



Theoretical premises of a “three in one” therapeutic approach to treat immunogenic and nonimmunogenic cancers: a narrative review

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Objective: We describe experimental and theoretical premises of a powerful cancer therapy based on the combination of three approaches. These include (I) *in situ* vaccination (intratumoral injections of CpG oligonucleotides and anti-OX40 antibody); (II) chronometric or metronomic low-dose cyclophosphamide (CMLD CP)-based chemotherapy; (III) cancer stem cell-eradicating therapy referred to as *Karanaban* (from the Sanskrit *kāraṇa* [“source”] + *ban* [“to kill”]).

Background: In murine models, the first two approaches are particularly potent in targeting immunogenic tumors for destruction. *In situ* vaccination activates a fully fledged anticancer immune response via an intricate network of ligand–receptor–cytokine interactions. CMLD CP-based chemotherapy primarily targets the suppressive tumor microenvironment and activates tumor-infiltrating effectors. In contrast, *Karanaban* technology, being aimed at replicative machinery of tumor cells (both stem-like and committed), does not depend on tumor immunogenicity. With this technology, mice engrafted with ascites and/or solid tumors can be successfully cured. There is a significant degree of mechanistic and therapeutic overlap between these three approaches. For instance, the similarities shared between *in situ* vaccination and *Karanaban* technology include the therapeutic procedure, the cell target [antigen-presenting cells (APC) and dendritic cells (DC)], and the use of DNA-based preparations (CpG and DNAmix). Features shared between CMLD CP-based chemotherapy and *Karanaban* technology are the timing and the dose of the cytostatic drug administration, which lead to tumor regression.

Methods: The following keywords were used to search PubMed for the latest research reporting successful eradication of transplantable cancers in animal models that relied on approaches distinct from those used in the *Karanaban* technology: *eradication of malignancy, cure cancer, complete tumor regression, permanently eradicating advanced mouse tumor, metronomic chemotherapy, in situ vaccination, immunotherapy*, and others.

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Conclusion: We hypothesize, therefore, that very potent anticancer activity can be achieved once these three therapeutic modalities are combined into a single approach. This multimodal approach is theoretically curative for any type of cancer that depends on the presence of tumor-inducing cancer stem cells, provided that the active therapeutic components are efficiently delivered into the tumor and the specific biological features of a given patient's tumor are properly addressed. We expect this multimodal approach to be primarily applicable to late-stage or terminal cancer patients who have exhausted all treatment options as well as patients with inoperable tumors.

Keywords: *In situ* vaccination; chronometric/metronome low-dose chemotherapy; *Karanaban* technology; immunity; cancer stem cells

Submitted May 28, 2021. Accepted for publication Oct 14, 2021.

doi: 10.21037/tcr-21-919

View this article at: <https://dx.doi.org/10.21037/tcr-21-919>

Introduction

Current experimental and clinical approaches to treat patients with various cancer types and degrees of disease progression include three basic techniques that do not depend on a specific target or molecule but rather rely on induction of an integral anticancer response. These are *in situ* vaccination, chronometric or metronomic low-dose cyclophosphamide (CMLD CP)-based chemotherapy, and a universal approach dubbed *Karanaban* technology, which primarily targets tumor-initiating stem cells (TISCs). It is becoming increasingly clear that taking into account the temporal dynamics of molecular events in the entire organism, organs, tissues, tumor, and tumor cells is the key to successful therapy (1). Also, the paradigm of the patient's individual immune status as a starting point for downstream immunotherapeutic interventions has been put posited (2). These novel concepts form the core of the proposed technology, which is based on the chronometric delivery of therapeutic agents to the tumor depending on the specific features of the tumor and immune cells as well as on the immune status of the patient.

This analysis was motivated by our experimental studies where we used the *Karanaban* technology to successfully treat mice engrafted with incurable and aggressive cancers, in addition to Krebs-2 and Ehrlich carcinomas. We searched PubMed for research papers reporting completely distinct curative approaches to treating similarly aggressive cancer models. We aimed to compare the underlying mechanisms and combine the strongest elements of each of the approaches into an integrated anticancer platform.

Our analysis of the literature indicated that there were very few studies reporting survival of laboratory animals

engrafted with solid or ascites forms of cancer beyond 90–150 days that could maintain their fertility and ability to produce healthy offspring. In the vast majority of cases, evidence of anticancer activity was established as inhibited or delayed tumor growth or longer time to death of cancer-engrafted animals.

We primarily focused on papers in which incurable forms of cancer were successfully treated in laboratory animals (3-8), and those with a completely developed curative approach were of particular interest (9-15).

An analysis of the available literature revealed three conceptually distinct approaches that are detailed in this review. These approaches are (I) *in situ* vaccination, aimed to expand the population of tumor-specific cytotoxic T cells and to initiate a systemic immune response that would have an abscopal effect and affect “nonvaccinated” cancer foci (3,10,16); (II) CMLD CP-based chemotherapy, aimed to inactivate the immunosuppressive activity of tumor-resident myeloid-derived suppressor cells (MDSCs) (5-8,17,18) and to restore the activity of anticancer immune cells; (III) our *Karanaban* technology, which induces the massive apoptosis of cancer cells and reduces cancer grafts by eradicating TISCs. Our analysis indicates that the first 2 approaches are applicable in the context of immunogenic tumors, whereas *Karanaban* demonstrates sustained activity regardless of the “hot” or “cold” immune status of the tumor (19).

