA via a spacer group, such as, for example, polyethylene glycol amine. Examples of conjugation reactions to γ -PGA, including that of folic acid and citric acid, can be found in WO 2014/155142 (published Oct. 2, 2014).

γ -PGA may comprise charge-modifying moieties. Such moieties may be selected from citric acid, ethylenediamine tetraacetic acid, 1,4,7,10-tetracyclododecane-N,N',N'',N'''-tetraacetic acid, and diethylenetriamine pentaacetic acid. Any combination of said moieties may be covalently joined to γ -polyglutamic acid, again, at the α -carbon carboxylic acid. Citric acid may be conjugated to the α -carbon carboxylic acid group of γ -PGA by forming an

ester linkage. (See, e.g., WO 2014/155142.) EDTA, DOTA, and DTPA may be joined to γ -PGA using, for example, spacer groups to join the amines of these moieties to the α -carbon carboxylic acid group of γ -PGA. Numerous options are available to one of skill in the art. The charge-modifying moieties can be used as sites for chelating Zn(II) ions, and the charge-modification will also affect transport and solubility of the ZnPGA complexes and as such can be used to tune the pharmaceutical effects of the carrier and the ZnPGA complexes.

γ -PGA may comprise both tumor-targeting and charge-modifying moieties so that the benefits and functionality of both types of moieties may be imparted to the γ -PGA carrier.

Compositions and formulations according to the invention may also comprise an NF- κ B inhibitor. As used herein, an NF- κ B inhibitor includes direct inhibitors as well as compounds that can inhibit the signaling cascade, or any compound that suppresses the effect of NF- κ B and thereby limits the proliferation or survival of tumor cells. Exemplary compounds that may be used as an NF- κ B inhibitor as defined herein include pyrrolidine thiocarbamate (PDTC) (NPL4), telmisartan (NPL9), olmesartan (NPL1), valsartan (NPL8), disulfiram (NPL4), or pharmaceutically acceptable salts thereof. These inhibitors may also be referred to as sensitizers, because they limit the viability of tumor cells and thereby sensitize them to the effect of other tumoricidal agents, such as the compositions and formulations of the subject invention. Example 4 shows the tumoricidal

effects of co-administration of PDTC and a formulation according to one embodiment of the invention.

Liquid Formulations

The zinc(II) and γ -PGA active ingredients can be formulated as a liquid. Suitable liquid formulations include a liquid solution, a liquid suspension, a syrup, and an oral spray. The liquid solutions can be taken orally or administered by injection, such intravenously or subcutaneously, or directly into or in the vicinity of a tumor, whereas liquid suspensions, syrups and sprays are generally appropriate for oral administration.

Methods of Preparing Liquid Dosage Forms

In some embodiments, zinc salt(s) and a γ -PGA carrier (unmodified γ -PGA or any and all forms of modified γ -PGA, as described above) may be prepared as a ZnPGA complex. Generally, the zinc salt(s) and γ -PGA carrier are combined and purified as described, for example, in Examples 1 and 2. The solution of the obtained ZnPGA complex may be diluted or substantially dried and reconstituted in more concentrated form for use in the procedure for preparing a liquid dosage form.

The method for preparing liquid dosage forms involves mixing together the desired amounts of (i) a ZnPGA complex and/or (ii) zinc salt(s) and γ -PGA carrier, along with suitable excipients.

A liquid solution formulation may be prepared with suitable carriers, diluents, buffers, preservatives, or other excipients suitably selected with regard to the form of administration. For example, intravenous formulations may be prepared buffered at a suitable pH and with isotonicity agents.

Compositions according to an embodiment of the invention can be formulated as a liquid suspension for use in methods according to an embodiment. For example, first, granulated compositions comprising mixtures of a Zn(II) salt and a γ -PGA carrier (including unmodified and/or all modified forms of γ -PGA) are prepared with a gastro-resistant binder included in the granulated solid. (See discussion below regarding methods of prepared solid formulations.) The γ -PGA carrier is prepared from γ -PGA having an average molecular weight in the range of about 5 kDa to about 500 kDa. The granulated solid is then suspended in an acidic liquid suitable for ingestion. The pH of the solution may be less than about pH 6 so that the granulated solid remains stable as a result of the gastro-resistant binder. In one embodiment the liquid suspension formulation also contains a thickening agent or viscosity enhancer, such that the granulated solids can remain suspended sufficiently and be efficiently ingested from the container.

