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(54) Title: HYALURONIC ACID IN THE TREATMENT OF CANCER

(57) Abstract: The present invention relates to a composition and method comprising purified HA, a second anti-neoplastic agent and a pharmaceutically acceptable carrier, wherein the purified HA and the second anti-neoplastic agent are administered to a mammal having cancer in an amount effective to treat the cancer.

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HYALURONIC ACID IN THE TREATMENT OF CANCER

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FIELD OF THE INVENTION

The invention relates to hyaluronic acid and a second anti-neoplastic agent in the treatment of cancer.

10

BACKGROUND OF THE INVENTION

Hyaluronic acid (hereinafter, "HA") is a glycosaminoglycan with repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine that exists as a high molecular mass polymer (10^6 to 10^7 Da) in its native form (Laurent et al. FASEB J. 6:2397, 1992). HA is a major non-structural component of connective tissue and is important for maintaining extracellular matrix architecture and for promoting cell motility, adhesion and proliferation (Entwistle, J. Cell. Biochem. 61:569, 1996).

The effects of both low molecular mass HA ($\leq 5 \times 10^5$ Da) and high molecular mass HA ($> 5 \times 10^5$ Da) on normal cells has been studied extensively (McKee et al. J. Biol. Chem. 272:8013, 1997; Hodge-Dufour et al. J. Immunol. 159:2492, 1997; Rooney et al. Int. J. Cancer 60:632, 1995). However, little is known about the effects of HA on malignant cells. *In vitro*, HA (> 0.320 mg/ml) inhibited proliferation of B16F10 murine melanoma cells by 50 to 90%. *In vivo*, HA (1 mg/ml), administered over 7 days by an Alzet osmotic pump into the immediate vicinity of a B16F10 murine melanoma tumor, reduced tumor volume $> 85\%$. *In vivo*, HA (> 750 mg/kg) administered with various other therapeutic agents over various periods of time, reduced or eliminated rectal, gastric, breast, prostate and endometrial cancers (PCT/CA/00283). *In vivo*, hyaluronan (HA) (7.5 mg/kg), administered with 2.5 mg/kg of the lipophilic, tubulin-stabilizing, chemotherapeutic drug paclitaxel (TAXOL[®]), decreased tumor mass of colon 26-cells seeded into BALB/c mice. It was proposed that the water-insoluble paclitaxel binds to hydrophobic patches on HA and that the HA binds to HA receptors on the surface of malignant cells and, thereby,

inhibit proliferation and induce apoptosis in cancer cells. As the HA and the toxic chemotherapeutic drug potentiate each other's effect on cancer cells, the standard dose of the toxic chemotherapeutic drug can be reduced without compromising the therapeutic effectiveness of the cancer treatment. Moreover, as HA is inexpensive and as most chemotherapeutic drugs are expensive, the combined use of HA and a chemotherapeutic drug can reduce significantly the cost of cancer treatment. The increase in dose effectiveness, decrease in toxicity and decrease in cost address long felt unfulfilled needs in the medical arts and provide important benefits for mammals, including humans.

10 Accordingly, it is desirable that the present invention provides a composition and method effective to treat cancer in a mammal, including a human.

It is also desirable that the present invention provides a composition and method that reduces the toxic side-effects of cancer treatments.

15 It is also desirable that the present invention provides a composition and method that reduces the cost of cancer treatments.

It is also desirable that the present invention provides a composition and method, wherein two or more anti-neoplastic agents act synergistically on cancer cells.

It is also desirable that the present invention provides a composition and method that inhibits proliferation of cancer cells.

20 It is also desirable that the present invention provides a composition and method that induces apoptosis in cancer cells.

It is also desirable that the present invention provides a composition and method that potentiates the effect of chemotherapeutic drugs on cancer cells.

25 It is also desirable that the present invention provides a composition and method that potentiates the effect of anti-neoplastic nucleic acids on cancer cells.

It is also desirable that the present invention provides a composition and method that potentiates the effect of anti-neoplastic bacterial DNAs on cancer cells.

30 It is also desirable that the present invention provides a composition and method that potentiates the effects of anti-neoplastic bacterial DNA-bacterial cell wall complexes on cancer cells.

It is also desirable that the present invention provides a composition and method that potentiates the effect of anti-neoplastic bacterial cell wall extracts on cancer cells.

35 It is also desirable that the present invention provides a a composition and method that potentiates the effect of anti-neoplastic synthetic oligonucleotides on cancer cells.

It is also desirable that the present invention provides a composition and method that stimulates the production of cytokines by immune system cells.

It is also desirable that the present invention provides a composition and method that stimulates the production of IL-6 by immune system cells.

5 It is also desirable that the present invention provides a composition and method that stimulates the production of IL-12 by immune system cells.

According to one aspect, the present invention provides a composition comprising purified HA, a second anti-neoplastic agent and a pharmaceutically acceptable carrier,

10 wherein the second anti-neoplastic agent is selected from the group consisting of *Mycobacterium phlei* DNA (M-DNA), *Mycobacterium phlei* DNA (M-DNA)-*Mycobacterium phlei* cell wall complex (MCC), a synthetic oligonucleotide or a combination thereof,

wherein the synthetic oligonucleotide is SEQ ID NO:1 OR SEQ ID NO:2.

15 In another aspect, the present invention provides a composition comprising a purified HA, an anti-neoplastic agent, and a pharmaceutically acceptable carrier or diluent, wherein said anti-neoplastic agent comprises *Mycobacterium phlei* DNA (M-DNA), *Mycobacterium phlei* DNA (M-DNA)-*Mycobacterium phlei* cell wall complex (MCC) a synthetic oligonucleotide, or a combination thereof.

20 In yet a further aspect, the present invention provides a use of HA and an anti-neoplastic agent in the manufacture of a medicament used to ameliorate or treat a neoplastic growth wherein said anti-neoplastic agent comprises *Mycobacterium phlei* DNA (M-DNA), *Mycobacterium phlei* DNA (M-DNA)-*Mycobacterium phlei* cell wall complex (MCC), a synthetic oligonucleotide, or a combination thereof

25 wherein the synthetic oligonucleotide is SEQ ID NO:1 OR SEQ ID NO:2.

