Results of multicenter double-blind placebo-controlled phase II clinical trial of preparation Panagen to evaluate its leukostimulatory activity and formation of the adaptive immunity response in patients with stage II-IV breast cancer

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Abstract

Introduction: We performed a multicenter, double-blind, placebo-controlled, phase II clinical trial of human dsDNA-based preparation Panagen in tablet form. In total, 80 female patients with stage II-IV breast cancer were recruited.

Methods: Patients received three consecutive FAC (5-fluorouracil, doxorubicin and cyclophosphamide) or AC (doxorubicin and cyclophosphamide) adjuvant chemotherapies (3 weeks per course) and 5 mg Panagen or placebo tablets daily (6 tablets every 2-3 hours, 30 mg/day) for 18 days in each chemotherapy course. Statistical analysis was performed using Statistica 6.0 software, and non-parametric analyses, namely Wilcoxon-Mann-Whitney and paired Wilcoxon tests. To describe the results, the following parameters were used: number of observations (n), median, interquartile range, and minimum-maximum range.

Results: Panagen displayed pronounced leukostimulatory and leukoprotective effects when combined with chemotherapy. In an ancillary protocol, anticancer effects of a tablet form of Panagen were analyzed. We show that Panagen helps to maintain the pre-therapeutic activity level of innate antitumor immunity and induces formation of a peripheral pool of cytotoxic CD8+ perforin+ T-cells. Our 3-year follow-up analysis demonstrates that 24% of patients who received Panagen relapsed or died after the therapy, as compared to 45% in the placebo cohort.

Conclusions: The data collected in this trial set Panagen as a multi-faceted “all-in-one” medicine that is capable of simultaneously sustaining hematopoiesis and stimulating anticancer immune response. Its unique feature is that it is delivered through the gastrointestinal tract and acts through the lymphoid system of intestinal mucosa. This medication can also boost individual anticancer adaptive immunity resulting from the activation of dendritic cells by the circulating tumor cell debris.

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Introduction

Programmed chemotherapies involve tightly scheduled and dosed administration of highly toxic substances, whose therapeutic efficacy is invariably accompanied with systemic damage to the body. Liver and hematopoietic cells are the first to suffer from such therapies. Hence, when cancer patients are treated with cytostatic drugs, they invariably receive adjuvant medications alleviating the deleterious effects of cytostatics. Leukostimulatory drugs are among such protective agents.

Several classes of drugs are currently used to stimulate leukopoiesis. The first group includes the drugs boosting cellular metabolism – dicarbamin, methyluracil, pentoxyl, leukogen, etc. The second group comprises colony-stimulating growth factor analogs, such as filgrastim (neupogen), sagramostim, lenograstim, molgramostim (leucomax), etc. Chemical leukostimulatory drugs (dicarbamin and alike) are used in patients receiving myelosuppressive chemotherapy. In particular, dicarbamin stimulates maturation of neutrophilic granulocytes thereby reducing the occurrence of leukopenia and neutropenia. To treat severe leukopenia, analogs of human G-CSF, such as filgrastim and alike, are also widely used. These medications act by inducing mobilization of hematopoietic stem cells and by modulating production and release of neutrophils into peripheral blood. This panel of G-CSF-derived drugs is therefore used to treat various forms of neutropenia in cancer patients receiving myelosuppressive chemotherapy.

Recently, one more class of drugs which is based on nucleic acids has been introduced into oncology practice (ridostin, derinat, polydan, desoxynatum, etc), as these drugs were reported to display, among others, leukostimulatory activity. To further support the therapeutic
potential of Panagen preparation, one must consider a group of drugs that are based on CpG-
modified DNA oligonucleotides, – these agents are used to induce adaptive antiviral and
anticancer immune response. When tested in mice, these drugs resulted in 50-60% suppression of
tumor growth.

By exploiting these research observations on the therapeutic activity of nucleic acids, we
proceeded to develop Panagen medication, which is based on the fragmented human dsDNA, and
is intended for use as an adjuvant leukostimulatory agent in cancer patients receiving multiple
lines of chemotherapy. We put forward and tested a novel concept of treating stage II-IV breast
cancer by combining standard chemotherapy course with Panagen. This strategy allows
protecting and activating the proliferation of hematopoietic stem cells along with expansion of
the population of CD8+perforin+ cytotoxic T-cells, i.e. it aids in developing anticancer immune
response in these patients.

Thus, Panagen is a multi-faceted drug, which is primarily characterized with
leukostimulatory activity and which functions to stimulate adaptive immune response across
multiple courses of chemotherapy. In contrast to the above-mentioned classes of drugs (in
particular, G-CSF- and CpG-ODN- based), Panagen is manufactured in a form of tablets with
gastro-resistant coating. This drug form is perfectly compatible with long-term therapy including
three or more consecutive courses of chemotherapy (up to one year) without running the risks of
adverse inflammatory and autoimmune reactions caused by the constant presence of dsDNA in
the bloodstream. The drug mode of action is notably distinct from the mobilizing effect of
colony-stimulating factors that induce abortive release of hematopoietic progenitors into the
bloodstream. It is rather based on the activation of mucosal mononuclear cells. This is
accompanied with secretion of stimulatory cytokines which induce proliferation of hematopoietic
stem cells. Of particular importance is that Panagen uniquely combines several therapeutic
features, thereby paving the way to novel clinical applications.
Below we describe the experimental data from both published reports and our own studies, which allowed us to design the strategy of cancer therapy with human dsDNA preparation Panagen as a leukostimulatory and leukoprotective agent, and as an activator of adaptive immune response.