In another embodiment of a liquid suspension, the granulated solid is prepared by first preparing a ZnPGA complex, where Zn(II) is complexed with the γ -PGA carrier. Examples of such preparations are provided in Examples 1 and 2, for example. Thereafter the ZnPGA can be granulated with a gastro-resistant binder, and other suitable excipients. Then, this granulated mixture can be prepared as liquid suspension as described immediately above.

In either of these embodiments for a liquid suspension formulation, the γ -PGA carrier generally is present in a concentration of about 0.01 wt% to about 10 wt%, and the Zn(II) is generally present in a concentration of about 0.001 wt% to about 10 wt%.

Liquid dosage formulations may also be prepared to include NF- κ B inhibitors. In embodiments that do not include such NF- κ B inhibitors in the formulation, the NF- κ B inhibitor may be co-administered using any other suitable formulation and form of administration.

Solid Formulations

The zinc salt and γ -PGA carrier can be formulated into oral dosage forms such as a tablet, a capsule, or related forms such as a minitab, a caplet, and the like. The dosage form is further formulated to include a gastro-resistant binder and/or gastro-resistant coating.

The zinc salt and γ -PGA carrier are combined with excipients suitable for use in a supplement product and suitable for making a particular dosage form, such as a tablet or a capsule, and the like. Typical excipients include fillers, binders, disintegrants, glidants, lubricants, as well as buffers, preservatives, anti-oxidants, flavoring agents, sweeteners, coloring agents, and the like. The amount and type of excipient to be added can be selected for various purposes, such as improved integrity of the dosage form, improved bioavailability, stability, manufacturing, coating, appearance, and/or compliance. Some excipients may serve more than one purpose and/or provide more than one improved characteristic.

Fillers may be water soluble or water insoluble, and one or more of each type may be combined. Examples of water soluble fillers include, without limitation, sugars such as glucose, fructose, sucrose, mannose, dextrose, galactose, and the like, and sugar alcohols, such as mannitol, sorbitol, xylitol, and the like, as known in the art. Examples of water insoluble fillers include, without limitation, waxes, long-chain fatty acids, talc, kaolin,

silicon dioxide, titanium dioxide, alumina, starch, powdered cellulose, microcrystalline cellulose, and the like, as known in the art.

Binders include, without limitation, cellulose derivatives such as carboxymethylcellulose calcium, carboxymethylcellulose sodium, cellulose acetate phthalate, ethyl cellulose, hydroxyethyl cellulose, hydroxyethylmethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, methyl cellulose, microcrystalline cellulose, polyvinyl pyrrolidone, as well as starches, saccharides, gelatin, natural or synthetic gums, and the like, as known in the art.

As described above, in some embodiments, gastro-resistant binders may be included in the formulation as a binder that functions to delay the release of the zinc(II) salt and γ -PGA until the intestine. When a gastro-resistant binder is used, it may be used in combination with other (non-gastro-resistant) binders.

Disintegrants include, without limitation, carmellose, carmellose sodium, croscarmellose sodium, crospovidone, alginates, low substituted hydroxypropyl cellulose, hydroxypropyl starch, partially pregelatinized starch, and the like, as known in the art.

Glidants include, without limitation, silicas, silicates, talc, calcium phosphate, and the like, as known in the art.

Lubricants include, without limitation, alkali metal or alkaline earth metal stearates, oleates, benzoates, acetates, chlorides, and the like, as known in the art.

Other types of excipients, such as buffers, preservatives, anti-oxidants, flavoring agents, sweeteners, coloring agents, are well-known and persons of

ordinary skill in the art can readily select and apply such components to the formulations.

Solid dosage formulations may also be prepared to include NF- κ B inhibitors. In embodiments that do not include such NF- κ B inhibitors in the solid formulation, the NF- κ B inhibitor may be co-administered using any other suitable formulation and form of administration.

Other types of active ingredients, such as vitamins, minerals, nutrients, and other nutritional or dietary supplements that are amenable to absorption in the intestine may also be added to the compositions and formulations described herein without departing from the scope of the invention, unless stated otherwise.

The compositions and formulations described herein may alternatively comprise, consist of, or consist essentially of zinc salt(s) and γ -PGA carrier and a gastro-resistant outer coating and/or a gastro-resistant binder, so long as it is consistent with the specification. The compositions and formulations may also lack or be substantially free of any component(s), e.g. active ingredient and/or excipient found in a prior art composition or that are otherwise not necessary to the disclosed invention.