All three approaches share some features, such as intratumoral delivery of drugs and systemic injection of moderate doses of cyclophosphamide (CP), which opens an opportunity to combine the strong attributes of these approaches.

We wondered which elements of these three curative

anticancer approaches were unique in terms of their therapeutic activity yet could be logically combined to produce a significantly enhanced synergistic effect of the novel integrative multimodal platform. Taken together, these elements should ensure complete lysis of tumor foci due to the cell-cycle dependent metronomic delivery of CP, reduction of the immunosuppressive activity of tumor-associated stroma, eradication of TISCs, and formation of the systemic antitumor immunity.

To reveal the latest progress in the area and generate the narrative review, a literature search was conducted in PubMed using keywords (*eradication of malignancy, metronomic chemotherapy, in situ vaccination, immunotherapy, and others*).

We present the following article in accordance with the Narrative Review reporting checklist (available at <https://dx.doi.org/10.21037/tcr-21-919>).

In situ vaccination

Relatively novel immunotherapeutic approaches known as *in situ* vaccination are based on the simultaneous activation of a broad range of immune cells within a small area of the tumor, which results in both a local antitumor immune response and abscopal effects (i.e., affecting both the injected tumor and distant noninjected metastatic lesions). This contrasts with the traditional approaches that are based on the systemic administration of anticancer drugs, which typically activates only a single arm of immunity.

Multiple technological platforms exploiting the concept of *in situ* vaccination are actively tested in the clinic. These include oncolytic viruses, modulation of activity of tumor-infiltrating myeloid cells and lymphocytes, ligand-receptor blockade [intratumor checkpoint, tumor necrosis factor (TNF) signaling], local irradiation, local radiofrequency, ultrasound and cryoablation, injections of transformed cells, cytokine activation, tissue electroporation, and combinations thereof (16,20-26).

Local immunotherapy has several important advantages over systemic immunotherapy. The first one is a significantly lower dose of the locally delivered drug, as compared to the dose required for systemic delivery, which in turn translates into lower toxicity. The second is that several independent approaches can be safely combined without the risk of developing uncontrolled toxicities (such as those observed following a cytokine storm) (16,20). The third is that high local concentrations of the drug can be achieved, which results in stronger immune responses.

Nonetheless, the ultimate goals of the technological platforms listed above include boosting the activity of antigen-presenting cells (APC), production of a broad range of antigens, and blocking the suppressive activity of the tumor-associated microenvironment (3,16). Technically, the approach includes intratumoral delivery of factors that irreversibly activate tumor-associated immune cells, which govern cancer cells lysis. As a result, a broad range of antigens and a vast repertoire of primed and activated effector CD8⁺ T cells are formed.

Several notable examples of such therapeutic approaches have been reported in the literature. These have demonstrated a pronounced efficacy in transplanted and induced cancer models. For instance, dendritic cell (DC) activation can be readily achieved by CpG oligonucleotides or TNF- α (3,27-29). Monoclonal antibodies as well as specific ligands have also been exploited as powerful anticancer agents. Antibodies that block programmed cell death protein 1/programmed death-ligand 1 (PD1/PD-L1; broadly referred to as checkpoint inhibitors) have been described to prevent PD-1-mediated inhibitory signaling in cytotoxic T cells, thereby maintaining their activity in the otherwise immunosuppressive tumor microenvironment (28,30,31). The glycolipid molecules *alpha*-galactosylceramide (α GalCer) and glucuronosylceramide have also been used in experimental cancer treatments. These molecules are analogous to the endogenous glycolipid known as isoglobotrihexosylceramide, which is a self-antigen complexed with the CD1d of APC. This complex is known to interact with a receptor found on the surface of natural killer T cells (NKT cells). This engagement leads to NKT activation and interferon gamma (IFN- γ) production (32).

Currently, antibodies and ligands for the molecules belonging to the TNF superfamily, for instance, anti-OX40 antibodies (OX40) (3,27-29,33-35), are being actively explored as antitumor modalities (36-38). Multiple research teams have attempted to exploit the interaction between OX40 and other TNF family receptors to suppress cancer progression. The approaches that showed some potency include antibodies, various protein fusions, and OX40 agonists, such as RNA aptamers (39-41) and small molecules (42). These molecules also activated T4⁺ lymphocytes, causing their proliferation and IFN- γ secretion, which, in turn, boosts the adaptive immune response. Experimental evidence indicates that broad anticancer immunity depends on the endowment of these molecules with Fc fragment moiety—either as a natural part of the antibody or as a fusion with the therapeutic protein of interest.

One of the most spectacular examples of how *in situ* vaccination can help eradicate tumors in mouse models has been published by Sagiv-Barfi and colleagues (3). In this work, the synergistic action of intratumorally administered CpG and agonistic anti-OX40 antibody cured multiple hard-to-treat immunogenic tumors, both spontaneous and established.

CMLD CP-based chemotherapy

Recent reports have highlighted the ability of CP to remodel the tumor microenvironment, which is known to severely suppress the activity of tumor-infiltrating immune cells. As it turns out, CP exerts a stimulating effect on such cells, and significant progress in understanding the mechanism(s) of CP activity in this context has been achieved for the low-dose metronomic regimen.

