Attenuated *Salmonella* Targets Prodrug Activating Enzyme Carboxypeptidase G2 to Mouse Melanoma and Human Breast and Colon Carcinomas for Effective Suicide Gene Therapy

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**Abstract**

**Purpose:** We engineered the oncolytic *Salmonella typhimurium* – derived bacterium VNP20009 as a vector to target delivery to tumors of the prodrug-activating enzyme carboxypeptidase G2 (CPG2) and to show enhanced antitumor efficacy on administration of different produgs.

**Experimental Design:** We characterized CPG2 expression in vectors by immunoblotting, immunofluorescence, and enzyme activity. We assessed prodrug activation by high-performance liquid chromatography. Target human tumor cell and bacterial vector cell cytotoxicity was measured by flow cytometry and colony-forming assays. Therapy was shown in two human tumor xenografts and one mouse allograft with postmortem analysis of bacterial and CPG2 concentration in the tumors.

**Results:** CPG2 is expressed within the bacterial periplasm. It activates produgs and induces cytotoxicity in human tumor cells but not in host bacteria. Following systemic administration, bacteria multiply within xenografts reaching $2 \times 10^7$/g to $2 \times 10^8$/g at 40 days postinoculation. The concentration of CPG2 in these tumors increases steadily to therapeutic levels of 1 to 6 units/g. The bacteria alone reduce the growth of the tumors. Subsequent administration of produgs further reduces significantly the growth of the xenografts.

**Conclusions:** The bacteria multiply within tumors, resulting in a selective expression of CPG2. The CPG2-expressing bacteria alone reduce the growth of tumors. However, in the presence of produgs activated by CPG2, this oncolytic effect is greatly increased. We conclude that bacterial oncolytic therapy, combined with CPG2-mediated prodrug activation, has great potential in the treatment of a range of cancers.

In suicide gene therapy, a variety of vectors has been used to introduce the genes encoding prodrug-activating enzymes selectively to tumor cells, rendering them sensitive to a prodrug, avoiding the toxicity usually associated with cytotoxic chemo-therapy. These include viruses, chemical complexes, naked DNA, and bacteria (1, 2). Most studies of bacterial vectors have involved *Clostridium* sp. spores with the innate property of germinating and proliferating only in the hypoxic milieu of the necrotic center of tumors (3, 4), although the bacterial species *Bifidobacteria*, *Lactobacillas*, and *Caulobacter* have also been employed in this way (5, 6). We report the use in an analogous context of the facultative anaerobic bacterium VNP20009 (7, 8). A similar *Salmonella*-derived vector has been used to target small interfering RNA (9). Our VNP20009 vector is a derivative of *Salmonella typhimurium* (ATCC 14028), attenuated by disruption of the genes *msbB* and *purI*. The *msbB* gene regulates the addition of a terminal myristoyl group to lipid A, which otherwise induces tumor necrosis factor-α, causing septic shock in infected patients (10). The *purI* gene is involved in purine biosynthesis. Thus, purI deletion mutants are auxotropic for purines but have been shown to proliferate in tumors (11), although the mechanism is unknown. Animal studies (12) and phase I clinical studies (13) have established the safe doses for systemic injection of VNP20009. The maximum tolerated dose was $3 \times 10^9$ colony-forming units (cfu)/m$^2$, with dose-limiting toxicities encountered in patients receiving $1 \times 10^9$ cfu/m$^2$, and focal tumor colonization observed in 3 of 24 patients (13). In the preclinical studies, intratumoral...
bacterial proliferation occurred to levels of 10^9 cfu/g, 10,000-fold higher than in the liver (the next most colonized tissue; ref. 10), these levels being too low to generate systemic toxicity. As a consequence of this selective colonization, perhaps involving immune responses (14), VNP20009 exerts an oncolytic effect and is effective in dogs with spontaneous cancers (15). We sought to enhance the efficacy further by engineering the bacteria to express the prodrug-activating enzyme carboxypeptidase G2 (CPG2; E.C. 3.4.17.11).