In yet a further aspect, the present invention provides a method comprising administering a composition comprising purified hyaluronic acid, a second anti-neoplastic agent and a pharmaceutically acceptable carrier to a mammal having cancer in an amount effective to treat the cancer,

30 wherein the second anti-neoplastic agent is *Mycobacterium phlei* DNA (M-DNA), *Mycobacterium phlei* DNA (M-DNA)-*Mycobacterium phlei* cell wall complex (MCC), an oligonucleotide or a combination thereof, and

wherein the oligonucleotide is SEQ ID NO: 1 or SEQ ID NO: 2.

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These and other features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiment and the appended claims.

DETAILED DESCRIPTION OF THE INVENTION

- 5 The present invention provides a composition comprising purified HA, a second anti-neoplastic agent and a pharmaceutically acceptable carrier, wherein the HA and the second anti-neoplastic agent act synergistically to potentiate each other's effect on cancer cells. The present invention also provides a method, wherein a composition comprising purified HA, a second anti-neoplastic agent and a pharmaceutically acceptable carrier is administered to a mammal having cancer in an amount effective to
10 treat the cancer.

- As used herein, "hyaluronic acid (HA)" refers to hyaluronan, hyaluronate, salts of HA, homologues, analogues, derivatives, complexes, esters, fragments and subunits of HA that have been tested and found suitable for use in a mammal, including a
15 human.

 As used herein, "anti-neoplastic agent" refers to any agent that inhibits the growth or metastases of a cancer.

 As used herein, "chemotherapeutic drug" refers to any drug approved by a regulatory agency of a country or a state government or listed in the U. S.

- 20 Pharmacopocia or other generally recognized pharmacopoeia to treat cancer in a mammal, including a human.

 As used herein, "non-lipophilic" refers to a chemotherapeutic drug having greater than zero solubility in water.

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Another object is to provide a composition and method that potentiates the effect of anti-neoplastic synthetic oligonucleotides on cancer cells.

Another object is to provide a composition and method that stimulates the production of cytokines by immune system cells.

5 Another object is to provide a composition and method that stimulates the production of IL-6 by immune system cells.

Another object is to provide a composition and method that stimulates the production of IL-12 by immune system cells.

10 These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiment and the appended claims.

DETAILED DESCRIPTION OF THE INVENTION

15 The present invention provides a composition comprising purified HA, a second anti-neoplastic agent and a pharmaceutically acceptable carrier, wherein the HA and the second anti-neoplastic agent act synergistically to potentiate each other's effect on cancer cells. The present invention also provides a method, wherein a composition comprising purified HA, a second anti-neoplastic agent and a pharmaceutically acceptable carrier is administered to a mammal having cancer in an amount effective to treat the cancer.

As used herein, "hyaluronic acid (HA)" refers to hyaluronan, hyaluronate, salts of HA, homologues, analogues, derivatives, complexes, esters, fragments and subunits of HA that have been tested and found suitable for use in a mammal, including a human.

25 As used herein, "anti-neoplastic agent" refers to any agent that inhibits the growth or metastases of a cancer.

As used herein, "chemotherapeutic drug" refers to any drug approved by a regulatory agency of a country or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia to treat cancer in a mammal, including a human.

30 As used herein, "non-lipophilic" refers to a chemotherapeutic drug having greater than zero solubility in water.

As used herein, "standard dose" refers to the dose or dose range suggested in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia to treat cancer in a mammal, including a human.

As used herein, "synergism" refers to the coordinated action of two or more agents on the growth or metastases of a cancer.

As used herein, "potentiates" refers to a degree of anti-cancer activity that is greater than additive.

As used herein "toxic" refers to the adverse side-effects of an anti-neoplastic agent as included in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia.

HA is highly viscous, highly electronegative and highly hydrophilic. Various methods for the isolation, purification and fractionation of HA are known to those skilled in the art. In addition, molecular mass fractions of purified HA can be purchased from commercial sources including, but not limited to, Fluka Chemical Corporation (Ronkonkoma, NY, USA), Genzyme Corporation (Cambridge, MA, USA), Lifecore Inc. (Chaska, MN, USA), Hyal Pharmaceutical Corporation (Mississauga, Ontario, Canada) and Bioniche Life Sciences, Inc. (Belleville, Ontario, Canada).

Anti-neoplastic agents include, but are not limited to, chemotherapeutic drugs, biologicals, immunostimulants, cytokines, antigens, antibodies, nucleic acids, synthetic oligonucleotides, vaccines, aptamers nucleic acids, antisense nucleic acids, immunomodulators, telomerase inhibitors, caspase activators, apoptosis inducers, cyclin inhibitors, CDK inhibitors, stable triple helix forming agents, genetically engineered, biologically engineered and chemically synthesized agents, agents that target cell death molecules for activation or inactivation, and combinations thereof.

Chemotherapeutic drugs include, but are not limited to, DNA-alkylating agents, DNA-cross-linking agents, antibiotic derivatives, topoisomerase inhibitors, tubulin stabilizers, tubulin destabilizers, antimetabolites, nitrogen mustard derivatives, steroids, hormone antagonists, protein kinase inhibitors, HMG-CoA inhibitors, metalloproteinase inhibitors, angiogenesis inhibitors, CDK inhibitors, cyclin inhibitors, caspase inhibitors, RNA, antisense RNA, DNA, antisense DNA, bacterial extracts, bacterial DNA, bacterial DNA-bacterial cell wall complexes, synthetic

oligonucleotides, molecular biologically modified viral and bacterial components, and combinations thereof.