**Choice of the drug active substance**

Our choice of human dsDNA as an active substance in Panagen was dictated by both our experimental data and the general knowledge of interplay between dsDNA fragments and the genome of a human cell.

When dsDNA preparations from various sources were compared, human dsDNA consistently displayed superior leukostimulatory and anti-tumor activity [1-9].

Use of the DNA preparations and molecular interaction between DNA fragments and cell genome are insufficiently studied, and mainly just in yeast or *in vitro*. Recombination of extracellular DNA fragments has been reported as a likely event taking place in the nuclei of immune cells and various stem cells. Notably, the termini of these molecules induce activation of double-stranded DNA repair and recombination response in the cells [2, 10-14]. Due to the presence of short stretches of homology on DNA ends, recombination may result in integration of exogenous DNA into the genome [15-25]. This translates to the conclusion that any xenogeneic DNA, even at low dosage, poses a threat to the integrity of the genome, as compared to the allogeneic DNA fragments, that are more likely to be considered as a suitable substrate for homologous recombination machinery, which in turn should lower the risk of introducing unwanted mutations.

The choice of specific size of DNA fragments to be used in the medication was based on the well-established fact that extracellular dsDNA is normally present in the human blood plasma and interstitial fluid at a concentration of 14-100 ng/ml, ranging 1-20 nucleosomal repeats in
size, which equals to approximately 200 – 6000 bp [26-31].

Human placental DNA was selected as a source of active substance. Our protocol for collecting and isolating human DNA assures it is free of steroid hormones, various types of polysaccharides, infectious agents (parasites, protists, bacteria, RNA- and DNA-viruses), which is rigorously quality controlled for each batch of the drug [32]. Furthermore, we make sure the drug is protein-free, as protein contaminations (for instance HMG proteins) are known to activate various types of immune and stem cells.

Major features of the Panagen active substance in light of its possible therapeutic applications

1. The fragments of exogenous extracellular dsDNA may interact and be internalized by various cell types without any transfection procedures. It was established that double-stranded fragmented DNA molecules (including those used in Panagen) can be delivered into cell compartments without transfection – in both unconnected / loosely connected cells and in the tissue context (such as Peyer’s patches and solitary lymphatic nodules) [33-42]. Specifically, this property has been demonstrated for bone marrow cells, including mouse and human CD34+ hematopoietic progenitors tested in vivo, ex vivo cultured mouse and human bone marrow cells, and ascites forms of mouse hepatoma and lung carcinoma. DsDNA fragments were also shown to be incorporated by human pluripotent ES cells ex vivo, and by human breast adenocarcinoma cell line MCF-7, and may interact with human dendritic cells obtained ex vivo [2, 10-14, 43, 44].

2. Leukostimulatory effect (targeting CD34+ hematopoietic stem cells) of a tablet form of the drug. DsDNA fragments have been reported many times to target hematopoietic progenitors and so to boost their proliferation [6-9]. Leukostimulatory effect of the tablet form of human dsDNA preparation was consistently demonstrated on dogs [45] and in phase I clinical trial on healthy volunteers. This stimulatory effect on proliferation is apparently due to the incorporation of dsDNA by immunocompetent cells of the gut-associated lymphoid tissue, which stimulates
their migration to the periphery [35-42] and concomitantly activates them to produce cytokines
via the system of cytosolic DNA sensors [14, 46, 47].

Activated intestinal lymphocytes leave the gut and migrate to distant body regions, including bone marrow, where they are believed to induce proliferation of hematopoietic stem cells or their more committed progeny via direct cell-cell contacts or through secretion of specific cytokines.

3. Activation of antigen-presenting dendritic cells and expansion of a population of cytotoxic perforin+CD8+ T cells contribute to the anticancer activity of Panagen. These features are based on the interaction of Panagen dsDNA fragments with dendritic cells, which in turn activates their antigen-presenting properties [1-5].

4. So-called “delayed death” phenomenon results from the selective targeting of CD34+ hematopoietic stem cells as they recover from the genotoxic stress caused by a cross-linking agent cyclophosphamide. Fragments of exogenous dsDNA reach the nuclear interior of bone marrow cells, including CD34+ hematopoietic stem cells (HSCs). Importantly, if this happens during a very specific “death window” interval, the introduced DNA fragments overwhelm and interfere with the ongoing dsDNA repair. Thus, the dsDNA breaks waiting for delicate resolution via homology-dependent recombination pathway become instantly and randomly end-joined by an error-prone SOS-repair system. This leads to the failure of CD34+ HSC to differentiate into lymphoid lineage. Within several days, functional depletion of the organism immune system occurs, and animals succumb to opportunistic infections and progressive inflammatory response [10, 12].

5. Synergistic action of Panagen and cytostatic drugs cyclophosphamide and doxorubicin. DNA-based immunomodulators have been shown to display synergistic effects with standard cytostatic drugs used in the clinics [48-50]. Consistently, we also reported that human dsDNA-based medication has a pronounced anti-cancer effect when combined with
cyclophosphamide and doxorubicin [1, 3, 5].