The gene encoding CPG2 was originally cloned from Pseudomonas sp. strain RS-16 (16). The enzyme is a Zn^2+ dependent 83-kDa homodimer. CPG2 cleaves folic acid to pteric acid and also activates a range of bifunctional alkylating agent and antibiotic prodrugs (17–22). CPG2 possesses several properties that make it suitable for suicide gene therapy. There is no human analogue, and no cofactors are required; it catalyzes a one-step activation, produces drugs that are cytotoxic to both cycling and quiescent cells, and can activate a large range of prodrugs. CPG2 catalyzes the hydrolysis of a deactivating glutamate moiety from various nitrogen mustard prodrugs to yield bifunctional alkylating agents that are more reactive than the prodrug and, being less polar, have greater penetration into cells where they produce DNA-DNA interstrand cross-links and cell death. A large number of these aromatic nitrogen mustard prodrugs have been synthesized. 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dried milk powder. Immunodetection of CPG2 by a previously described (29) rabbit polyclonal antiserum was followed by a donkey anti-rabbit antibody conjugated to horseradish peroxidase. Detection was by enhanced chemiluminescence reagent (Amersham Pharmacia).

**Determination of CPG2 enzymic activity in bacteria**

Bacteria were lysed with a nonionic detergent (“Bug Buster” Novagen) and CPG2 activity in the extract was determined by a kinetic method using methotrexate as substrate (29). Briefly, aliquots were added to cuvettes containing assay buffer and methotrexate, and the rate of decrease in ΔA20 was measured spectrophotometrically. CPG2 (1 unit) is defined as the amount that degrades 1 mmol methotrexate/min.

**Immunofluorescence**

Bacteria were harvested by centrifugation, washed once in PBS, and fixed with 3% paraformaldehyde. For permeabilization, cells were resuspended in 0.1% Triton X-100, washed, and resuspended in PBS/lysozyme (100 μg/mL)/5 mmol/L EDTA. Samples were incubated with anti-CPG2 rabbit antiserum followed by labeled anti-rabbit antibody (Alexa 488 nm; Molecular Probes/Invitrogen). The DNA was counterstained with TO-PRO-3 dye (Molecular Probes/Invitrogen) and the samples were viewed using a confocal microscope.

**Cell fractionation**

Bacteria were lysed using osmotic shock. Briefly, bacteria were resuspended in a hypersonic solution containing 20% buffered sucrose (pH 8) on ice for 10 min, centrifugally pelleted, resuspended in plain buffer, and incubated on ice for another 10 min at which stage the outer cell membrane ruptures. The inner cell membrane and cytoplasm (spheroplasts) were centrifugally harvested at 4,000 rpm. The periplasm and outer membrane in the supernatant were harvested at 50,000 × g for 30 min. The amount of CPG2 in each subcellular fraction was determined by immunoblotting. Gel loading was standardized by adjusting the percentage of the total harvest for each subcellular fraction.

**Assessment of cytotoxicity**

**Cell cycle analysis by flow cytometry.** WiDr cells were seeded in six-well plates (1 × 10^6 per well) 16 h before addition of prodrug 1 (40 μmol/L) and bacterial vectors at concentrations as indicated. After 90 min, the cells were washed three times with fresh medium containing penicillin (12 μg/mL), streptomycin (20 μg/mL), and chloramphenicol (10 μg/mL) and incubated for a further 2 days. This dose-time of 40 μmol/L × 90 min = 3600 μmol/L min is commensurate with the ALIC of 5,763 μmol/L min resulting from the safe dose of 537.6 mg/m^2 in phase I clinical trial (25). Adherent and detached cells were harvested, washed in PBS, and fixed in 70% ethanol. The cells were washed in PBS and resuspended in PBS containing RNase (10 μg/mL) and propidium iodide (40 μg/mL). The samples were incubated at 37°C for 30 min and analyzed by flow cytometry (FACSCalibur; Becton Dickinson; software package: CellQuest). Data from 20,000 cells were plotted as counts (abscissa) versus fluorescence intensity (ordinate).

**Colony-forming assay.** WiDr cells were exposed to prodrug in the presence or absence of vectors as above. After the initial 90-min exposure, cells were replated in antibiotic-containing medium at limiting dilutions, allowed 14 days growth, fixed in 70% cold ethanol, and stained with crystal violet (1%, w/v). Colonies were scored and cell survival was expressed as percentage of control.