As a monotherapy, standard high-dose CP injection in mice results in long-term (6-20) reduction of the numbers of circulating immune cells in the peripheral blood, bone marrow, and spleen (43,44). CP also stimulates a protumor activity of tumor stroma cells, thereby leading to tumor growth and progression (45). This weakened immune control of the tumor ultimately results in the formation of TISC-derived cellular clones resistant to CP (6,43,44,46-52,53). On the other hand, low-dose metronomic delivery of CP has been shown to stimulate immune cells, particularly tumor-infiltrating cells such as natural killer cells (NK), NKT, and DCs. Importantly, such a treatment regimen switches the polarization of tumor-associated macrophages and may result in tumor regression (17,54).

The antitumor activity of CMLD CP-based chemotherapy is largely involves the remodeling of MDSCs and the mature myeloid cell network. Basically, it represents a regulatory type of therapy that remodels the tumor stroma and prepares the tumor for efficient targeting by the factors of innate and adaptive immunity. This feature of CP is presently considered the core of its therapeutic anticancer activity. Chemotherapy based on CMLD CP makes it possible to directly kill cancer cells and repeatedly disrupt the suppressive tumor milieu (45). Notably, low-dose metronomic chemotherapy is known to work well only for CP but not for other cytostatic drugs.

Presently, three major avenues of CP-based chemotherapy are being actively explored. These include various regimens of chronometric or metronomic low-dose delivery, synergistic activity of CP and CpG oligonucleotides, and the combination of CP with various antibodies and cytokines.

Several studies have focused on testing the sequence, dose, and frequency of chronometric or metronomic low-dose chemotherapy based on the administration CP alone or in combination with CpG. These empirical studies share one goal: to cure the laboratory animals of cancer. For instance, Manrique and colleagues comprehensively investigated various regimens and doses of chemotherapeutic drugs, types of CpGs, injection schedules, sites of injections, and duration of chemotherapy courses (18). The optimal scheme was based on intraperitoneal injection of 200 mg/kg CP every 7 days for 4–6 courses, and CpG (5 mg/kg) was delivered on day 3 following CP injection, as this time was essential for the recovery of bone marrow after CP chemotherapy (which was then followed with a 4-day rest period). It was found that the CP dose of 100 mg/kg was ineffective; the injection site was a nonfactor; 5-fluorouracil, irinotecan, sunitinib, temozolomide, gemcitabine, docetaxel, paclitaxel, oxaliplatin, and doxorubicin—both as a monotherapy and in combination with a metronomic co-delivery with CpG—were either ineffective, toxic, or poorly tolerated.

In other studies, complete curing of mice engrafted with immunogenic tumors was observed as a result of developing a similar drug delivery schedule (5,17). CP was injected every 6 or 12 days at a dose of 140–200 mg/kg (up to 4 courses), and CpG (100 mg/kg per single intratumoral injection) was administered simultaneously with CP. Interestingly, daily low-dose CP-based chemotherapy had little, if any, antitumor effect, while chronometric or metronomic injection of CP every 6 days resulted in the recruitment of innate immune cells to the tumor and stimulated their activity (5,17).

Chemotherapy based on CMLD CP results in the activation of various cells of innate immunity, leading to broad and multipronged anticancer effects. Activation of tumor-infiltrating lymphocytes is associated with stronger expression of various markers, such as Nkp46, Nkg2d, Prf1, Gzmb (NK1.1), Cd207 and Cd74 (DCs), and Cd86 (F4/80 and Emr1, macrophages). Interleukin 15 (IL-15) and IL-18 are among the upregulated genes responsible for the development, proliferation, and cytotoxicity of NK cells. In the tumor, stronger B220 expression associated with killer interferon-producing DCs can be observed following CP treatment. These cells kill neighboring tumor cells, and engulf and present tumor antigens, thereby bridging the innate immune cell killing and activation of adaptive T and B cell responses (55). CP induces secretion of CCL21 by the tumor stroma, which attracts peripheral immune cells

such as macrophages, NKs, NKTs, and DCs, which in turn act together to destroy the tumor (45,56-61). Low-dose CP treatment leads to an increase in DC progenitors in bone marrow. These cells then mature into active DCs, which can activate adaptive immunity. Higher levels of Tlr7 and Csf1 contribute to the maturation of DCs (56). Finally, Icam2 and Icam1, expressed by endothelial and immune cells, are required for the transendothelial migration of immune cells to the tumor site (5,17,61).

There are two major cell types present in the tumor stroma that display protumor features; these include regulatory T cells (Tregs) and MDSCs. Nontoxic low-dose chemotherapy with CP renders cancer cells immunogenic due to elimination of suppressive Tregs and reversion of MDSC functionality towards an antitumor phenotype.

CD25+ FOXP3 + T lymphocytes are Tregs. They secrete TGF-beta 1, IL-10, IFN- γ , and IL-35, while expressing CTLA-4. The aforementioned cytokine and CTLA4/CD68 interaction may suppress the activity of effector T cells and DCs. Chemotherapy based on CMLD CP is highly effective at breaking the suppressive function of lymphocytes and may eliminate such cells from the tumor microenvironment. Moreover, one of the striking features of CMLD CP-based chemotherapy is that Tregs and other T cell subpopulations are differentially affected. This is likely due to the failure of repair machinery in Tregs to restore chromatin breaks following interstrand crosslink induction by CP (62,63). Consequently, CP treatment results in the loss of Treg inhibitory activity or complete eradication of Tregs from the tumor, whereas effector T cells are spared and may thereby mediate a potent immune response.