Pharmaceutically acceptable carriers include liquid carriers, solid carriers, or both. Liquid carriers include, but are not limited to, water, saline, physiologically acceptable buffers, aqueous suspensions, oil emulsions, water in oil emulsions, water-in-oil-in-water emulsions, site-specific emulsions, long-residence emulsions, sticky-emulsions, microemulsions and nanoemulsions. Preferred aqueous carriers include, but are not limited to, water, saline and physiologically acceptable buffers. Preferred non-aqueous carriers include, but are not limited to, a mineral oil or a neutral oil including, but not limited to, a diglyceride, a triglyceride, a phospholipid, a lipid, an oil and mixtures thereof. Solid carriers are biological carriers, chemical carriers, or both and include, but are not limited to, particles, microparticles, nanoparticles, microspheres, nanospheres, minipumps, bacterial cell wall extracts, and biodegradable or non-biodegradable natural or synthetic polymers that allow for sustained release of the composition (Brem et al. J. Neurosurg. 74: 441, 1991).

Cancers include, but are not limited to, squamous cell carcinoma, fibrosarcoma, sarcoid carcinoma, melanoma, mammary cancer, lung cancer, colorectal cancer, renal cancer, osteosarcoma, cutaneous melanoma, basal cell carcinoma, pancreatic cancer, bladder cancer, brain cancer, ovarian cancer, prostate cancer, leukemia, lymphoma and metastases derived therefrom.

Preferably, the molecular mass of the HA used is between about 1×10^3 and 1×10^7 Da, more preferably between about 5×10^4 and 1×10^6 Da, and most preferably between about 1×10^4 and 8×10^5 Da. Preferably, the amount of HA administered per dose is from about 0.001 to 25 mg/kg, more preferably from about 0.01 to 15 mg/kg, and most preferably from about 0.1 to 10 mg/kg. The amount of anti-neoplastic agent administered per dose depends on the anti-neoplastic agent used and is preferably about 5 to 75% of the standard dose, more preferably from about 5 to 50% of the standard dose, and most preferably from about 5 to 10% of the standard dose.

Routes of administration include, but are not limited to, oral, topical, subcutaneous, transdermal, subdermal, intra-muscular, intra-peritoneal, intra-vesical, intra-articular, intra-arterial, intra-venous, intra-dermal, intra-cranial, intra-lesional,

intra-tumoral, intra-ocular, intra-pulmonary, intra-spinal, placement within cavities of the body, nasal inhalation, pulmonary inhalation, impression into skin, electrocorporation, osmotic minipumps, and through a cannula to the site of interest.

Depending on the route of administration, the volume per dose is preferably
5 about 0.001 to 100 ml per dose, more preferably about 0.01 to 50 ml per dose, and most preferably about 0.1 to 30 ml per dose. The dose can be administered in a single treatment or in multiple treatments on a schedule and over a period of time appropriate to the cancer being treated, the condition of the recipient, and the route of administration. Moreover, the HA can be administered before, at the same time as, or
10 after administration of the anti-neoplastic agent as long as both are administered within a 24 hour time period.

In an example, 100 mg of $<1.5 \times 10^4$ Da HA + 4 mg/kg of the antimetabolite fluorinated pyrimidine 5-fluorouracil (hereinafter, "5-FU"; standard dose 12mg/kg) are administered intravenously to a mammal having cancer in a number of doses and
15 over a period of time effective to treat the cancer. In another example, 100 mg of $5.0-7.5 \times 10^5$ Da HA + 2 mg/kg of 5-FU are administered intratumorally to a mammal having a cancer in a number of doses and over a period of time effective to treat the tumor. In another example, 100 mg of $5.0-7.5 \times 10^5$ Da HA + 1.2 mg/kg of 5-FU are administered intratumorally to a mammal having a cancer in a number of doses and
20 over a period of time effective to treat the tumor. In another example, 100 mg of $1-3 \times 10^5$ Da HA + 10 mg/m² of the alkylating agent cisplatin (PLATINOL®; hereinafter, "CIS"; standard dose 100 mg/m²) is administered intravenously to a mammal having cancer in a number of doses and over a period of time effective to treat the cancer. In another example, 100 mg of $3-5 \times 10^5$ Da HA + 36 mg/m² of the DNA cross-linker carboplatin (PARAPLATIN®; standard dose 360 mg/m²) is administered
25 intravenously to a mammal having a cancer in a number of doses and over a period of time effective to treat the cancer.

The amount of HA per dose, the particular second anti-neoplastic agent used, the amount of the second anti-neoplastic agent per dose, the dose schedule and the
30 route of administration should be decided by the practitioner using methods known to those skilled in the art and will depend on the type of cancer, the severity of the cancer, the location of the cancer and other clinical factors such as the size, weight

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and physical condition of the recipient. In addition, *in vitro* assays may be employed to help identify optimal ranges for HA + anti-neoplastic agent administration.

The following examples will serve to further illustrate the present invention without, at the same time, however, constituting any limitation thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

10

Example 1

Cells

All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and were cultured in the medium recommended by the ATCC.

15

Table 1 shows the cell lines, their origins and their properties.

Table 1

Cell lines

CELL LINE	ORIGIN	PROPERTIES
MCF-7	Human breast cancer	Caspase 3-negative; estrogen-dependent
PC-3	Human prostate cancer	p53 mutated; androgen-independent (hormone refractory)
LNCaP	Human prostate cancer	TGF-beta 1 receptor-negative; androgen-dependent
Du-145	Human prostate cancer	Fas-resistant; Rb-mutated; p53 mutated; androgen independent
T-24	Human bladder cancer	p53 mutated
RT-4	Human bladder cancer	N.D.
UMUC-3	Human bladder cancer	P-glycoprotein over-expression
HT-1376	Human bladder cancer	p53 and p21 (waf-1) mutated
HT-1080	Human fibrosarcoma	N.D.
B-16F1	Murine melanoma	N.D.

Example 2

20

Reagents

HA, purified from *Streptococcus sp.*, was obtained from Lifecore Inc. (Chaska, MN, USA) and was dissolved in sterile saline at 0.8 mg/ml (CYSTISTAT[®],

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Bioniche Life Sciences Inc., Belleville, Ontario, Canada) or at 10.0 mg/ml (SUPLASYN®, Bioniche Life Sciences Inc., Bellville, Ontario, Canada).