**Determination of prodrug activation in ppmCPG2-VNP20009ad**

VNP20009 or ppmCPG2-VNP20009ad was suspended in PBS/1% glucose/2 mmol/L MgSO4, and prodrug 2 was added to 1 mmol/L. Aliquots were spun down and the supernatants were frozen in liquid nitrogen. The samples were quickly thawed, and aliquots (50 μL) were injected onto a C18 column (Whatman Partisphere 4.6 × 100 mm), eluted with a gradient of 20% to 90% methanol/100 mmol/L ammonium acetate (pH 7) over 15 min, and monitored from 250 to 350 nm. Chromatograms were integrated at 300 nm.

**In vivo therapy**

All experiments were conducted in accordance with UK Home Office regulations and UK Coordinating Committee on Cancer Research guidelines (38). Xenografts or allografts were established in nude (nu/nu) female CD1 or C57 black female mice (20-22 g) by s.c. inoculation (0.2 mL) in the right flank of MDA-MB-361 human breast carcinoma or WiDr human colon carcinoma cells (10^5 and 8 × 10^6, respectively) or B16-F10 mouse melanoma cells (4 × 10^3) in PBS. After 8 (WiDr), 10 (B16-F10), or 12 (MDA-MB-361) days, mice were allocated by stratified distribution into control and treated groups. ppmCPG2-VNP20009ad (2 × 10^6 cfu) were diluted in PBS/2 mmol/L MgSO4 and injected into the tail veins. Prodrugs were dissolved in DMSO and diluted 20-fold in 1.26% (w/v) sodium bicarbonate just before injection. Each course of prodrug treatment consisted of three i.p. injections over a 24-h period to a total preestablished maximum tolerated dose of 1,500, 1,200, and 600 mg/kg for prodrugs 2, 3, and 4, respectively. Courses of prodrug were administered on days 7, 14, 28, 35, and 42 (±1 day) after vector. Animals were culled if the tumor exceeded 1.5 cm in any dimension, or if a scab formed, as demanded by the UK Home Office license requirement (38). At cull, tumors were excised and analyzed for CPG2 enzyme activity and for the presence of colony-forming bacterial cells. Similar measurements were performed in separate time-course experiments and the data were pooled.

**Determination of CPG2 enzyme activity and bacterial cfu in tumors**

Tumors were homogenized in PBS/2 mmol/L MgSO4 at 10% (w/v). CPG2 enzyme activity was determined as described previously (39). Briefly, homogenates were incubated with methotrexate for 30 min, and the reaction was stopped with acidified methanol. The concentration of the reaction product diaminomethylpteroyl acid in the centrifuged supernatant was determined by high-performance liquid chromatography. An external calibration curve was constructed by spiking control homogenates with standard enzyme. Bacterial cfu were determined by serially diluting homogenates in growth medium and plating on agar plates. Dilutions that gave 10 to 200 colonies after overnight culture were scored. The results were expressed as enzyme units or cfu per wet weight gram of tumor.

**Statistical analysis**

All analyses were performed using the functions described in Results using GraphPad Prism version 4.02 for Windows, GraphPad Software. Computed values are quoted with their 95% confidence intervals in brackets.

**Results and Discussion**

**Characterization of ppmCPG2.** We transformed VNP20009 with the plasmid pTrcOMPAppCPG2 encoding ppmCPG2 and ampicillin resistance, generating ppmCPG2-VNP20009. Expression of CPG2 of expected monomeric apparent molecular weight (~42 kDa) in transformed bacteria was confirmed by immunoblotting (Fig. 1A) and enzymic activity by methotrexate digestion (Fig. 1B). The subcellular localization of CPG2 in ppmCPG2-VNP20009 was verified by immunofluorescence of intact or permeabilized cells (Fig. 1C). Staining was observed only after permeabilization, indicating internal expression of ppmCPG2. The majority of the CPG2 was present in the periplasm as confirmed by immunoblotting of the cytoplasmic, periplasmic, and outer membrane subcellular fractions (Fig. 1D).