Methods of Preparing Solid Dosage Forms

The active ingredients, zinc salts and γ -PGA, and the selected excipients may be sized, declumped, or powderized individually or in combination. The various components may be combined by dry mixing, or granulated by wet or dry granulation, spray, extrusion, rolling, or fluidized bed granulation, and thereafter may optionally be milled, or other such techniques as known in the art.

In some embodiments, zinc salt(s) and a γ -PGA carrier (unmodified γ -PGA or any and all forms of modified γ -PGA, as described above) may be prepared as a ZnPGA complex. Generally, the zinc salt(s) and γ -PGA carrier are combined and purified as described, for example, in Examples 1 and 2. For convenience, the solution of the obtained ZnPGA complex may be substantially dried and used as a dry or substantially powder in the procedure for prepared a solid dosage form.

The method for preparing solid dosage forms involves mixing together the desired amounts of (i) a ZnPGA complex and/or (ii) zinc salt(s) and γ -PGA carrier, and the excipients, which comprise one or more filler and/or one or more binder and/or one or more disintegrant and/or one or more lubricating agent and/or one or more glidant. When a granulating step is included, then any of the excipients may be added, in whole or in part, before, during, or after the granulating step. In some embodiments some or all of a lubricating agent are mixed in after a granulating step. In some of these embodiments, a glidant is also mixed in after the granulating step.

Where the granulation step involves using a solvent, such as water, or an organic solvent, or an aqueous organic solution to wet the blend of components as they are granulated, the resulting product is usually dried to remove residual solvent. Examples of organic solvents include ethanol and isopropanol, and the like, as known in the art. Preferably, substantially all of an organic solvent is removed in a drying step. When water is part of the solvent used in a granulation step, preferably no more than 10 wt%, or no more than 5 wt%, or no more than 2 wt% of the water is left after drying and proceeding to the next step.

The mixed or granulated solids may be formed into tablets by tableting the solids using compression, compaction, or molding. Thereafter, in some embodiments, the tablets are coated with a gastro-resistant coating, as described above. Generally, the gastro-resistant substance and, optionally, other excipients (e.g., plasticizer, emulsifier are dissolved or dispersed into an aqueous or organic solvent and then applied using any of numerous methods known in art, including spray coating, fluidized bed coating, pan coating, and the like. In some embodiments, the tablets are coated for purposes of appearance, mechanical stability, chemical stability, and the like, but without a gastro-resistant material included in the coating.

Alternatively, the mixed or granulated solids may be filled into a capsule or caplet, and enclosed inside. The term capsule includes soft capsules, hard capsules, gelcaps, vegetable capsules, and may be one-piece or two-piece capsules. Enterically-coated capsules are available (e.g., enteric capsule drug delivery technology), or the capsules may filled, enclosed, and then coated with the gastro-resistant coating by the methods mentioned above using a solution or dispersion of the substance, optionally with other excipients. In other embodiments, the mixed or granulated solids comprise a gastro-resistant binder material, and such solids can be loaded in capsules with or without an enteric coating.

The size and shape of either tablets or capsules is not particularly limited. It is expected that the desired dosage amounts of zinc salts and γ -PGA can be formulated into a tablet or capsule that is not unduly large.

Dosing and Administration

The dosage forms described herein may be administered to provide a therapeutically effective amount of zinc to achieve the desired biological response in a subject. A therapeutically effective amount means that the amount of zinc delivered to the patient in need of treatment through the combined effects of the Zn, the γ -PGA, and any modifications to the γ -PGA, the form of any ZnPGA complex, the presence or absence of an NF- κ B inhibitor, and/or the delivery efficiency of the dosage form, and the like, will achieve the desired biological response.

The desired biological response include the prevention of the onset or development of a tumor or cancer, the partial or total prevention, delay, or inhibition of the progression of a tumor or cancer, or the prevention, delay, or inhibition of the recurrence of a tumor or cancer in the subject, such as a mammal, such as in a human (also may be referred to as a patient).

All tumor types that are susceptible to PARP1-mediated necrosis are contemplated to be indications that can be treated according to the methods of treatment disclosed herein. Examples 4, 5 and 6 demonstrate the efficacy of treatments according to embodiments of the disclosed methods using embodiments of the disclosed compositions and pharmaceutical formulations. The results demonstrate effective treatments in mouse cancer cells and in human cancer cells *in vivo*, and in human subjects.