Mycobacterium phlei-DNA (hereinafter, "M-DNA") and M-DNA-*Mycobacterium phlei* cell wall complex (hereinafter, "MCC") were prepared as in US
5 Application No. 09,129,312 (incorporated by reference herein).

Example 3

Preparation of HA of $\leq 5.0 \times 10^5$ Da

HA of $\leq 5.0 \times 10^5$ Da was prepared from HA of $5.0-7.5 \times 10^5$ Da by digestion with hyaluronidase type IV-S derived from bovine testes (Sigma-Aldrich Canada,
10 Oakville, Ontario, Canada) for 60 minutes at 37°C, by sonication on ice (Branson Sonifier Model 450, Danbury, CT, USA) for 20 minutes at maximal intensity, or by autoclaving (Amsco-Steris International, Model 2002, Mentor, OH, USA) for 30 minutes.

The HA obtained was electrophoresed in 0.5% agarose gels prepared in TAE
15 buffer (40 mM Tris, 20 mM acetic acid and 2.0 mM EDTA, pH 7.9) for 3 hours at 100 V (Lee et al. Anal. Biochem. 219:278, 1994). The molecular mass distribution of the HA was visualized using 0.005% of the cationic dye Stains-All (1-Ethyl-2-[3-(1-ethylnaphthol[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphthol[1,2-d]thiazolium
Bromide; Sigma-Aldrich, Oakville, Ontario, Canada) and the gel photo was scanned
20 using 1D software (Advance American Biotechnology, Fullerton, CA, USA). The molecular mass of HA was $< 1.5 \times 10^4$ Da after hyaluronidase, about $1.0-3.0 \times 10^5$ Da after sonication, and about $3.0-5.0 \times 10^5$ Da after autoclaving.

Example 4

Measurement of cell proliferation

25 Cell proliferation was measured using dimethylthiazoldiphenyltetrazolium (MTT) reduction (Mosman et al. J. Immunol. Methods 65:55, 1983). Unless otherwise stated, 100 µl of 5 mg of MTT (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 1 ml of PBS was added into each well. After 4 h, medium was removed from each well, 1.0 ml of acid-isopropanol (0.04 N HCl in isopropanol) was added
30 and reduced MTT was solubilized by mixing. Absorbency of the reaction product was measured at a wavelength of 570 nm using a multiplate spectrophotometer reader (Elx800 Model, Bio-TEK Instruments Inc., Winooski, Vermont, USA).

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Example 5

Inhibition of cell proliferation with HA

Unless stated otherwise 1.0×10^5 cells/ml were seeded in 6-well flat-bottom tissue culture plates and were maintained for 48 h at 37°C in a 5% CO_2 atmosphere.

5 PC-3, LNCaP and Du-145 human prostate cancer cells; MCF-7 human breast cancer cells; HT-1080 human fibrosarcoma cancer cells; B16F1 murine melanoma cells; and, UMUC-3, RT-4, HT-1376 and T-24 human bladder cancer cells were incubated with 0.8, 8.0 and 80 $\mu\text{g/ml}$ of $5.0\text{--}7.5 \times 10^5$ Da HA (Table 2).

Table 2

10 Inhibition of cell proliferation with $0.5\text{--}7.5 \times 10^5$ Da HA

CELLS	% INHIBITION		
	0.8 $\mu\text{g/ml}$	8.0 $\mu\text{g/ml}$	80.0 $\mu\text{g/ml}$
PC-3	15	26	30
LNCaP	7	13	26
Du-145	6	15	38
MCF-7	19	24	35
HT-1080	4	4	36
B16F1	20	20	30
UMUC-3	10	10	2
RT-4	12	13	5
HT-1376	0	3	1
T-24	10	8	0

As shown in Table 2, inhibition of proliferation of PC-3, LNCaP, Du-14, MCF-7, HT-1080 and B16F1 cancer cells increased with increasing concentrations of HA, whereas inhibition of proliferation of UMUC-3, RT-4, HT-1376 and T-24 cancer cells did not
 15 increase with increasing concentrations of HA.

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PC-3, LNCaP and Du-145 human prostate cancer cells were incubated with 8.0 µg/ml of $5.0-7.5 \times 10^5$ Da HA or of $<1.5 \times 10^4$ Da HA (Table 3).

Table 3

Inhibition of cell proliferation

CELLS	% INHIBITION	
	$5.0-7.5 \times 10^5$ Da HA 8.0 µg/ml	$<1.5 \times 10^4$ Da HA 8.0 µg/ml
PC-3	26	5
LNCaP	13	24
Du-145	15	37

5

PC-3 cancer cell proliferation was inhibited more by $5.0-7.5 \times 10^5$ Da HA, whereas LNCaP and Du-145 cancer cell proliferation was inhibited more by $<1.5 \times 10^4$ Da HA.

Example 6

10 *HA potentiation of the anti-neoplastic effect of M-DNA and MCC*

Unless stated otherwise 2.0×10^4 cells/ml were seeded in 24-well plates and were maintained for 48 h at 37° C in a 5% CO₂ atmosphere. MTT was used at 50 µl per well.

15 T-24 human bladder cancer cells, PC-3 and Du-145 human prostate cancer cells, and MCF-7 human breast cancer cell were incubated with saline or with 80.0 µg/ml of $5.0-7.5 \times 10^5$ Da HA + 1.0 µg/ml of M-DNA (Table 4).

Table 4

HA potentiation of M-DNA inhibition of cell proliferation

CELLS	% INHIBITION $5.0-7.5 \times 10^5$ Da HA	
	Saline	80.0 µg/ml
T-24 + saline	0	0
T-24+ M-DNA	28	36
PC-3 + saline	0	30
PC-3 + M-DNA	10	50
Du-145 + saline	0	38
Du-145+ M-DNA	11	58
MCF-7+ saline	0	35
MCF-7 + M-DNA	13	52

As shown in Table 4, HA potentiated the anti-neoplastic effect of M-DNA on T-24, PC-3, Du-145 and MCF-7 cancer cells.