The largest population of immune cells forming the tumor stroma is composed of MDSCs, which comprise CD11+Gr-1+ myeloid cells displaying the intermediate differentiation phenotype. MDSCs constitute 30% of the total number of bone marrow cells and 4% of all mononuclear cells of the spleen. Murine MDSCs are represented by two major subpopulations. Anti-Gr-1 antibodies (R B6-8C5) bind the same epitope on two distinct molecules, Lyc6G and Lyc6C, which are found on MDSCs. Thereby, two MDSC fractions known as granulocyte/polymorphonuclear MDSCs (CD11b+Lyc6G+Lyc6Clow, similar to neutrophils in terms of morphology) and monocyte MDSCs (CD11b+Lyc6G-Lyc6Chigh, similar to monocytes) can be found. The first fraction may constitute up to 80% of all MDSCs. Monocyte MDSCs are largely represented by tumor-associated macrophages and may constitute up to 50% of the weight of tumor-associated

stroma. In growing solid tumors, monocyte MDSCs are known to quickly differentiate into M2 tumor-associated macrophages (64-66). Both cell populations of MDSCs actively suppress immune cells, mostly tumor-infiltrating T cells. Various mechanisms are involved and include interaction between TIM-3 and galectin-9, ADAM17 and CD62L, arginase 1, iNOS, TGF-beta 1, IL-10, COX-2, VEGF, TGF beta 1, and indoleamine-2-3-dioxygenase (IDO) among other factors (67-69).

Chemotherapy based on CMLD CP switches the properties of MDSCs from protumor to antitumor status. This primarily occurs via repolarization of tumor-resident M2 macrophages towards a M1 phenotype, which is associated with active secretion of lysozymes and stronger antibody-dependent cellular cytotoxicity (ADCC)-mediated phagocytic activity (61,70,71). Antitumor properties of MDSCs can be significantly enhanced by combining CMLD CP-based chemotherapy with CpG oligonucleotides, antitumor vaccine, or cytokines (72-75).

Thus, CMLD CP-based chemotherapy—used as a monotherapy or in combination with CpG, vaccines, or cytokines—has the following therapeutic features that can destroy the tumor.

- ❖ Being a cytostatic drug, CP directly kills cancer cells by inducing aberrant mitosis and apoptosis.
- ❖ Besides displaying direct cytotoxicity, CMLD CP-based chemotherapy (both as a monotherapy and as combinations with various immunomodulating agents) activates innate and adaptive immunity, which contributes to tumor eradication.

Major events occurring during this therapy are as follows:

- (I) Recruitment of immune cells from the periphery into the tumor focus.
- (II) Activation of professional properties of tumor-infiltrating cells of innate and adaptive immunity.
- (III) Elimination of tumor-associated T-reg.
- (IV) Conversion of MDSCs from the protumor to antitumor phenotype, with M2-to-M1 transition of macrophages being prominent in this process.

This multipronged attack against the tumor forms the foundation of the high efficacy of CMLD CP-based chemotherapy in cancer patients.

Karanaban technology

Studies performed in the laboratory of induced cell processes at the Institute of Cytology and Genetics, Siberian

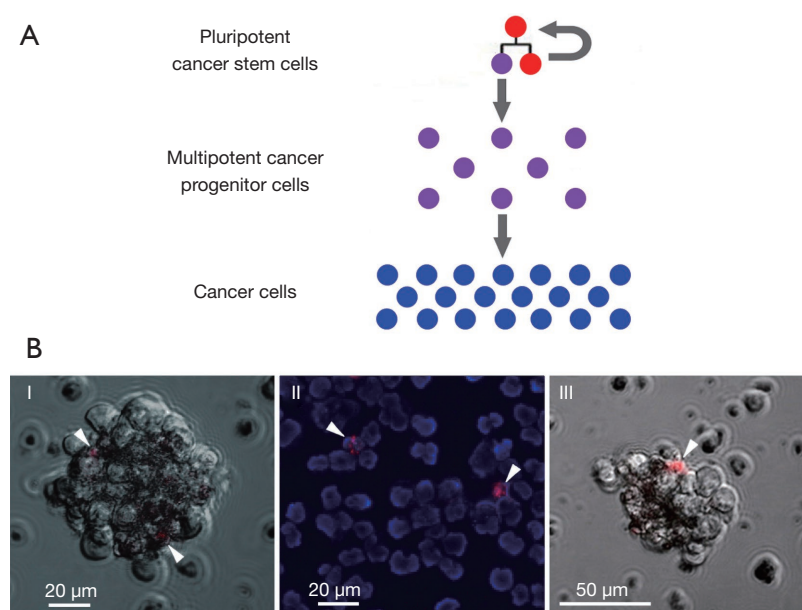


Figure 1 Cancer stem cells. (A) A model of cellular hierarchy within the tumor, with a self-perpetuating population of pluripotent cancer stem cells found on the top. (B) Various cancers encompass a subpopulation of DNA-internalizing cancer cells, referred to as TAMRA+ cancer stem cells (arrowheads): (I) sphere-forming cell line arising from human Epstein-Barr virus-induced lymphoma; (II) mouse Krebs-2 ascites; (III) a human lymphoma cell cluster. Cells (10^6) were incubated with 0.1 mkg TAMRA-labeled DNA fragment (human Alu repeat, about 500 bp) for 1 hour at room temperature in the dark.