Achieving a therapeutically effective amount will depend on the formulation's characteristics, any will vary by gender, age, condition, and genetic makeup of each individual. Individuals with inadequate zinc due to, for example, genetic causes or other causes of malabsorption or severe

dietary restriction may require a different amount for therapeutic effect compared to those with generally adequate levels of zinc.

The subject is generally administered an amount of zinc from about 1 mg up to about 300 mg zinc per day. For example about 25 mg, or 50 mg, or 75 mg, or 100 mg, or 150 mg, or 200 mg zinc per day. Multiple dosage forms may be taken together or separately in the day. The oral dosage forms generally may be administered without regard to meal time. Treatment generally continues until the desired therapeutic effect is achieved. Low dosage levels of the compositions and formulations described herein may also be continued as a treatment according to an embodiment of the invention if a tumor regresses or is inhibiting, for the purpose of preventing, delaying, or inhibiting its recurrence, or used as a preventative treatment.

EXAMPLES

Example 1: Preparation and characterization of ZnPGA at pH 7.0 using phosphate-precipitation method for removing non-bound excess zinc.

To prepare ZnPGA, 55 mg PGA (50,000 Da molecular weight) was dissolved in 5 mL 10 mM MES buffer, pH 7.0, containing 10 mM ZnSO₄ at room temperature, and then sonicated while placed on ice for 10 minutes. Then, 0.5 mL 200 mM phosphate buffer, pH 7.0, was added to the solution to precipitate free zinc ions, and the mixture was filtered through a 0.2 µm syringe sterilization filter. The zinc content was measured using ICP-MS and by 4-(2-pyridylazo)-resorcinol assay. The final stock ZnPGA contained 1% (wt/vol) PGA and 400 µg/mL bound zinc ions. Stock ZnPGA solutions were prepared fresh on each day of administration.

Example 2: Preparation and characterization of ZnPGA at pH 7.0 using dialysis method for removing non-bound excess zinc.

To prepare ZnPGA, 55 mg PGA (50,000 Da molecular weight) was dissolved in 5 mL 10 mM MES buffer, pH 7.0, containing 10 mM ZnSO₄ at room temperature, and then sonicated while placed on ice for 10 minutes. Then, the solution was dialyzed on ice against 1L 10 mM MES, pH 7.0, for 2 hours, successively three times, for a total of 3 volumes over 6 hours. The recovered solution was filtered through a 0.2 µm syringe sterilization filter. The zinc content was measured using ICP-MS and by 4-(2-pyridylazo)-resorcinol assay. The final stock ZnPGA contained 0.9% (wt/vol) PGA and 380 µg/mL bound zinc ions. Stock ZnPGA solutions were prepared fresh on each day of administration.

Example 3: *In vitro* flow cytometric analysis (FACS) of ZnPGA-induced cell death in human cancer cells with different drug resistance genotypes.

The mode of cell death induced by ZnPGA, whether apoptosis or necrosis, was examined in three human cancer cell lines with different drug resistance genotypes: H460 lung cancer (WT p53 apoptosis gene with no reported drug resistance), T98G neuroblastoma (mutated p53 and multidrug resistance protein 1 "MRP1" expression), and MES-SA Dx5 sarcoma (WT p53 and P-glycoprotein "PgP" multidrug resistance protein expression). Briefly, each cell line was prepared into 10,000 monolayer adherent state in late exponential growth phase per ATCC-suggested methods and media (RPMI-1640, F-12K, McCoy's 5A, EMEM, DMEM, etc), supplemented with 10% fetal

bovine serum (FBS) and 1% penicillin/streptomycin in a CO₂ incubator at 37 °C and 5% CO₂ using 96-well plates with a medium volume of 200 µL per well. The prepared cells on the 96-well plates were treated with different concentrations of ZnPGA for 24 hours, followed by FACS characterization of the cell state.