**Choice of the tablet form of the drug and the strategy of drug administration**

The full potential of Panagen activities which include leukostimulatory activity, activation of dendritic cells and stimulation of adaptive antitumor immunity, can only be exploited upon its long-term and continuous administration, so that it can efficiently act upon immune cells, particularly antigen-presenting cells. It has been reported in the literature and established in our own experiments that long-term presence of large amounts of dsDNA in the bloodstream of humans and experimental animals results in multiple inflammation foci in various organs and in activation of autoimmunity [10, 51-56]. This has rendered the systemic route of administration – which is typically used in drugs with similar features (leukostimulation, leukoprotection and activation of protective immunity activation) quite problematic.

Yet, it was also known that dsDNA fragments administered per os can reach the immune cells residing in mucosal lymphatic system, where they activate them to produce a variety of cytokines and migrate elsewhere in the body [35-42]. So, we hypothesized that dsDNA fragments administered as tablets with gastro-resistant coating (Panagen) should activate immune cells in the gut, and this route of delivery could be exploited to ultimately target HSCs and antigen-presenting cells.

Our preclinical study performed in dogs [45] and phase I clinical trial of a tablet form of Panagen on 20 healthy volunteers indicated that this drug form stimulated leukopoiesis to the same extent as did intraperitoneal injections. Based on these data, we proceeded to phase II clinical trial on stage II-IV breast cancer patients.

Earlier studies of human dsDNA preparation Panagen have established it as a leukostimulatory agent. Taking into account its described synergistic activity with cytostatic drugs cyclophosphamide and doxorubicin to potently inhibit tumor growth in experimental
animals, we developed a new therapeutic scheme of cytostatic treatment of human malignancies.

As was confirmed in multiple studies, leukostimulatory activity of dsDNA preparation is caused by the stimulation of bone marrow cell proliferation, in particular HSCs. This stimulatory effect may result from either internalization of dsDNA fragments by bone marrow progenitors or production of pro-proliferative cytokines by mononuclear cells activated by dsDNA fragments [10, 14, 35-42].

In terms of inducing adaptive anticancer immune response, the major steps of dsDNA therapeutic activity in combination with cross-linking and anthracycline cytostatics are as follows:

- Human dsDNA potently activates dendritic cells [1-5].
- Upon oral administration, dsDNA reaches the immune cells of intestinal mucosa and stimulates their professional properties [35-42].
- DsDNA fragments turn on the system of cytosolic sensors, thereby leading to production of specific cytokines by immune cells [14, 46, 47].
- Cyclophosphamide metabolite, phosphoramide mustard, induces formation of interstrand crosslinks in cancer cells, which leads to their death and production of tumor cell debris.
- Cyclophosphamide interferes with the functions of T-regulatory lymphocytes, resulting in their temporary depletion and functional suppression. In contrast, dendritic cells and cytotoxic T-cells are less sensitive to cyclophosphamide. Tumor cells then lose their cellular and humoral protection, whereas immunocompetent cells stop receiving inhibitory signaling from T-regulatory lymphocytes. This combination of factors makes it possible for the immune system to target the tumor [57-68].
- Anthracycline cytostatics, such as doxorubicin, induce membrane translocation of calreticulin in apoptotic cancer cells. This is interpreted as an “eat me” signal by the antigen-presenting cells, dendritic cells in particular. Similarly to cyclophosphamide, this
results in formation of tumor cell debris [69-72].

Taking into account the above-listed properties, we put forward a scientific basis for the following novel therapeutic strategy. Use of cyclophosphamide leads to the physical disintegration of tumor cells and debris formation. Doxorubicin also causes tumor cells to undergo apoptosis and induces membrane translocation of calreticulin in dying cells, which serves as an «eat me» signal for dendritic cells. These treatments converge to form immunogenic tumor cell debris. Additionally, cyclophosphamide selectively targets T-regulatory lymphocytes, and potently inhibits their functions or directly kills them. This leaves tumor cells unprotected from the immune system surveillance. Our studies indicate that following cyclophosphamide treatment and tumor cell lysis, administration of exogenous DNA will stimulate activation of antigen-presenting properties of dendritic cells. This will be accompanied by the suppression of T-regulatory lymphocytes that will no longer restrain the immune system from attacking the tumor. This combined action of cyclophosphamide and doxorubicin will enhance antigen uptake by activated dendritic cells that in turn will launch specific anti-cancer immune response.

There are three key points in this strategy, as applied to the clinical practice:

1. Tablet form of the drug is administered 48 hours post cyclophosphamide treatment. This assures safety of the drug by avoiding the cell-destructive period, known as the “death window”.

2. The drug is administered continuously throughout the courses of chemotherapy. It is prescribed as a leukostimulatory medication used intermittently, continuously and massively, which mediates sustained activation of mucosal immune cells, and so results in increased proliferation of HSCs and their immediate committed progeny. Temporary drug withdrawal throughout the chemotherapy courses may only be required so as to avoid the “death window”.

3. Uninterrupted administration of the drug across multiple lines of chemotherapy allows
combining its leukostimulatory potential with activation of antigen-presenting
dendritic cells resident in the human mucosa. Cytostatic background further
contributes to the maturation and release of CD4+CD8+perforin+ cytotoxic T-cells
into peripheral blood, which is generally accepted as developing adaptive immune
response.