Branch of the Russian Academy of Sciences (ICG SB RAS) have established a mechanism for the synergistic activity of CP and a composite double-stranded (dsDNA) preparation (DNAmix) against TISCs. These efforts have culminated in the development of a novel anticancer modality referred to as *Karanaban* technology (कारणहन्, from the Sanskrit *kāraṇa* [“source”] + *han* [“to kill”]). This approach primarily targets TISCs and induces massive apoptosis of committed tumor cells (6-8).

Karanaban technology is based on the three discoveries.

- ❖ Low-differentiated TISCs were found to share a universal molecular marker: they are capable of internalizing fragments of extracellular dsDNA. This feature can be conveniently exploited by using tetramethylrhodamine (TAMRA)-labeled DNA probes (*Figure 1*).
- ❖ Once internalized, these DNA fragments can potently interfere with the repair of interstrand DNA crosslinks produced by an earlier treatment with CP. This results in either direct TISC killing or in their loss of tumor-inducing properties. Without this supply of new tumor cells, the tumor is destroyed by the immune cells of an organism (3).

Within a given tumor, following CP treatment(s), synchronization of TISCs in their cell cycle can be achieved. Targeting them during a sensitive period of the cell cycle via the chronometric or metronomic delivery of cytotoxic agents may translate into the complete eradication of TISCs.

The process of repairing interstrand crosslinks after exposure to a crosslinking cytostatic, the main events occurring in the population of tumor cells following the application of *Karanaban* technology, and principles of CP and DNAmix administration within the *Karanaban* framework have been previously conceptualized in the figure published in a previous work (19).

This technology includes the following several steps:

- (I) The percentage of low-differentiated TISCs within the tumor are measured (internalization of TAMRA+ DNA probe followed by FACS or microscopy analysis).
- (II) The temporal profile of interstrand crosslink repair induced by the prior treatment with CP or mitomycin C (MMC) is clarified.
- (III) The day when therapy-sensitive TAMRA+ TISCs synchronously enter the G1 phase of a cell cycle is

identified—this occurs after 3 consecutive S/G2/M accumulation steps and is based on the information on the dynamics of the repair cycle.

- (IV) DNAmix (6) is added to the therapy exactly at the timepoint that corresponds to the switching from the nucleotide excision repair (NER) to the homologous recombination (HR) phases so that a subfraction of the DNAmix molecules interferes with NER, and the rest of the molecules block HR. This prevents TISCs from counteracting the therapeutic "strike" and either kills them or renders them nontumorigenic. In parallel, addition of the DNAmix leads to a massive lysis of committed cancer cells.

This technology has been successfully tested on several experimental mouse and human cancers and in 3 pilot clinical cases (6,8,76-79).

Shared features of *in situ* vaccination, metronomic low-dose CP-based chemotherapy, and *Karanahan* technology, and the advantages of their combination into a single universal anticancer therapy

The rationale behind this review is to open a discussion regarding whether the basic mechanisms underlying these 3 technologies can be safely combined into a novel approach that would be capable of inducing potent systemic anticancer immunity, reverting the immunosuppressive tumor microenvironment, inducing the apoptosis of differentiated cancer cells, and precipitating the eradication of TISCs.

In our opinion, this approach would perfectly complement the above three technologies and compensate for each of their weaknesses. Specifically, *in situ* vaccination would be improved by the introduction of an element that would render it efficient in the context of nonimmunogenic "cold" tumors. In turn, *Karanaban* technology incorporating the major features of the CMLD CP-based chemotherapy, namely the 6- to 7-day interval between the injections and the use of CP, would benefit from the induction of systemic antitumor immunity downstream of the receptor/ligand-mediated APC activation.

DNAmix and CP treatments are core elements of the *Karanaban* technology and CMLD CP-based chemotherapy

At the core of *Karanaban* technology and CMLD CP-

based chemotherapy is the use of DNAmix and CP. Their combination is rationally motivated and has been shown to be effective in "incurable" cancers, such as Krebs-2, U-87 xenografts, and B cell lymphomas in mice (6,8,78,80,81).

It has been incontrovertibly shown that systemic anticancer immunity is boosted by such therapy (6,8,77). Nonetheless, *Karanaban* technology is largely based on the eradication of TISCs and induction of massive apoptosis of committed cancer cells, rather than on anticancer immunity. This is supported by the following experimental data.

Only cancer cells comprising a TAMRA+ population of TISCs are capable of stable engraftment in mice. Cancer cells that are TISC-depleted (cell sorting or selective TISC killing by CP and DNAmix) fail to engraft in syngeneic or immunodeficient mice (7,78). Notably, there is no contribution of the host immunity, as cytostatic drug treatment occurs *ex vivo* (another cross-linking cytostatic drug, MMC) prior to engraftment. Likewise, cell sorting has also been performed in cancer cell lines cultivated *in vitro*, so the observed failure to engraft can be attributed to the mouse immune system.