HT-1376, RT-4 and T-24 human bladder cancer cells were incubated with 0.8 $\mu\text{g/ml}$ of $5.0\text{-}7.5 \times 10^5$ Da HA + 1.0 $\mu\text{g/ml}$ of MCC (Table 5).

5

Table 5

HA potentiation of MCC inhibition of cell proliferation

CELLS	% INHIBITION	
	Saline	$5.0\text{-}7.5 \times 10^5$ Da HA at 0.8 $\mu\text{g/ml}$
HT-1376 + saline	0	3
HT-1376 + MCC	21	34
RT-4 + saline	0	13
RT-4 + MCC	16	37
T-24 + saline	0	8
T-24 + MCC	31	45

As shown in Table 5, HA potentiated the anti-neoplastic effect of MCC on H-1376, RT-4 and T-24 cancer cells.

10

Example 7

HA potentiation of the anti-neoplastic effect of synthetic oligonucleotides

MCF-7 human breast cancer cells (2.5×10^5 cells/ml) were incubated as in Example 6 with 0.0, 0.01 and 0.1 $\mu\text{g/ml}$ of $5.0\text{-}7.5 \times 10^5$ Da HA + 100 $\mu\text{g/ml}$ of the synthetic 6 base oligonucleotide GG(GT)₁GG (SEQ ID NO:1) or + 100 $\mu\text{g/ml}$ of the synthetic 27 base oligonucleotide (GT)₁₃G (SEQ ID NO:2) (Table 6). MTT was used at 50 μl per well.

15

Table 6

HA potentiation of inhibition of synthetic oligonucleotide inhibition of cell proliferation

SEQUENCES 100 $\mu\text{g/ml}$	% INHIBITION $5.0\text{-}7.5 \times 10^5$ Da HA		
	0.0 $\mu\text{g/ml}$	0.01 $\mu\text{g/ml}$	0.1 $\mu\text{g/ml}$
	0	6	5
GG(GT) ₁ GG-(6 bases) - SEQ ID NO:1	20	34	33
(G ₁ T) ₁₃ G-(27 bases) - SEQ ID NO:2	44	51	59

20

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As shown in Table 6, 0.01 $\mu\text{g/ml}$ HA and 0.1 $\mu\text{g/ml}$ HA potentiated the anti-neoplastic activity of 6 base GG(GT)₁GG (SEQ ID NO:1) and 0.01 $\mu\text{g/ml}$ HA potentiated the antineoplastic activity of 27 base (G₁T)₁₃G (SEQ ID NO:2).

Example 8

5 *HA potentiation of the anti-neoplastic effect of chemotherapeutic drugs*

RT-4 human bladder cancer cells and MCF-7 human breast cancer cells were incubated as in Example 6 with 0.0, 0.008 or 0.08 $\mu\text{g/ml}$ of $5.0\text{--}7.5 \times 10^5$ Da HA + 0.1 $\mu\text{g/ml}$ of CIS, + 1.0 $\mu\text{g/ml}$ of 5-FU or + 10 $\mu\text{g/ml}$ of 5-FU (Table 7).

Table 7

10 HA potentiation of chemotherapeutic drug inhibition of cell proliferation

CELLS	% INHIBITION 5.0-7.5 x 10 ⁵ Da HA		
	Saline	0.008 $\mu\text{g/ml}$	0.08 $\mu\text{g/ml}$
RT-4 + saline	0	0	0
RT-4 + CIS at 0.1 $\mu\text{g/ml}$	2	13	15
RT-4 + 5-FU at 1.0 $\mu\text{g/ml}$	14	18	19
MCF-7 + saline	0	12	12
MCF-7 + 5-FU at 10 $\mu\text{g/ml}$	18	32	31

As shown in Table 7, 0.008 $\mu\text{g/ml}$ and 0.08 $\mu\text{g/ml}$ HA potentiated the anti-neoplastic effect of 0.1 $\mu\text{g/ml}$ CIS and of 1.0 $\mu\text{g/ml}$ 5-FU on RT-4 cancer cells and the anti-neoplastic effect of 10 $\mu\text{g/ml}$ of 5-FU on MCF-7 cancer cells.

15 Example 9

CD44 cell surface receptors and HA inhibition of proliferation

CD44 is a cell surface HA receptor that has multiple variants (Screaton et al. Proc. Natl. Acad. Sci. USA, 89:12160, 1992). CD44 variants are selectively expressed in human tumors and are over-expressed on numerous tumor cell lines (Naot et al. Adv. Cancer Res. 71:241, 1997). It has been suggested that CD44 receptors on cancer cells enables HA to deliver effective amounts of the highly lipophilic chemotherapeutic drug paclitaxel (TAXOL®) into cancer cells at low paclitaxel dosage amounts because the paclitaxel binds to the HA that, in turn, binds to the CD44 receptors (PCT/CA98/00660).

25 To determine if HA binding to CD44 receptors correlates with inhibition of cell proliferation, CD44 receptor expression was detected by flow cytometry (FCM)

using the fluorescent anti-HA receptor monoclonal antibody FITC-CD44 (clone G44-26 (C26), Pharmingen, Mississauga, Ontario, Canada). Briefly, cells were pelleted by centrifugation at 180 x G for 5 min at RT, washed twice in PBS and incubated with FITC-CD44 at the concentration recommended by the manufacturer for 20 min at 4° C in the dark. The cells were then washed twice in PBS by centrifugation and cell fluorescence was measured at 488 nm excitation and 530 nm emission (FL1 detector). Data were analyzed on a FACSCALIBUR using the program CELLQUEST (Becton Dickinson, San Jose, CA, USA).