The proposed mode of action of the tablet form of Panagen relies on targeting the gut
mucosa-resident lymphoid cells by dsDNA fragments. The active substance is encapsulated and
delivered to the small intestine. The coating then disintegrates, and the substance is dissolved in
the intestinal lumen. Dissolved dsDNA fragments reach mononuclear cells found in Peyer’s
patches, in lymphoid follicles of vermiform appendix and in solitary follicles, where they activate
the cells via a cascade of dsDNA sensors. Upon activation, various types of immune cells
normally resident in gut-associated lymphoid tissue migrate into the bloodstream and reach
immunocompetent organs. Immune cells then activate proliferation and mobilization of HSCs
and their immediate committed progeny, via cell-cell contacts or secreted cytokines.

Gut-associated dendritic cells also migrate into the bloodstream upon activation. When
they eventually reach and become anchored in the lymphoid organs (such as mesenterium), they
are faced with cancer antigens in the form of tumor cell debris. All these events culminate in
induction of anticancer adaptive immune response.

Here, we report on the results of phase II clinical trials of a human dsDNA-based
preparation Panagen.

Materials and Methods

DNA quantification in blood plasma of patients receiving tablet form of Panagen medication
Levels of DNA in blood plasma were determined according to the method described by
Spirin [73].
**Brief outline of phase II clinical trial protocol**

We performed a multicenter, double-blind, placebo-controlled, phase II clinical trial of preparation Panagen in compliance with the following documentation:

- Approval of the Ethics Committee of the Federal Service on Surveillance in Healthcare and Social Development of Russian Federation (Protocol No. 7 of 04/08/2008) to carry out clinical trial of preparation Panagen.

- Approval of the Ministry of Health and Social Development of the Russian Federation (MH&SD) No. 209 of 05/12/2008 to perform phase I clinical trial of preparation Panagen.


- Approval of the MH&SD No. 47 of 03/12/2010 to perform phase II clinical trial of preparation Panagen.

- Protocol adaptation following the MH&SD decision, letter No. 276822-31-1 of 09/08/2011.

- Registration certificate “Medical Drugs of Russia” for the active pharmaceutical ingredient of the preparation Panagen No. 004429/08 of 06/09/2008.

- Product monograph for the active pharmaceutical ingredient of Panagen No. 42-
The clinical studies were approved by the local ethics committees at the Novosibirsk Municipal Hospital No 1 and the Irkutsk Regional Oncology Dispensary, where clinical trials were subsequently performed. The studies were carried out in compliance with the World Medical Association Declaration of Helsinki. Written informed consent to participate in the study was obtained from each of the patients, which specified open publication of the results presented as reports or otherwise. All patients were also insured. The study recruited 80 female high-risk category patients with stage 2 breast cancer or patients with stage III-IV breast cancer who were advised to undergo chemotherapy. All patients were sequentially randomized, i.e. the patient was assigned to one of the two groups irrespective of the time she joined the trial. The first group comprising 57 patients received Panagen, of which 6 patients were later excluded from the study for various reasons, the second group received placebo (with 23 patients recruited, of which 6 were excluded during the trial). For ethical reasons, the second group was maximally down-sized so as to reliably meet the statistical significance threshold.

The patients received standard FAC chemotherapy (fluorouracil 500 mg/m², doxorubicin 50 mg/m², cyclophosphamide 500 mg/m² – all i.v. for 1 day) or AC chemotherapy (doxorubicin 50 mg/m² and cyclophosphamide 500 mg/m² i.v. for 1 day). The patients completed three courses of chemotherapies (3 weeks per course), and each course began on the day of chemotherapy (day1).

Patients received 5 mg Panagen or placebo tablets daily (6 tablets every 2-3 hours, 30 mg/day). The tablets were given to the patients 48 hours post-chemotherapy (day 3) and the course continued for 17 more days until day 20 post-chemotherapy. If the next round of chemotherapy was delayed, the patients stayed on Panagen or placebo. The patients stopped taking tablets 1 day before the next course of chemotherapy. Delay of next course of chemotherapy of up to one week was considered acceptable. All patients from the placebo cohort
received standard-of-care therapy as required by the MH&SD of Russian Federation.

The clinical trial was conducted in two medical centers, Novosibirsk Municipal Hospital No 1 (20 patients on FAC and 31 patients on AC regimens), and Irkutsk Regional Oncology Dispensary (29 patients on FAC regimen).

For more method details see Additional file 1.

Statistical analysis was performed using Statistica 6.0 software, and non-parametric analyses, namely Wilcoxon-Mann-Whitney and paired Wilcoxon tests. To describe the results, the following parameters were used: number of observations (n), median, interquartile range, and minimum-maximum range.