In our study, complete elimination of Krebs-2 tumors in mice could only be achieved via CP/DNAmix-based targeting of TISCs. This approach was also effective in a xenogeneic setting, with human U-87 glioblastoma cells being used as a model (7,78). Our studies have firmly established that single or metronomic injections of CP and DNAmix are inefficient in treating such animals. This only becomes possible if the treatments are given to the animals at specific time points (depending on the dynamics of the DNA repair in each particular tumor) and when TISCs are completely eradicated once they become synchronized in a sensitive phase of the cell cycle. Even nonimmunogenic tumors can be eliminated this way. The same treatments performed without taking into account the repair dynamics invariably fail to fully cure the animals and lead to relapses (6,8) despite activating the host immunity.

Indeed, *Karanaban* technology conveniently combines all the basic indicator parameters identified empirically for CMLD CP and CpG-based chemotherapy.

- (I) 300 mg/kg of CP is given during the first 2 days, on days 5–9 an additional (final) dose of 100 mg/kg is given *vs.* 140–200 mg/kg is given every 6–7 days (18);
- (II) DNAmix is used as an activator of DCs *vs.* CpG activates the same APCs (5,17).
- (III) This means that use of *Karanaban* technology leads to the following key events, which are either unique or analogous to those observed

after CMLD CP-based chemotherapy: TISCs are eradicated, differentiated cancer cells undergo apoptosis, suppressive activity of Tregs and MDSCs is abrogated, tumor-infiltrating lymphocytes are activated, and immune cells are recruited from the periphery.

Principle of a combination of the three technologies

Our analysis indicates that there are three major effects that may ultimately target the tumor for destruction.

- (I) CpG combines with antibodies blocking checkpoint inhibitors or TNF family molecules. This treatment stimulates cytolytic activity of tumor-infiltrating immune cells and professional properties of DCs. It also helps destroy cancer cells and presents the broad range of cancer antigens to DCs “on site”, which results in the induction of adaptive immunity.
- (II) CMLD CP-based chemotherapy primarily targets the cellular components of a suppressive tumor microenvironment (Tregs, MDSCs). It also activates tumor-infiltrating immune cells and helps recruit periphery-derived, otherwise nonsuppressed immune cells into the tumor. When used in combination with CpG oligonucleotides, the effects are additively enhanced compared to those derived from a CP-only regimen.
- (III) In *Karanaban* technology, the first 3 CP injections over the course of 48–72 hours block proliferation of cancer cells and result in their massive apoptosis. When DNAmix is delivered during the specific timepoint of NER/HR phase, this additively enhances the apoptosis of committed cancer cells, drives TISCs towards an apoptotic pathway (as interstrand DNA crosslink repair is blocked), synchronizes TISCs in the G2/M(G1) phase of the cell cycle, activates the professional properties of DCs, and completely eradicates TISCs from the tumor. Injection of CP and DNAmix on days 5–9 provides the final “strike” to the surviving TISC population. In this setting, CP has a multifaceted activity: it alleviates tumor-infiltrating immune cell suppression, recruits peripheral immune cells, and activates all of the tumor-resident immune cells.

A combination of technologies makes sense when the weak aspects of one technology are compensated for by the

strengths of the others and when the resulting approach is superior to any single technology used alone. There are 4 main aims of this combination: TISC eradication, apoptosis of differentiated cancer cells, alleviation of immune suppression, and activation of antitumor immunity.

Karanaban technology includes CMLD CP-based chemotherapy and has all the features of CMLD CP-based chemotherapy and CpG oligonucleotides. This indicates that *Karanaban* technology may entirely substitute CMLD CP-based chemotherapy, which targets Tregs and MDSCs for destruction, activates tumor-infiltrating immune cells including antigen-presenting DCs, and recruits peripheral immune cells into the tumor. The unique feature of *Karanaban* technology is that it allows complete TISC eradication, which leaves the tumor highly sensitive to the activated immune response.

Inclusion of immune checkpoint blockers or TNF family-specific antibodies (3) in DNAmix injections makes it possible to launch a fully fledged antitumor immune response mediated by ligand-receptor pathways. In this case, CpG is substituted for the DNAmix preparation, which essentially mimics all the effects of CpG.

There are several reasons why *Karanaban* technology may serve as an excellent unifying platform for two other anticancer approaches. The combination of *Karanaban* technology and *in situ* vaccination includes the use of DNAmix instead of CpG. An efficient dose of either agent may result in similar stimulation of the professional properties of DCs (82), the first component of *in situ* vaccination. In the context of *Karanaban* technology, DNAmix induces total apoptosis of committed cancer cells and launches the molecular events leading to the eradication of TISCs. The second component of *in situ* vaccination, namely the injection of OX40-specific antibodies, facilitates the stronger activation of the T cell response and may complement the therapeutic activity of the *Karanaban* technology with an adaptive immune response.

Once *Karanaban* technology and CMLD CP-based chemotherapy are combined, the common themes are (I) the similar therapeutic doses of CP and (II) the similar timing between the first 2 injections of CP in accordance with CMLD CP-based chemotherapy {days 0-[6-7]}, and the timing between tightly grouped, cell-cycle dependent triple injections of CP and the final “strike” injection that eradicates TISCs in accordance with *Karanaban* technology {days 0-[4-9]}.