Briefly, cells in each well of 96-well plates were harvested into microcentrifuge tubes and washed in 100 µL cold phosphate-buffered saline (PBS), pH 7.4. Next, each sample was centrifuged and resuspended in cold 100 µL binding buffer (10 mM HEPES, 140 mM NaCl, 2 mM CaCl₂ at pH 7.4). For staining, 5 µL AlexaFluor 488® annexin V (Annexin V: Cat#A13200, Invitrogen) and 5 µL of 100 µg/mL propidium iodide (PI) in binding buffer were added to each for staining at room temperature for 15 min. At the end of the incubation, the samples were placed on ice after adding 400 µL binding buffer to each sample until immediate FACS reading. FACS was performed with an excitation wavelength at 488 nm and reading the absorbance signals at 530 nm and 575 nm, at a 100 µL/min flow-rate.

The results are shown in Figure 2, which shows *in vitro* cell-state quadrant analyses on the Annexin V and PI binding to the treated cells. The data demonstrated that ZnPGA dose-dependently and consistently induced necrotic cell death in all three cell lines with three different drug resistance genotypes after the 24 hour exposure. In Figure 2, the upper panels show results from the treatment of non-resistant H460 human lung cancer cells (WT p53 and no drug resistance protein expression), the middle panels show a multidrug resistant MES-SA Dx5 human sarcoma (WT p53 and PgP multidrug resistance protein expression), and the lower panels show a multidrug

resistant T98G human neuroblastoma (mutated p53 and MRP1 multidrug resistance protein expression). The dose of ZnPGA increases across each row in the figure.

Example 4: *In vivo* growth-inhibition effect of orally supplemented ZnPGA against LL2 murine lung cancer in the lungs of immunocompetent C57BL allograft model.

A monodisperse suspension of murine lewis lung carcinoma (LL2) cells were obtained by trypsinization of its *in vitro* culture and prepared in cold PBS at 2×10^5 cell/mL. A 0.5 mL suspension of LL2 cells was injected through the tail veins of C57BL/6 female mice, and the injected mice were sacrificed 16 days later for observation of pulmonary tumor growth. Oral drug treatment was started the day after tumor injection via dilution into the drinking water at the indicated doses. The animals were sacrificed 16 days after the cancer injection, and their lungs were observed for the growth of LL2 tumors.

Figure 3 shows that doses of 160 $\mu\text{g/mL}$ zinc in drinking water via ZnPGA over 15 days of treatment led to marked reduction of visibly solid LL2 tumor growth. Furthermore, supplementing the ZnPGA solution with the NF- κ B inhibitor PDTC virtually eliminated the LL2 tumor growth in the orthotopic allograft model of murine lung cancer, suggesting a particular synergy between the NF- κ B inhibitor PDTC and ZnPGA in their antitumor effects.

Example 5: *In vivo* growth-inhibition effect of orally supplemented ZnPGA against H460 human lung cancer in immuno-incompetent athymic Nu/Nu female mice subcutaneous xenograft model.

H460 single-cell suspension was prepared by trypsinization of its *in vitro* culture at logarithmic growth phase and prepared in serum-free cold RPMI-16040 medium at 10^7 cells/mL. Subcutaneous xenografts of the human tumor on immuno-incompetent athymic Nu/Nu female mice were created by subcutaneously injecting 0.1 mL of the H460 cell suspension in the skin near the right flank of the mice. Oral drug treatment was started on the day following tumor injection. One group of three subjects received saline in drinking water, a second group of three subjects received cisplatin (5 mg/kg) intraperitoneally once weekly, and a third group of three subjects received ZnPGA (160 µg/mL zinc) via drinking water. Starting 14 day post-injection, the long and short dimensions of palpable tumor mass (length and width, respectively) were measured every two days using a digital caliper. The experiment continued for 28 days. The tumor volume was obtained by the formula, $V = \text{length} \times \text{width}^2 \times 1/2$.

Figures 4 shows the results of the experiment. Consistent with the *in vitro* and the allograft orthotopic LL2 lung cancer model study results in C57BL mice of Examples 3 and 4, respectively, administering 160 µg/mL zinc in drinking water via ZnPGA led to marked inhibitory effects on the growth of subcutaneously xenografted H460 human lung cancer. Importantly, the tumor-suppressive effect of the orally supplemented ZnPGA was similar or better than those of the intraperitoneally injected cisplatin.

The foregoing invention having been described in detail and by way of example and illustration, those of skill in the art will appreciate the range of compositions and methods disclosed herein and embraced by the claims.

Example 6: Clinical observations of patients receiving granularized mixture of zinc(II) salt and γ -polyglutamic acid.