**MTT assay using human peripheral blood mononuclear cells**

Cytotoxicity of human peripheral blood mononuclear cells (PBMCs) obtained by fractionation of patient peripheral blood on the density gradient of ficoll-urografin (d=1.077 g/ml) was assayed against the MCF-7 tumor cell line. Tumor cells were placed in 96-well plates (5×10^4 cells/well). Next, 5×10^4, 10×10^4 or 25×10^4 PBMCs per well (1:1, 1:2 or 1:5 ratios) were added. Cell mixtures were incubated in RPMI-1640 supplemented with gentamicin sulphate (100 mkg/ml) in 5% CO_2 at 37°C for 21 h. Co-incubation was terminated by adding MTT solution to 0.5 mg/ml and reactions were allowed to stay for 3 more hours. Cells were centrifuged at 4000 rpm for 10 min (Eppendorf Centrifuge 5810 R). Supernatant was decanted and blue-colored formazan crystals were dissolved in 100 mkl DMSO. Optical density was read using «Multiskan RC» at 570 nm with background subtraction measured at 620 nm. Results of the MTT assay were processed using Microsoft Excel 2002. Cytotoxicity index (CI) was calculated as follows:

$$CI(\%) = \left[1 - \frac{OD_{e+t} - OD_e}{OD_t}\right] \times 100,$$

where:

- OD_{e+t} – optical density value in experimental wells (co-incubated effector and target cells);
- OD_e – optical density value in wells with effector cells;
OD<sub>t</sub> – optical density value in wells with target cells.

**Cytokine production by PBMCs isolated from recruited patients**

To gain insight into the dynamics of cytokine production upon dsDNA administration, we used samples of peripheral blood from patients recruited into our trial. Blood samples were taken at three control timepoints, namely, at initial point – 1-3 days prior the first round of chemotherapy; at intermediate point – 1 day before the second course of chemotherapy; and at the final point – upon completion of the therapy (i.e. after completion of the third three-week course of Panagen).

To assay spontaneous cytokine production, peripheral venous blood was collected in heparin-containing vacutainers, and fresh 1 ml blood samples were reserved for assaying spontaneous cytokine production. In parallel, we also measured mitogen-induced cytokine production. For this purpose, we used a commercial kit “Cytokine-Stimul-Best” containing a mix of mitogens (PHA, Con A, LPS, - at 4, 4 and 2 mkg/ml each). The samples were incubated at 37°C for 24 hours. Cells were centrifuged at 10000 rpm for 3 min (Eppendorf Centrifuge 5810 R), the supernatants were transferred into new tubes, snap-frozen and stored at -70°C until further processed for quantification of cytokine production. Concentrations of IFN-γ, IFN-α, TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-2, IL-17, VEGF, MCP, IL-18, IL-4, GM-CSF, G-CSF and IL-1 receptor antagonist (IL-1RA) in the samples were measured using solid-phase sandwich ELISA kits manufactured by the JSC «Vector-Best» (Novosibirsk, Russia).

SigmaStat statistical software package (Systat Software Inc., San Jose, CA, USA) was used for statistical data analysis. Two-way ANOVA followed by the Holman-Sidak test was used to analyze data in 2 groups x 3 intervals matrix to find dependencies between independent factors. A value of p<0.05 was considered as statistically significant. Results are presented as Mean±SE in their absolute units of measure (ng/µl). Absolute units of concentration were log10-converted.
Results and discussion

DNA content in the blood plasma samples of patients receiving tablet form of Panagen

Panagen medication tested in the clinical trial is manufactured in the form of gastro-resistant tablets. This gastro-resistant coating dissolves at neutral pH in intestines, and so the active substance – fragmented human dsDNA – is liberated into intestinal lumen, where it reaches the mononuclear cells of Peyers patches [42]. Analyses of blood plasma samples from healthy donors (phase I of clinical trial) receiving Panagen tablets daily for three months showed no increase in extracellular DNA concentration. Fasting blood samples were collected in the morning, 8 hours post-Panagen tablet. Daily dose was 30 mg, in six tablets (5mg each) taken throughout the day, approximately 1 tablet every 2-3 hours (Figure 1). Furthermore, we detected no changes in DNA concentration in blood plasma 2 hours after swallowing 2-3 Panagen tablets (data not shown).

Effects of Panagen on hematopoietic progenitors

Leukostimulatory effects of Panagen drug based on fragmented human dsDNA were analyzed throughout three consecutive courses of FAC or AC chemotherapies in patients with stage II-IV breast cancer (Additional files 2-4).

Our primary goal at this step was to understand how the drug modulates different blood lineages under the increasing detrimental pressure of repeated chemotherapies. To do so, we measured specific blood lineage cell counts in peripheral blood at control points after 1, 2 and 3 rounds of chemotherapy in patients on Panagen vs placebo, and determined whether these values were significantly different. We assumed that positive effect of Panagen would be demonstrated if significant differences in blood cell counts are observed in at least one control point. This
seemingly liberal definition of a positive effect was dictated by several factors. First, we found no published data describing and substantiating the specific time-points to assay the dynamics of hematopoiesis in response to gastrointestinal tract delivery of a drug – hence we were free to choose the control points. Second, neutrophils are known to quickly migrate from the periphery to their destination points, which makes it rather challenging to reliably measure their stimulated proliferation by analyzing peripheral blood samples. We also monitored the frequencies of stage I-IV neutropenia-related events throughout the chemotherapy courses, as well as the dynamics of CD34+/45+ HSCs, which was essentially a blind search in the absence of the documented time-course data.