CD44 expression and the inhibition of proliferation by 5.0-7.5 x 10⁵ Da HA were measured using Jurkat T cell leukemia cells; MCF-7 human breast cancer cells; RT-4, T-24, HT-1376 and UMUC-3 human bladder cancer cells; and, PC-3, Du-145 and LNCaP prostate cancer cells (Table 8)

Table 8

CD44 cell surface expression and HA inhibition of cell proliferation

CELLS	CD44 expression in mean fluorescent units		5.0-7.5 x 10 ⁵ Da HA - 80 µg/ml
	Unstained cells	Cells + anti-CD44	% inhibition of proliferation
HT-1376	4	200	1
RT-4	4	60	5
T-24	3	1800	0
UMUC-3	3	1800	2
PC-3	6	400	30
Du-145	3	450	38
MCF-7	3	70	35
Jurkat T	3	3	0
LNCaP	6	6	26

As shown in Table 8, expression of CD-44 receptors by cancer cells did not correlate with inhibition of proliferation by HA. PC-3 cancer cells express CD44 receptors, whereas LNCaP cancer cells do not express CD44 receptors (Lokeshwar et al. *Anticancer Res.* 15:1191, 1995). However, as shown in Table 8, HA inhibition of proliferation of PC-3 cancer cells (30%) and of LNCaP cancer cells (26%) was not significantly different.

Example 10

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Induction of cytokine production

Peripheral blood mononuclear cells (hereinafter, "PBMCs") were isolated from the blood of 5 healthy humans by Ficoll-Hypaque (Amersham Pharmacia Biotech, Baie d'Urfée, Québec, Canada) density gradient centrifugation of whole blood. Stimulation of IL-6 and IL-12 production by the immune system cells was determined using the appropriate commercial ELISA (BioSource, Camarillo, CA, USA). Results are expressed as the "fold" (x) increases in cytokine production by treated cells compared to control cells.

PBMCs were incubated with 1 mg/ml of $5.0-7.5 \times 10^5$ Da and production of IL-6 and IL-12 was determined (Table 9).

Table 9
Cytokine production

Individual	IL-6	IL-12
1	3.8x	25.2x
2	1.8x	7.1x
3	4.1x	26.5x
4	1.4x	10.0x
5	6.3x	8.5x

As shown in the Table 9, HA stimulated both IL-6 and IL-12 production by immune system cells.

PBMCs, isolated from the blood of individual #1, were incubated with 0.008, 0.04, 0.2 and 1 mg/ml of $5.0-7.5 \times 10^5$ Da (Table 10)

Table 10
Cytokine production

$5.0-7.5 \times 10^5$ Da HA (mg/ml)	IL-6	IL-12
0.008	2.4x	4.9x
0.040	3.5x	13.8x
0.200	2.9x	8.5x
1.000	3.8x	25.2x

As shown in the Table 10, HA at 0.008, 0.04, 0.2 and 1 mg/ml, stimulated both IL-6 and IL-12 production by immune system cells.

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Example 11

Effect of saline, HA, M-DNA, MCC, HA+M-DNA and HA+MCC on PC3 tumors in mice

PC-3 human prostate cancer cells are implanted subcutaneously into 224 male nude BALB/c mice. The mice are divided into 28 groups of 8 mice (Table 11).

Table 11

Effect of saline, HA, M-DNA, MCC, HA+M-DNA and HA+MCC

	Saline	M-DNA or MCC 0.02 mg/kg	M-DNA or MCC 0.2 mg/kg	M-DNA or MCC 2.0 mg/kg
Saline	Group 1	Groups 5 and 17	Groups 9 and 21	Groups 13 and 25
HA at 16.0 mg/kg	Group 2	Groups 6 and 18	Groups 10 and 22	Groups 14 and 26
HA at 1.6 mg/kg	Group 3	Groups 7 and 19	Groups 11 and 23	Groups 15 and 27
HA at 0.16 mg/kg	Group 4	Groups 8 and 20	Groups 12 and 24	Groups 16 and 28

Saline, HA, M-DNA, MCC, HA+M-DNA and HA+MCC are administered intravenously on day 0 and at 3-day intervals for 4 weeks (10 injections) at which time the mice are sacrificed and tumor mass and number of metastases are determined. Groups 2 to 28 mice have less tumor mass and fewer metastases than Group 1 mice. Groups 6-8, 10-12, 14-16, 18-20, 22-24 and 26-28 have less tumor mass and fewer metastases than Groups 5, 9, 13, 17, 21 and 25 mice.

Example 12

Effect of saline, HA, M-DNA, MCC, HA+M-DNA and HA+MCC on LNCaP tumors in mice

LNCaP human prostate cancer cells (CD44 null) are implanted subcutaneously into 224 male nude BALB/c mice. The mice are divided into 28 groups of 8 mice (Table 12).

Table 12

Effect of saline, HA, M-DNA, MCC, HA+M-DNA and HA+MCC

	Saline	M-DNA or MCC 0.02 mg/kg	M-DNA or MCC 0.2 mg/kg	M-DNA or MCC 2.0 mg/kg
Saline	Group 1	Groups 5 and 17	Groups 9 and 21	Groups 13 and 25
HA at 16.0 mg/kg	Group 2	Groups 6 and 18	Groups 10 and 22	Groups 14 and 26
HA at 1.6 mg/kg	Group 3	Groups 7 and 19	Groups 11 and 23	Groups 15 and 27
HA at 0.16 mg/kg	Group 4	Groups 8 and 20	Groups 12 and 24	Groups 16 and 28

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Saline, HA, M-DNA, MCC, HA+M-DNA and HA+MCC are administered intravenously on day 0 and at 3-day intervals for 4 weeks (10 injections) at which time the mice are sacrificed and tumor mass and number of metastases are determined. Groups 2 to 28 mice have less tumor mass and fewer metastases than Group 1 mice. Groups 6-8, 10-12, 14-16, 18-20, 22-24 and 26-28 have less tumor mass and fewer metastases than Groups 5, 9, 13, 17, 21 and 25 mice. These results show that treatment outcome does not depend on CD44 expression by the cancer cells.