Completion of the *Karanaban* technology therapeutic regimen leads to the induction of total apoptosis in

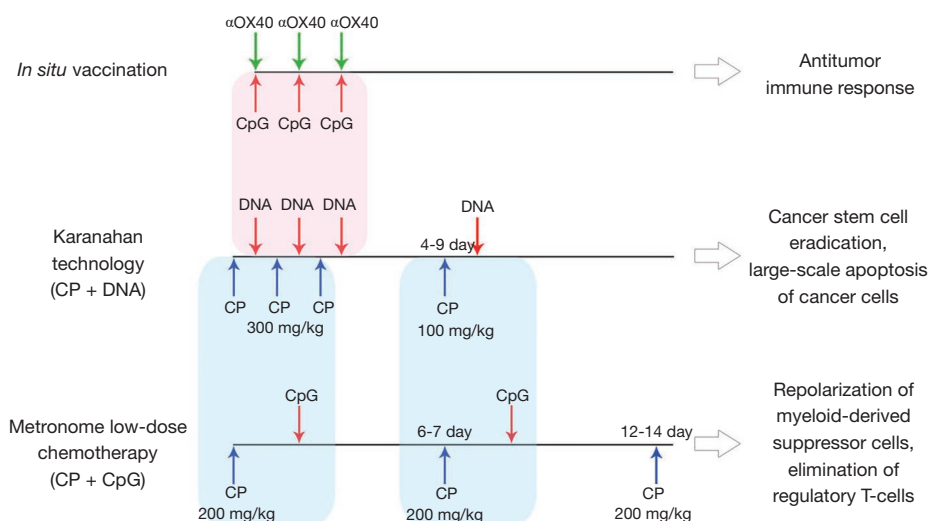


Figure 2 Side-by-side comparison of the three technologies. Shaded areas denote overlapping features of the approaches. α OX40, anti-OX40 antibodies; CpG, short synthetic single-stranded DNA molecules containing CpG motifs; CP, cyclophosphamide.

committed cancer cells and launches the events resulting in TISC elimination. The third injection of CP {days 0-[4-9]-[12-14]} (unless the tumor has been completely lysed by the preceding treatments according to *Karanaban* technology protocol) will serve to further strengthen the effect by eliminating the suppressive activity of the remaining tumor-associated stroma (*Figure 2*).

Once *Karanaban* technology is combined with just a single element of *in situ* vaccination platform and a single element of CMLD CP-based chemotherapy, 3 independent pathways to target the tumor are simultaneously induced. The cumulative therapeutic effect of such a combined approach leaves little likelihood that the primary tumor and the distant metastases will survive (*Figure 3*).

Anticipated obstacles in the clinical implementation of the novel "three in one" anticancer platform

Combining the three technologies may indeed cause problems with adapting the resulting platform to real-world clinical practice. This platform has a target population of stage 4 cancer patients for two reasons. First, this would be consistent with the current health care regulations in most countries. Second, our own studies of early-stage cancer samples indicate that such tumors display a discrete proliferative activity (83), which may complicate the accurate assessment of the cell cycle or repair duration

as an input. Several technical issues exist as well. One is that some tumors are not accessible for intratumor injections due to their size and localization. In such cases, guided drug delivery may be considered. Second, different metastases may be heterogeneous in terms of their cell cycle parameters, which are key for the efficacy of *Karanaban*. This would necessitate the analysis of multiple biopsy specimens and a highly coordinated injection schedule. Third, delivery of the CP metabolite, phosphoramidate mustard, is not uniform across different organs and tissues. Our limited clinical trial experience (data not shown) indicates that CP injections spare bone metastases. This is typically interpreted as the failure of phosphoramidate mustard to efficiently reach this tissue, which is consistent with its poor blood supply. *Karanaban* monotherapy results in only partial response of liver-localized metastases, which may be attributable to insufficient concentration of the CP metabolite once it is produced in the liver and returns back from the circulation throughout the body. Ways to address this issue would be either to increase the CP dose or to provide ultrasound-guided delivery inside or adjacent to the tumor of directly alkylating drugs (nitrogen mustard, MMC). Injection of antibodies would facilitate the activation of T cell immunity, helping to eradicate a difficult-to-target cancer.

Activation of immunocompetent cells such as neutrophils and NK cells is of critical importance. Accurate timing of CP and DNAmix injection results in massive apoptosis of