The analysis performed thus far summarizes the following therapeutic features of Panagen in the context of three courses of FAC/AC chemotherapies. We demonstrate that absolute cell counts for lymphocytes, neutrophils and monocytes in control points on day 21 after 1, 2 and 3 chemotherapy courses are significantly different between Panagen and placebo-treated patient cohorts (Figure 2). In order to mitigate the confounding effects from individual patients on statistical analysis of Panagen leukostimulatory activity (as assayed by cell counts in peripheral blood, by timing and magnitude of cell proliferation), the patients were grouped into Panagen-responders and non-responders (Figure 3, see appropriate parts of Additional files 2-4). Patients whose cell counts were higher at a given time point than on day 14 or day 21 after the first course of chemotherapy (set as 100%) were classified as responders. Most of the blood parameters in the group of Panagen-responders were significantly higher than in the placebo cohort. Notably, 52% of patients positively responded on Panagen therapy throughout the 3 courses of chemotherapy as measured in control points. This approach allowed us to accurately delineate the leukostimulatory effect of Panagen with minimal contribution of individual patient-specific effects.

Notably, blood test parameters in placebo group display statistically significant
differences when compared to the first control point (Figure 2). If one compares the cell count
curves for placebo and Panagen patient groups, most of the data points (for leukocytes,
neutrophils and lymphocytes) display pronounced decline by the end of the third round of
chemotherapy in placebo-, but not in Panagen-treated patients where they remain at initial levels
(Figure 2). These data are consistent with a protective effect of Panagen on leukocyte
progenitors.

In both FAC and AC chemotherapies, we observed progressively fewer neutropenias in
Panagen patients facing the increasingly negative effects of chemotherapies, as compared to the
placebo cohort, where the frequency of neutropenias increased (Figure 4) (Additional file 2, p.

Panagen alters the timing of cyclophosphamide-induced abortive release of CD34+/45+
HSCs into peripheral blood. Notably, Panagen also significantly increases the number of HSCs
mobilized into the bloodstream (Additional file 5).

As it follows from our analysis, Panagen potently stimulates erythropoietic lineage in
patients on FAC protocol. Hemoglobin levels generally dropped in patients on chemotherapy, yet
this was only observed in 63% patients receiving Panagen vs 100% placebo-treated patients.
Further, in 23% patients receiving Panagen, we saw an increase in platelet counts by day 21
(Additional file 2, p. 27-29).

Panagen has hepatoprotective activity counteracting the activities of chemotherapeutic
drugs cyclophosphamide, doxorubicin and fluorouracil (Additional file 2, p. 32-38; Additional
file 3, p. 38-50; Additional file 4, p. 16-24). Panagen suppresses the effects of drug-induced
immunodeficiency in patients on the AC protocol of breast cancer chemotherapy (Additional file
3, p. 51-52). Panagen activity positively correlates with regeneration of surface epithelium, which
is likely due to increased proliferation of basal cells in the skin (Additional file 2, p. 39-42).
**Activation of adaptive immune response**

When combined with cytostatic drugs, Panagen increases the number of CD8+perforin+ cytotoxic T cells in peripheral blood, which serves as a major marker of mounting adaptive immune response (Additional file 6).

In our earlier studies, we established that fragmented genomic DNA is actively targeting dendritic cells and potently induces their allostimulatory activity and maturation both *ex vivo* and *in vivo* [2, 4]. We also showed that when combined with cyclophosphamide, fragmented dsDNA preparation displays pronounced anti-cancer activity *in vivo* in mice with tumor grafts [1]. When fragmented dsDNA was injected in tumor-engrafted mice following cyclophosphamide or cyclophosphamide and doxorubicin administration, significant antitumor activity was observed [5]. We speculate that the most likely scenario describing suppression of tumor growth in these *in vivo* experiments involves activation of key immune system components, namely that of adaptive immunity, which is primarily characterized by production of CD8+perforin+ cytotoxic T cells [3]. We can not formally exclude yet another option, i.e. two-pronged targeting of cancer cells by immune system and via direct cytotoxic activity of dsDNA preparation [74].

In order to firmly establish whether the anticancer immune response does unfold upon combined use of fragmented exogenous dsDNA and cytostatics, we surveyed basic cell types involved in adaptive immunity. Namely, we assayed the dynamics of plasmacytoid and myeloid dendritic cells, T-regulatory lymphocytes and CD8+ perforin+ cytotoxic T-cells. Our analysis failed to uncover pronounced trends when measuring dendritic cell and T-regulatory lymphocyte counts.

We observed significant increase in CD8+ perforin+ cytotoxic T-cells in the peripheral blood of patients receiving Panagen vs placebo on day 21 following 1 course of chemotherapy, notably 58% patients on FAC protocol (7 out of 12) and 16% patients on AC protocol (3 out of 19) responded (Figure 5). This supports the activating role of Panagen on progression of
adaptive immune response when it is combined with standard FAC and AC chemotherapeutics.

Clearly, Panagen potently protects PBMCs known to mediate innate anticancer immunity and counterbalances the negative effects from three courses of aggressive chemotherapy. We further analyzed Panagen activity to maintain and enhance proliferation of PBMCs in the context of innate anticancer immunity. We chose to analyze non-specific cytotoxic activity of patient-derived PBMCs against human adenocarcinoma cell line MCF-7. Our results indicate that Panagen has protective and stimulatory activity towards PBMCs. Cytotoxic indices of PBMCs in patients receiving Panagen were significantly higher than those observed in the placebo group (Figure 6).