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Induction of cytotoxicity by ppCPG2-VNP20009 plus prodrug 1.
The ability of ppCPG2-VNP20009 to mediate prodrug-specific
cytotoxicity was assessed by flow cytometry. Human colon
carcinoma cells (WiDr) were treated with increasing doses of
ppCPG2-VNP20009 or the empty vector EV-VNP20009 with or
without prodrug 1. The cells were subsequently stained with
propidium iodide for flow cytometric cell cycle analysis. The
majority of the control cells or cells treated with EV-VNP20009
were in G1 (Fig. 2A, left). There was a shift in the profile of cells
-treated with ppCPG2-VNP20009 plus prodrug 1 (Fig. 2A, right).
The majority of the cells are in mid-late S-G 2-M, with an
increased proportion seen in a sub-G1 population arising from
dead cells. This pattern is characteristic of the disruption of the
cell cycle that precedes cell death in cells exposed to DNA cross-
linking agents (40). The perturbation was responsive to
bacterial doses from $1 \times 10^6$ to $2.5 \times 10^7$ cfu. At higher doses
of ppCPG2-VNP20009 from $2.5 \times 10^7$ to $6.25 \times 10^8$ cfu, the
majority of cells (>95%) were in non-G1 compartments
(Fig. 2B). EV-VNP20009 bacteria produced no such effect. No
cell cycle shift was observed with supernatant bacterial
medium, showing that CPG2 is not shed into the medium
(data not shown).

We then performed a clonogenic survival assay. WiDr cells
were treated with ppCPG2-VNP20009 in the presence or ab-
ence of prodrug 1, replated at limiting dilutions, and allowed
to form colonies. Tumor cells treated with prodrug alone or
with EV-VNP20009 in the presence of prodrug 1 showed no
effect on cell survival (Fig. 2C). In cells treated with ppCPG2-
VNP20009 plus prodrug 1, we detected a bacterial dose-
responsive reduction in cell viability, illustrating prodrug
activation-specific cytotoxicity. Thus, we show that ppCPG2-
VNP20009 can act as a vector for CPG2 and induce prodrug
sensitivity in infected mammalian cell cultures.

Production of active drug and effect on bacterial viability.
ppCPG2-VNP20009 requires ampicillin selection to maintain
expression. This would be a drawback in vivo where selection
would be difficult. We therefore moved the ompACPG2 gene construct to the plasmid pYA3332 and used the
resulting plasmid pYA3332 ompA-CPG2 to transform the
bacterium VNP20009asd, generating the effectively stable
vector ppCPG2-VNP20009asd, which produced similar levels
of ppCPG2 activity to ppCPG2-VNP20009. To investigate the
in vivo therapeutic efficacy of the suicide gene therapy, we
selected the benzoyl glutamate prodrugs 2 to 4 that have
previously been proven effective in transfected xenograft
models (23) and investigated prodrug activation with
ppCPG2-VNP20009asd. Prodrug 2 has the benefits for
chromatographic studies of being more stable than prodrug 1,
easier to separate, and detect by UV absorbance and yielding
a readily detectable drug (2a). Both prodrug 2 and drug 2a are
unstable in aqueous medium, but their respective degradation
product peaks could be identified by incubation of authentic
compounds in plain buffer. When we incubated prodrug 2 with
cultures of VNP20009 or ppCPG2-VNP20009asd, a time-
dependent loss was observed (Fig. 3A), which is faster for cul-
tures of ppCPG2-VNP20009asd. However, whereas prodrug 2

![Image](image-url)
and its degradation products are seen in supernatant fractions from both cultures of VNP20009 and ppCPG2-VNP20009asd, drug 2a and its degradation products are seen only in the supernatant from ppCPG2-VNP20009asd. All retention times (2, 3.8 min, products 2.5 and 7.0 min; 2a, 5.5 min, products 4.1 and 9.2 min) and spectra matched those from authentic compound. We conclude that prodrug 2 accesses the periplasm and is converted to the cognate drug 2a, which is then released into the surrounding medium. The rates of loss of the prodrug are plotted against time and fitted to an exponential decay (Fig. 3A). The rate constants differ significantly from each other [F(DFn,DFd): 5.9 (1,8); P = 0.041]. From the difference, we then calculated the extent of activation-specific prodrug 2 loss (Fig. 3A).