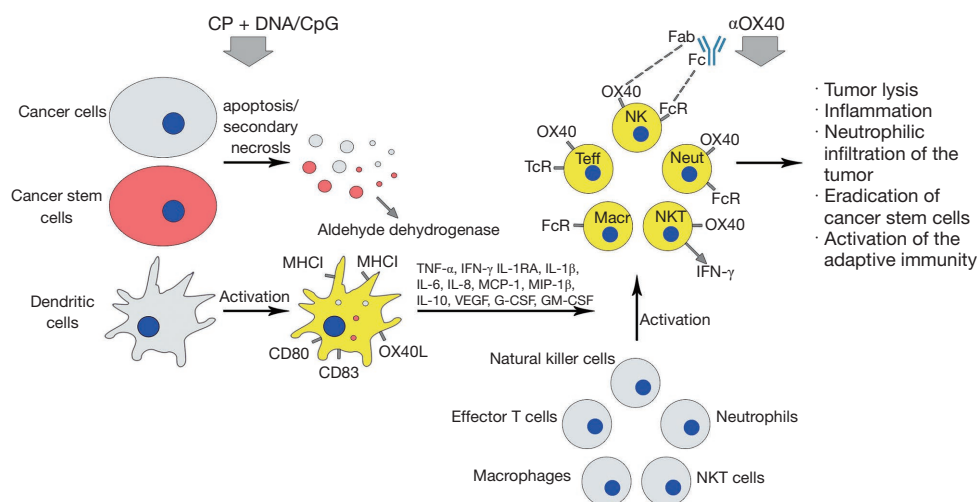


Figure 3 The progression of events induced by the integrated “three-in-one” technology. Successive intratumoral CP and DNAmix administration, strictly tied to the DNA repair timing, initiates the lysis of tumor cells that results in the formation of cellular debris consisting of apoptotic and necrotic components, elimination of cancer stem cells, disruption of the suppressive properties of tumor-associated stromal cells, development of local inflammation, and activation of the antigen-presenting properties of dendritic cells, which begin to secrete a wide range of cytokines (TNF- α , IFN- γ , IL-1RA, IL-1 β , IL-6, IL-8, MCP-1, MIP-1 β , IL-10, VEGF, G-CSF, and GM-CSF). These cytokines and direct cell–cell interactions activate tumor-infiltrating immune cells: effector T cells (Teff), NK, Neut, NKT, and Macr, which begin to express OX40 and FcR receptors on their surface. Anti-OX40 antibodies initiate a cascade of cellular and humoral reactions causing the development of adaptive immunity. CP, cyclophosphamide; NK, natural killer cells; Neut, neutrophils; NKT, natural killer T cells; Macr, OX40, macrophages; anti-OX40 antibodies.

cancer cells and subsequent secondary necrosis and tumor lysis, essentially forming a large inflammatory region that attracts neutrophils from the periphery. Chronometric delivery of CP also moderately potentiates NK cells (84) and antigen-presenting DCs. This results in the secretion of proinflammatory cytokines and growth factors, such as IL-1, TNF- α , IFN- γ , and G-CSF (85,86). DC activation in turn stimulates NKT cells and provides additional production of IFN- γ (29,87). To summarize, all these events may culminate in a cytokine storm and systemic inflammatory reaction (6,84,88). IFN- γ and G-CSF/GM-CSF stimulate surface expression of Fc receptors on neutrophils and NK cells. Consequently, delivery of OX40-specific antibodies will not only boost T cell response but will also actively involve neutrophils and NK cells via Fc/FcR-mediated ADCC (33,89–92). Further upsurge of inflammation should follow (33,93) and may manifest as multiple necrotic foci due to uncontrolled and nonspecific cytotoxic activity of neutrophils and NK cells, small vessel thrombosis, systemic inflammation, and multiple organ failure leading to death. Additional studies are required to comprehensively assess the likelihood of such a scenario. To

overcome the above issues, transparent and easy-to-follow guidelines are needed for performing efficient resuscitation procedures and preventing such adverse reactions. This set of interventions may include the use of thrombolytic drugs and blood dialysis, as well as the administration of drugs that block renal, hepatic, and pulmonary insufficiency observed under severe sepsis conditions.

Conclusions

We hypothesize that very potent anticancer activity can be achieved once these three therapeutic modalities are combined into a single approach.

One factor that is key to the combination of the three technologies is timing. The mechanisms underlying any of these technologies should not interfere with each other. Specifically, one must estimate the time required to activate DCs using DNAmix, the time to activate innate and adaptive immunity by OX40-specific antibodies, the time needed for TISC eradication, and finally, the time required for total cancer cell apoptosis.

In principle, this “three-in-one” platform is envisaged

to efficiently destroy any tumor that is dependent on the pool of TISCs. Whether or not it will work depends on how the technical issues and biological features of the tumor in a given patient are addressed. We strongly believe that this platform will be broadly used in terminal-stage cancer patients who are given maintenance support and in those patients with inoperable tumors. Tumor eradication or reduction of tumor burden open an opportunity for proceeding to the next line of therapy or therapy consolidation.

Acknowledgments

The authors are sincerely grateful to Andrey A. Gorchakov for the brilliant translation of the manuscript.

Funding: This work was supported by the Russian Ministry of Science and Higher Education via the Institute of Cytology and Genetics (State Budget Project No. 0259-2021-0013), the Russian Foundation for Basic Research (Project No. 18-29-09045), and "Karanahan" LLC.

Footnote

Reporting Checklist: The authors have completed the Narrative Review reporting checklist. Available at <https://dx.doi.org/10.21037/tcr-21-919>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/tcr-21-919>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Cite this article as: Proskurina AS, Ruzanova VS, Ostanin AA, Chernykh ER, Bogachev SS. Theoretical premises of a "three in one" therapeutic approach to treat immunogenic and nonimmunogenic cancers: a narrative review. *Transl Cancer Res* 2021;10(11):4958-4972. doi: 10.21037/tcr-21-919