**Effects of Panagen on patient cytokine profiles**

The most noteworthy changes were observed in the production of cytokines that predominantly mediate an acute phase of innate immune response (TNF-α, IL-1RA, IL-6, MCP-1). In contrast, no significant changes were observed in the production of cytokines that regulate the development of adaptive immunity (IL-2, IFN-γ, IL-4, IL-10, IL-17) or other cytokines (IL-8, IL-1, GM-CSF, G-CSF, and VEGF) (Additional file 7).

Opposing trends in the production of TNF-α, IL-2, IL-1, IL-1RA, IL-18, IL-10 and IFN-γ between Panagen-treated and placebo groups have been observed. The production of above-mentioned cytokines in the Panagen group of patients decreased, while the production of the same cytokines in the placebo group increased. In general, decreased ability to secrete cytokines is associated with immune suppression. However, it is important to keep in mind that increase in systemic cytokine production is not necessarily a good sign. Uncontrolled systemic secretion of cytokines is one of the major pathogenic outcomes of septic shock and systemic inflammatory response syndrome [75, 76]. It is usually associated with the rapid and severe increase of circulating levels of IL-6, IL-8, MCP-1, MIP-1β, IFN-γ, GM-CSF (also known as “cytokine
storm”) due to polyclonal activation of immunocompetent cells [77].

Spontaneous production of cytokines by whole blood cultures recapitulates immune reactivity in vivo more accurately as it mimics natural milieu as well as chronic effects of endogenous factors (Panagen) prior to blood collection. On the other hand, mitogen stimulation helps address the functional state of the immune cells and their ability to respond to a new stimulus.

Progressive increase in the spontaneous production of TNF-α, IL-1, IL-1RA, IL-6, IL-18, and IL-10 was restricted to placebo group. Likewise, increase in the mitogen-activated production of TNF-α, IL-2, IL-4, IL-17, GM-CSF was only observed in the placebo group. In contrast, production of TNF-α and IL-2 in Panagen-treated patients progressively declined. Moreover, those changes were statistically significant (Additional file 7). In respect to physiological significance, it is however essential to remember that an increase in systemic concentration of these two cytokines is often associated with initial stages of a cytokine storm. Collectively, these results argue for cytoprotective properties of Panagen.

Changes in cytokine production observed could be quantitative as well as qualitative in nature since these were analyzed in whole blood cell cultures. It is important to bear in mind possible sequestration of cytokine-producing monocytes as well as lymphocytes (especially memory ones) from periphery to the tumors, and more importantly, the favorable prognosis of such a process, as was shown in breast cancer patients earlier [78, 79].

**Long-term follow-up analysis**

We compared the frequency of relapses in patients of both study groups 3 years following the therapy in Novosibirsk Municipal Hospital No 1 (18 FAC patients and 26 AC patients). In Panagen vs placebo cohorts, 24% and 45% of patients, respectively, relapsed or died (Figure 7, Additional file 8).
Conclusions

1. Human dsDNA-based drug Panagen shows leukoprotective and leukostimulatory activity when assessed throughout three consecutive FAC and AC chemotherapies.

2. Panagen efficiently protects the cells involved in innate anticancer immunity from detrimental effects of FAC and AC chemotherapies.

3. Panagen induces adaptive immune response, as assayed by production of CD8+perforin+ cytotoxic T cell population.

The data collected in this trial set Panagen as a multi-faceted “all-in-one” medicine that is capable of simultaneously sustaining hematopoiesis and stimulating anticancer immune response. Its unique feature is that it is delivered through the gastrointestinal tract and acts through the lymphoid system of intestinal mucosa. This medication can also boost individual anticancer adaptive immunity resulting from the activation of dendritic cells by the circulating tumor cell debris.

List of abbreviations

AC chemotherapy – chemotherapy including doxorubicin and cyclophosphamide; dsDNA – double-stranded DNA; FAC chemotherapy – chemotherapy including 5-fluorouracil, doxorubicin and cyclophosphamide; HSCs – hematopoietic stem cells; PBMCs – peripheral blood mononuclear cells

Competing interests

The authors declare that they have no competing interests.
Authors' contributions

ASP performed the analysis, interpreted the data, and drafted the manuscript. TSG carried out clinical work with patients and drafted the manuscript. EAA carried out the molecular studies. EVD carried out the molecular studies. KEO performed the design of the study and provide the technical conditions for perform works. VPN performed the analysis and interpreted the data. NAP participated in the design of the study. SVS carried out clinical work with patients and participated in study design. ERC participated in the design of the study. AAO performed the analysis and interpreted the data. OYL carried out experiments to estimate the adaptive immunity induction. VVD helped in the data interpretation. DMP carried out clinical work with patients. GSS helped in the data interpretation. NAV and TGR carried out cytokine analysis. VAR carried out production of DNA preparation. SSB conceived the study, participated in its design, and coordinated and drafted the manuscript. MAS participated in the study design and coordination. All authors read and approved the final manuscript.

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**Additional files**

**Additional file 1.** Progress of phase II clinical trials of Panagen.
Additional file 2. Results of the completed phase II double-blind multicenter placebo-controlled clinical trial to evaluate the safety and leukostimulatory activity of Panagen in breast cancer patients. The study was performed at the Oncology Department of Novosibirsk Municipal Hospital No 1 and included 18 patients receiving FAC therapy (14 patients additionally received Panagen, 4 patients received placebo).