Example 13

Effect of saline, HA, M-DNA, MCC, HA+M-DNA and HA+MCC on B16F1 tumors in mice

B16F1 cancer cells are implanted intravenously into 224 female C57BL/6 mice. The mice are divided into 28 groups of 8 mice (Table 13).

Table 13

Effect of saline, HA, M-DNA, MCC, HA+M-DNA and HA+MCC on B16F1 tumors

	Saline	M-DNA or MCC 0.02 mg/kg	M-DNA or MCC 0.2 mg/kg	M-DNA or MCC 2.0 mg/kg
Saline	Group 1	Groups 5 and 17	Groups 9 and 21	Groups 13 and 25
HA at 16.0 mg/kg	Group 2	Groups 6 and 18	Groups 10 and 22	Groups 14 and 26
HA at 1.6 mg/kg	Group 3	Groups 7 and 19	Groups 11 and 23	Groups 15 and 27
HA at 0.16 mg/kg	Group 4	Groups 8 and 20	Groups 12 and 24	Groups 16 and 28

Saline, HA, M-DNA, MCC, HA+M-DNA and HA+MCC are administered intratumorally in saline on day 0 and at 3 day intervals for 4 weeks (10 injections) at which time the mice are sacrificed and tumor mass and number of metastases are determined. Groups 2 to 28 mice have less tumor mass and fewer metastases than Group 1 mice. Groups 6-8, 10-12, 14-16, 18-20, 22-24 and 26-28 have less tumor mass and fewer metastases than Groups 5, 9, 13, 17, 21 and 25 mice.

Example 14

Effect of saline, HA, CIS, 5-FU, HA+CIS and HA+5-FU on PC3 tumors in mice

PC-3 cancer cells are implanted subcutaneously into 224 male nude BALB/c mice. The mice are divided into 28 groups of 8 mice (Table 14).

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Table 14

Effect of saline, HA, CIS, 5-FU, HA+CIS and HA+5-FU on PC3 tumors

	Saline	CIS or 5-FU 0.1 mg/kg	CIS or 5-FU 1.0 mg/kg	CIS or 5-FU 10.0 mg/kg
Saline	Group 1	Groups 5 and 17	Groups 9 and 21	Group 13 and 25
HA at 16.0 mg/kg	Group 2	Groups 6 and 18	Groups 10 and 22	Group 14 and 26
HA at 1.6 mg/kg	Group 3	Groups 7 and 19	Groups 11 and 23	Group 15 and 27
HA at 0.16 mg/kg	Group 4	Groups 8 and 20	Groups 12 and 24	Groups 16 and 28

Saline, HA, CIS, 5-FU, HA+CIS and HA+5FU are administered intravenously in saline on day 0 and at 3 day intervals for 4 weeks (10 injections) at which time the mice are sacrificed and tumor mass and number of metastases are determined. Groups 2 to 28 mice have less tumor mass and fewer metastases than Group 1 mice. Groups 6-8, 10-12, 14-16, 18-20, 22-24 and 26-28 have less tumor mass and fewer metastases than Groups 5, 9, 13, 17, 21 and 25 mice.

10

Example 15

Effect of saline, HA, CIS, 5-FU, HA+CIS and HA+5-FU on LNCaP tumors in mice

LNCaP cancer cells (CD44 null) are implanted subcutaneously into 224 male nude BALB/c mice. The mice are divided into 28 groups of 8 mice (Table15).

Table 15

15 Effect of saline, HA, CIS, 5-FU, HA+CIS and HA+5-FU on LNCaP tumors

	Saline	CIS or 5-FU 0.1 mg/kg	CIS or 5-FU 1.0 mg/kg	CIS or 5-FU 10.0 mg/kg
Saline	Group 1	Groups 5 and 17	Groups 9 and 21	Group 13 and 25
HA at 16.0 mg/kg	Group 2	Groups 6 and 18	Groups 10 and 22	Group 14 and 26
HA at 1.6 mg/kg	Group 3	Groups 7 and 19	Groups 11 and 23	Group 15 and 27
HA at 0.16 mg/kg	Group 4	Groups 8 and 20	Groups 12 and 24	Groups 16 and 28

Saline, HA, CIS, 5-FU, HA+CIS and HA+5FU are administered intravenously in saline on day 0 and at 3 day intervals for 4 weeks (10 injections) at which time the mice are sacrificed and tumor mass and number of metastasis are determined. Groups 2 to 28 mice have less tumor mass and fewer metastases than Group 1 mice. Groups 6-8, 10-12, 14-16, 18-20, 22-24 and 26-28 have less tumor mass and fewer metastases

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than Groups 5, 9, 13, 17, 21 and 25 mice. These results show that treatment outcome does not depend on CD44 expression by the cancer cells.

Example 16

Effect of saline, HA, CIS, 5-FU, HA+CIS and HA+5-FU on B16F1 tumors in mice

5 B16F1 cancer cells are implanted subcutaneously into 224 female C57BL/6 mice. The mice are divided into 28 groups of 8 mice (Table 16).

Table 16

Effect of saline, HA, CIS, 5-FU, HA+CIS and HA+5-FU on B16F1 tumors

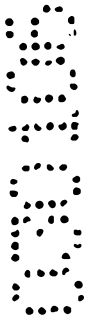
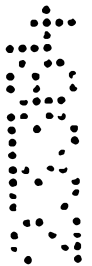
	Saline	CIS 0.01 mg/kg or 5-FU 1.0 mg/kg	CIS 0.1 mg/kg or 5-FU 10.0 mg/kg	CIS 1.0 mg/kg or 5-FU 100.0 mg/kg
Saline	Group 1	Groups 5 and 17	Groups 9 and 21	Group 13 and 25
HA-N at 16.0 mg/kg	Group 2	Groups 6 and 18	Groups 10 and 22	Group 14 and 26
HA-N at 1.6 mg/kg	Group 3	Groups 7 and 19	Groups 11 and 23	Group 15 and 27
HA-N at 0.16 mg/kg	Group 4	Groups 8 and 20	Groups 12 and 24	Groups 16 and 28

10 Saline, HA, CIS, 5-FU, HA+CIS and HA+5FU are administered intravenously in saline on day 0 and at 3 day intervals for 4 weeks (10 injections) at which time the mice are sacrificed and tumor mass and number of metastases are determined. Groups 2 to 28 mice have less tumor mass and fewer metastases than Group 1 mice.