To establish whether prodrug activation would adversely affect the bacteria, two assays were performed. The CPG2-expressing bacteria ppCPG2-VNP20009 were incubated with the dose of prodrug 1 used to induce cytotoxicity in WiDr cells (40 μmol/L). No significant difference in bacterial viability between treated cells and those untreated or treated with prodrug vehicle alone is found (Fig. 3B). In a separate experiment, VNP20009 bacteria were exposed to various concentrations of the active drug 4a arising from one of the prodrugs (4) used in vivo (Fig. 3C). There is very little reduction in the viability of the bacteria, although fitting a linear regression forced through 0,100 indicates a significant cytotoxic response (t,DF: 2.8,5; P = 0.038). The therapeutic in vivo dose of prodrug 4 (200 mg/kg) even if immediately converted to drug 4a would...
only correspond to a concentration of 300 μmol/L. By interpolation from Fig. 3C, this would result in negligible toxicity to the bacteria. We conclude therefore that doses of prodrug giving rise to cytotoxicity in tumor cells in vivo would not affect the bacterial vectors.

**In vivo tumor colonization by ppCPG2-VNP20009asd and CPG2 expression.** We determined the numbers of live ppCPG2-VNP20009asd− bacteria recovered from the MDA-MB-361, WiDr, and B16-F10 tumors and plotted them against the time from the original inoculation. Bacterial colonization of the tumors proceeds similarly in all three tumor types, initially increasing rapidly then tending toward a maximum (Fig. 4). The best fit to the data was a hyperbola, enabling a prediction of the maximum bacterial load (nonoverlapping 95% confidence intervals in brackets), which was approximately 7 times higher in B16-F10 (1.7 × 10^8 cfu/g; 8.3 × 10^7-3.5 × 10^8) than in WiDr (2.5 × 10^7; 1.0 × 10^7-6.3 × 10^7) but with the corresponding value for MDA-MB-361 (2.1 × 10^6), having a very broad 95% confidence interval. In WiDr, these values for bacterial load per gram are significantly lower than those per milliliter, producing the greatest cell kill in six-well plates (Fig. 2C). However, in vitro, the bacteria are distributed throughout the medium with only a small proportion being in close contact with the cells. The time to half-maximal colonization is longer in MDA-MB-361 [6.2 days (3.3-9.2)] than in WiDr [1.8 days (1.2-2.5)] or B16-F10 [1.2 days (0.8-1.6)]. During the same period, the amount of CPG2 enzyme activity in the tumors was also determined (Fig. 5). After an average initial lag (expressed as the X intercept when Y = 0) of 4.2 days that did not differ significantly among the three cell lines, the CPG2 activity increased linearly. The greatest rate of accumulation of CPG2, calculated as the slope, was seen in B16-F10 [Fig. 5C; 0.29 units/g/d (0.22-0.35)] followed by WiDr [Fig. 5B; 0.11 units/g/d (0.069-0.153)] and MDA-MB-361 [Fig. 5A; 0.037 units/g/d (0.015-0.058)]. The lack of a plateau in CPG2 activity to mirror that in bacterial load suggests that either CPG2 expression continues to build in the existing bacteria after they have become stationary or there is death and release of CPG2 from some bacteria but sufficient division to...
In vivo therapy of human breast and colon carcinoma and mouse melanoma tumors. Tumor doubling times were calculated from fits to rising exponentials (nonoverlapping 95% confidence intervals in brackets). As expected for an oncolytic vector, ppCPG2-VNP20009asδ- alone inhibits the growth of all three tumor types. However, in MDA-MB-361 (Fig. 6A), it takes until day 14 for there to be any apparent difference between the tumor growth in the controls and any of the treated groups, which probably reflects the slower rate of bacterial colonization and CPG2 accumulation seen in this tumor type (Fig. 4A) compared with WiDr (Fig. 4B) and B16-F10 (Fig. 4C). Subsequent to this initial nonresponsive phase, doubling times from day 14 were calculated by fitting the data to a rising exponential, constraining the start value to the group average for day 0. In this later phase, the control tumors grow with a