Additional file 3. Results of the phase II double-blind multicenter placebo-controlled clinical trial to evaluate the safety and leukostimulatory activity of Panagen in breast cancer patients. The study was performed at the Oncology Department of Novosibirsk Municipal Hospital No 1 and included 26 patients receiving AC therapy (19 patients additionally received Panagen, 7 patients received placebo).

Additional file 4. Results of the phase II double-blind multicenter placebo-controlled clinical trial to evaluate the safety and leukostimulatory activity of Panagen in breast cancer patients. The study was performed at the Irkutsk Regional Oncology Dispensary and included 23 patients receiving FAC therapy (18 patients additionally received Panagen, 5 patients received placebo).

Additional file 5. Analysis of CD34+/45+ progenitor cell dynamics at control time points throughout the three cycles of chemotherapy.


Additional file 7. Effects of Panagen on patient cytokine profiles.

Additional file 8. Long-term follow-up analysis.

Figure legends
Figure 1. DNA concentration in blood plasma of healthy volunteers not receiving Panagen (control, n=15) and following daily oral administration of 30 mg Panagen for 1 and 3 months (n=9).

Figure 2. Dynamic changes in blood cell counts ($\times10^9$ cells/L) throughout the clinical trials at the initial pre-therapy timepoint and on day 21 post each chemotherapy course. I – FAC regimen in Novosibirsk Municipal Hospital No 1, II – AC regimen in Novosibirsk Municipal Hospital No 1, and III – FAC regimen in Irkutsk Regional Oncology Dispensary. Significantly higher values are observed for Panagen (dashed orange line) vs placebo (black solid line) groups of patients (Wilcoxon-Mann-Whitney test), as well as within each group relatively to the initial level before the therapy (Wilcoxon paired test). For patients who received Panagen, increased value is marked with up-facing arrow, for patients from placebo group, decreased value is highlighted by downward-facing arrow. Red asterisk (*) denotes significant values with $p<0.05$, blue hash symbol (#) marks statistically significant difference with $p<0.11$.

Figure 3. Changes in stimulation indices (%) for blood cell types throughout three chemotherapy courses. Stimulation index is expressed as a ratio of values registered in second and third control time points (days 14 and 21) to the appropriate value observed in the control point of the first chemotherapy course (set as 100%). Patients were subgrouped into Panagen-responders, Placebo and Panagen-non-responders. The number of patients per group is indicated for each time point. Red line denotes 100% level, i.e. the values reported in control time points (days 14 and 21) after the first chemotherapy. Values that show statistically significant difference between Panagen-responders and Placebo patient groups (Wilcoxon-Mann-Whitney test) with $p<0.01$ (**), $p<0.05$ (*) and $p<0.09$ (#) are highlighted.

Figure 4. Frequency of grade I-IV neutropenia-related events in patients on day 14 of three courses of FAC and AC chemotherapies.

Figure 5. Arbitrary content (%) of CD8+ perforin+ cytotoxic T-cells in peripheral blood of
patients undergoing FAC or AC chemotherapy on day 21 after the first course, relatively to the initial baseline level (100%, red line). Values were normalized to the placebo group values. Median values, quartile range 25-75% (box) and minimum-maximum range are given for each group; n – the number of patients per group. Significant differences from Placebo with p<0.05 (Wilcoxon-Mann-Whitney) are marked with red asterisk.

**Figure 6.** Comparative analysis of cytotoxicity indices in PBMCs of patients on day 21 following the third round of chemotherapy. Largely responsible for innate anticancer immunity, PBMCs retain their specific functions at the levels observed before the therapy in patients receiving Panagen throughout three courses of chemotherapy (p<0.05). Unlike in Panagen cohort, PBMCs from placebo group patients display three-fold decrease in cytotoxicity indices by the end of the third chemotherapy course relatively to the initial level.

**Figure 7.** 3-year follow-up analysis of Panagen clinical trial. Percentage of relapse events and deaths of patients relatively to the total number of patients.
Figure 1

DNA in plasma, ng/ml

Control | Panagen 1 month | Panagen 3 months
Figure 2

Leukocytes

Neutrophils

Monocytes

Lymphocytes
Figure 3
Figure 4

Comparison of Neutropenia Frequency

FAC

placebo
Panagen

After 1st chemotherapy
After 2nd chemotherapy
After 3rd chemotherapy

AC

After 1st chemotherapy
After 2nd chemotherapy
After 3rd chemotherapy

Neutropenia frequency
Figure 5
Figure 7

Death or cancer relapse rate among patients, %

Placebo (5 of 11 patients)

Panagen (8 of 33 patients)
Additional files provided with this submission:

Additional file 1: Additional file 1.pdf, 78K
Additional file 2: Additional file 2.pdf, 1289K
http://www.biomedcentral.com/imedia/1476826291316877/supp2.pdf
Additional file 3: Additional file 3.pdf, 1512K
Additional file 4: Additional file 4.pdf, 672K
Additional file 5: Additional file 5.pdf, 376K
Additional file 6: Additional file 6.pdf, 663K
Additional file 7: Additional file 7.pdf, 273K
Additional file 8: Additional file 8.pdf, 21K