15 While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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



EDITORIAL NOTE

APPLICATION NUMBER - 23364/01

The following Sequence Listing pages 1/1 to 1/1 are part of the description. The claims pages follow on pages 20 to 22 .

SEQUENCE LISTING

<110> Bioniche Life Sciences Inc.

Phillips, Nigel

Filicn, Mario

<120> Hyaluronic Acid in the Treatment of Cancer

<130> 6857-22

<150> US 60/173,375

<151> 1999-12-28

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gtgtgtgtgt gtgtgtgtgt gtgtgtg

27

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A composition comprising purified HA, a second anti-neoplastic agent and a pharmaceutically acceptable carrier,
wherein the second anti-neoplastic agent is selected from the group consisting of *Mycobacterium phlei* DNA (M-DNA), *Mycobacterium phlei* DNA (M-DNA)-*Mycobacterium phlei* cell wall complex (MCC), a synthetic oligonucleotide or combination thereof, and
wherein the synthetic oligonucleotide is SEQ ID NO:1 or SEQ ID NO:2.
2. The use of purified HA and an anti-neoplastic agent in the manufacture of a medicament used to ameliorate or treat a neoplastic growth wherein said anti-neoplastic agent comprises *Mycobacterium phlei* DNA (M-DNA), *Mycobacterium phlei* DNA (M-DNA)-*Mycobacterium phlei* cell wall complex (MCC), a synthetic oligonucleotide, or a combination thereof,
wherein the synthetic oligonucleotide is SEQ ID NO:1 or SEQ ID NO:2.
3. The use of claim 2, wherein the medicament stimulates immune system to produce cytokines.
4. The use of claim 3, wherein the cytokines are IL-6 or IL-12.
5. The composition of claim 1, wherein the purified hyaluronic acid is hyaluronan, hyaluronate, a salt of hyaluronic acid, a homologue, an analogue, a derivative, a complex, ester, fragment or subunit of hyaluronic acid that is suitable for use in a mammal.
6. The composition of claim 1, wherein the pharmaceutically acceptable carrier is a liquid carrier, a solid carrier or a combination thereof.
7. The composition of claim 6, wherein the liquid carrier is water, saline, a physiologically acceptable buffer, an aqueous suspension, an oil emulsion, a water-in-oil emulsion, a water-in-oil-in-water emulsion, a site-specific emulsion, a long residence emulsion, a sticky emulsion, a microemulsion, a nanoemulsion, a mineral oil, neutral oil or mixture thereof.

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8. The composition of claim 6, wherein the solid carrier is a chemical carrier, a biological carrier, a particle, a microparticle, a nanoparticle, a microsphere, a nanosphere, a minipump, a bacterial cell wall extract, a biodegradable or non-biodegradable natural or synthetic polymer that allow for sustained release of the composition, or a mixture thereof.
9. The composition of claim 1, wherein the purified hyaluronic acid has a molecular mass of between about 1×10^3 and 1×10^7 Daltons.
10. The composition of claim 1, wherein the purified hyaluronic acid has a molecular mass of between about 5×10^4 and 1×10^6 Daltons.
11. The composition of claim 1, wherein the purified hyaluronic acid has a molecular mass of between about 1×10^4 and 8×10^5 Daltons.
12. The use of claim 2, wherein the medicament is administered to treat cancer in a mammal, wherein the cancer is squamous cell carcinoma, fibrosarcoma, sarcoma, melanoma, mammary cancer, lung cancer, colorectal cancer, renal cancer, osteosarcoma, cutaneous melanoma, basal cell carcinoma, pancreatic cancer, bladder cancer, brain cancer, ovarian cancer, prostate cancer, leukemia or lymphoma.
13. The use of claim 12, wherein the administering is oral, topical, subcutaneous, transdermal, subdermal, intra-muscular, intra-peritoneal, intra-vesical, intra-articular, intra-arterial, intra-venous, intra-dermal, intra-cranial, intra-lesional, intra-tumoral, intra-ocular, intra-pulmonary, intra-spinal, nasal inhalation, pulmonary inhalation, impression into skin, electroporation, through an osmotic minipump, or through a cannula to the site of interest.
14. The use of claim 2, wherein the amount of purified HA administered per dose is about 0.001 to about 25 mg per kg body weight.
15. The use of claim 2, wherein the amount of purified HA administered per dose is about 0.01 to about 10 mg per kg body weight.

16. A method comprising administering a composition comprising purified hyaluronic acid, a second anti-neoplastic agent and a pharmaceutically acceptable carrier to a mammal having cancer in an amount effective to treat the cancer,
- 5 wherein the second anti-neoplastic agent is *Mycobacterium phlei* DNA (M-DNA), *Mycobacterium phlei* DNA (M-DNA)-*Mycobacterium phlei* cell wall complex (MCC), an oligonucleotide or a combination thereof, and
 wherein the oligonucleotide is SEQ ID NO: 1 or SEQ ID NO: 2.
- 10 17. A composition according to claim 1 substantially as hereinbefore described with reference to the examples and/or the preferred embodiments and excluding, if any, comparative examples.
- 15 18. A use according to claim 2 substantially as hereinbefore described with reference to the examples and/or the preferred embodiments and excluding, if any, comparative examples.
19. A method according to claim 16 substantially as hereinbefore described with reference to the examples and/or the preferred embodiments and excluding, if
20 any, comparative examples.

Dated this twenty-seventh day of January 2006

Bioniche Life Sciences Inc.

Patent Attorneys for the Applicant:

F B RICE & CO