doubling time of 20.8 days (24.3-18.1) compared with 31.0 days (37.9-26.2) in the vector alone treated and a much slower average of 83 days (64.5-120.0) when tumors additionally received any of the three prodrugs 2, 3, or 4, there being no significant difference between the effects of these prodrugs. Significant difference exists between the curves where indicated [F(DFn,DFd): 51.4 (2,356); P < 0.0001]. In the WiDr (Fig. 6B) and B16-F10 (Fig. 6C) tumors, there was, by comparison, no initial unresponsive phase (Fig. 4B and C); hence, the rising exponentials used to generate doubling times were applied from the first time point, constraining the start value to the group average for day 0. This more immediate response is commensurate with the more rapid onset of bacterial colonization and CPG2 accumulation in WiDr and B16-F10 (Figs. 3B and C and 4B and C). In WiDr xenografts, the control tumor or those treated with ppCPG2-VNP20009asδ- alone or with ppCPG2-VNP20009asδ- plus prodrug 4 do not give significantly different doubling times, having a joint average of 9.9 days (8.2-12.2) and are shown as a single group. However, those treated with ppCPG2-VNP20009asδ- plus prodrug 3 have a longer doubling time of 15.6 days (14.6-16.9). Significant difference exists between the curves where indicated [F(DFn,DFd): 50.1 (3,323); P < 0.0001]. These three prodrugs alone have no effect on the growth of either MDA-MB-361 or WiDr xenografts (23). B16-F10 control tumors grew more rapidly than human tumors, the doubling time being 2.3 days (2.5-2.1), with a more marked reduction in growth rate following treatment with ppCPG2-VNP20009asδ- alone of 5.3 days (5.5-5.0). Treatment with ppCPG2-VNP20009asδ- plus prodrugs produced further significant extensions of doubling times of 6.2 days (6.6-5.8) for prodrug 4 and to 9.4 days (10.4-8.5) for prodrug 3. Significant difference exists between the curves [F(DFn,DFd): 90.6(3,307); P < 0.0001]. Toxicity defined by >10% body weight reduction was not encountered. Evidence of therapeutic tumor necrosis was observed as frequent scab formation; however, according to UK Home Office license requirements on humane regulations (38), this necessitated premature culling before the scientifically relevant endpoint of tumor size had been reached; hence, meaningful survival data could not be acquired. In WiDr and B16-F10 but not in MDA-MB-361, in combination with ppCPG2-VNP20009asδ-, prodrug 3 is more effective than prodrug 4. In a separate experiment (data not shown), prodrugs 3 and 4 alone were shown to have no effect on B16-F10 tumor growth, with all six of the treated and control tumors reaching the humane limit (1,000 mm³) by 15 to 18 days, having grown slightly slower than in the present experiment. We have shown previously in transfected xenograft models that the choice of prodrug to produce maximum effect varies between tumor types and degree of CPG2 expression within that tumor (23). We observed the same pattern here in a targeted system, B16-F10 especially, which emphasizes the versatility of CPG2 as a prodrug-activating enzyme. In each case, a comparison of the tumor volume to cfu per gram enables an approximation of the total bacterial load, indicative of at least 100-fold more bacteria in the tumor than that administered to the whole animal, showing that the observed increase is a result of bacterial growth in the tumors.

In summary, we have shown that attenuated oncolytic Salmonella can act as a vector for the prodrug-activating enzyme

![Fig. 6. Growth of human tumor xenografts (A, breast carcinoma MDA-MB-361; B, colon carcinoma WiDr) and a mouse allograft (C, mouse melanoma B16-F10; controls [open circles] ] after i.v. injection of ppCPG2-VNP20009asδ- (open squares) followed by five weekly i.p. doses of prodrugs 2 (filled inverted triangle), 3 (filled diamond), or 4 (filled circle). Broken lines, 95% confidence interval.](www.aacrjournals.org)
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Frank Friedlos, Panos Lehouritis, Lesley Ogilvie, et al.

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