Oral administration of a supplement-grade enteric-release coated tablet formulation made from granulized zinc sulfate and γ -polyglutamic acid mixture lead to (1) clinical regression of third drug-refractory early gastric cancer in a female patient with two previous histories of cancer, and (2) clinical regression of first primary early gastric cancer in a male patient without previous treatment or disease history.

CLAIMS

I claim:

1. A method of inducing PARP1-mediated tumor necrosis in a tumor in a patient, the method comprising administering a therapeutically effective amount of a Zn(II) salt and a γ -polyglutamic acid carrier to the patient with the tumor;

wherein said γ -polyglutamic acid carrier comprises γ -polyglutamic acid and/or a tumor-targeting γ -polyglutamic acid derivative and/or a charge-modified γ -polyglutamic acid derivative and/or a tumor-targeting charge-modified γ -polyglutamic acid derivative.

2. The method according to claim 1, wherein said tumor has a drug-resistant phenotype.

3. The method according to claim 2, wherein said drug-resistance phenotype is dysfunctional p53.

4. The method according to claim 2, wherein said drug-resistance phenotype is MDR1 overexpression.

5. The method according to claim 2, wherein said drug-resistance phenotype is MRP1 overexpression.

6. The method according to any one of claims 1-5, wherein said Zn(II) salt and said γ -polyglutamic acid carrier are administered in a therapeutic amount in combination with a therapeutic amount of an NF- κ B inhibitor.
7. The method according to any one of claims 1-6, wherein said therapeutic amount of said Zn(II) salt and said γ -polyglutamic acid carrier are administered together to said patient in a solid dosage form or in a liquid dosage form.
8. The method according to claim 7, wherein said solid dosage form is selected from a tablet, a minitab, a hard capsule, a soft capsule, a caplet, a gelcap, an oral disintegrating films, granules, pellets, a paste, and a powder sachet.
9. The method according to claim 7, wherein said liquid dosage form is selected from a liquid solution, a liquid suspension, a syrup, and an oral spray.
10. The method according to claim 7, wherein said administering step is selected from an oral administration, a topical administration, and an injection administration.
11. A pharmaceutical composition comprising (i) a pharmaceutically acceptable Zn(II) salt, (ii) γ -polyglutamic acid containing a tumor-targeting moiety and/or a charge-modifying moiety, and (iii) optionally further comprising γ -polyglutamic acid.

12. The pharmaceutical composition according to claim 11, wherein said tumor-targeting moiety is selected from folic acid and RGD peptide, and either or both of said moieties are covalently joined to γ -polyglutamic acid.

13. The pharmaceutical composition according to claim 11 or 12, wherein said charge-modifying moiety is selected from citric acid, ethylenediamine tetraacetic acid, 1,4,7,10-tetracyclododecane-N,N',N'',N'''-tetraacetic acid, and diethylenetriamine pentaacetic acid, and any combination of said moieties are covalently joined to γ -polyglutamic acid.

14. The pharmaceutical composition according to any of claims 11-13, further comprising γ -polyglutamic acid.

15. The pharmaceutical composition according to any one of claims 11-14, wherein a substantial portion of said Zn(II) salt is a bound complex of the Zn(II) ion with γ -polyglutamic acid and/or said tumor-targeting moiety and/or said charge-modifying moiety.

16. The pharmaceutical composition according to any one of claims 11-14, wherein (i) said Zn(II) salt and (ii) said γ -polyglutamic acid polymers are mixed together in a solid mixture.

17. The pharmaceutical composition according to any one of claims 11-16, wherein said composition further comprises an NF- κ B inhibitor.

18. The pharmaceutical composition according to any one of claims 11-17, wherein said composition is formulated as a solid dosage form.

19. The pharmaceutical composition according to claim 18, wherein said solid dosage form further comprises a gastro-resistant binder and/or a gastro-resistant outer coating.

20. The pharmaceutical composition according to any one of claims 11-17, wherein said composition is formulated as a liquid dosage form.

21. The pharmaceutical composition according to claim 20, wherein said liquid dosage form is a suspension of a pharmaceutical composition that further comprises a gastro-resistant material.

22. A method for treating a tumor in a patient, the method comprising administering a therapeutically effective amount of the pharmaceutical composition according to any one of claims 11-21 to the patient with the tumor.

23. The method according to claim 22, wherein said tumor has a drug-resistant phenotype selected from dysfunctional p53, MDR1 overexpression, and MRP1 overexpression.