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Novel nitric oxide generating compound glycidyl nitrate enhances the therapeutic efficacy of chemotherapy and radiotherapy

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ABSTRACT

Selective release of nitric oxide (NO) in tumors could improve the tumor blood flow and drug delivery for chemotherapeutic agents and radiotherapy, thereby increasing the therapeutic index. Glycidyl nitrate (GLYN) is a NO generating small molecule, and has ability to release NO on bioactivation in SCC VII tumor cells. GLYN-induced intracellular NO generation was significantly attenuated by NO scavenger carboxy-PTIO (cPTIO) and NAC. GLYN significantly increases tumor blood flow, but has no effect on the blood flow of normal tissues in tumor-bearing mice. When used with cisplatin, GLYN significantly increased the tumor growth inhibition effect of cisplatin. GLYN also had a modest radiosensitizing effect in vitro and in vivo. GLYN was well tolerated and there were no acute toxicities found at its effective therapeutic doses in preclinical studies. These results suggest that GLYN is a promising new drug for use with chemotherapy and radiotherapy, and provide a compelling rationale for future studies of GLYN and related compounds.

1. Introduction

Research to identify novel therapeutic sensitizers for chemotherapy and radiotherapy for the treatment of cancer that are more efficacious and less toxic than sensitizers to date has led us to study a series of novel energetic compounds. Energetic compounds are designed to store and release chemical energy under specific conditions [1]. Many of these compounds contain multiple nitro groups and a subset of these is biologically inert, but can release nitrogen oxide, particularly nitric oxide, on bioactivation in targeted tissues. We recently reported that one such compound, 1-bromoacetyl-3,3-dinitroazetidine (RRx-001), is efficacious as a stand-alone chemotherapeutic drug and a radiosensitizer, with a very favorable toxicity profile [2]. RRx-001 is currently being evaluated in Phases I/II clinical studies. Its activity is due in part to the formation of nitric oxide [3]. Here we report the anti-cancer activity of glycidyl nitrate (GLYN), chosen for its ability to release nitric oxide on bioactivation, for its potential to bind to DNA [4,5] and for its lack of acute toxicity at effective therapeutic doses in preclinical studies.

Nitric oxide has a variety of biological effects that affect tumor angiogenesis, metastasis, apoptosis, blood flow and immunological responses [6,7], many of which are mediated by cGMP-dependent signaling. NO can be directly cytotoxic to tumors via the generation of intermediates such as peroxynitrite and N_2O_3 that can stimulate proapoptotic pathways [8,9]. NO can also affect DNA damage repair pathways by inhibiting the repair enzymes [10]. NO in high micromolar concentrations has been reported to be a hypoxic radiosensitizer [6,11–16]. In contrast, at much lower levels, NO can have a radioprotective bystander effect [17,18]. Therefore, selective release of relative high concentrations of NO in tumor, as compared to normal tissue, could provide for an increased therapeutic index for radiation therapy.

NO has also been reported to directly mediate the effects of some chemotherapeutic drugs [19,20] and to act as a chemosensitizer in preclinical models [21–24], which could be due in part to effects of NO on tumor vasculature and permeability. Results from a Phase II clinical study in prostate cancer patients suggested that an NO donor mediated vasodilation in tumors, resulting in a significant reduction in PSA [25]. Unfortunately, therapeutic strategies targeting NO signaling with a variety of drugs including NO donors and NO mimetics have been limited by a variety of toxicities, including systemic hypotension. Here we report results of studies with GLYN, demonstrating that it is a well tolerated promising chemoradiosensitizer in a preclinical tumor model.

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2. Materials and methods

2.1. Materials

Glycidyl nitrate (GLYN) used in these studies was obtained from ATK Aerospace System as a solution in DMSO. GLYN has a molar mass of 119.08 (Fig. 1). GLYN was dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich) and then diluted with DMEM culture medium (Invitrogen) to obtain a desired concentration for study. The final DMSO concentration in 10 mM GLYN solution was 0.9% (v/v). Carboxy-PTIO potassium salt (cPTIO) and N-acetyl-L-cysteine (NAC) were purchased from Sigma–Aldrich.

2.2. Cell lines

The human colorectal cancer cell line HT29, melanoma cell line M21 and the murine squamous cell carcinoma SCC VII cell line were grown and maintained in DMEM medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 ug/ml streptomycin in a 37 °C humidified incubator with a mixture of 95% air and 5% CO_2. The identity of the cells studied was regularly verified throughout the course of the studies by observation of the growth pattern and cell morphology in vitro and in vivo. All experiments were performed on exponentially growing cells with cell population doubling times of approximately 20–36 h.

2.3. Measurement of nitric oxide production

Intracellular NO was measured by using a nitric oxide fluorometric assay kit (BioVision) and a fluorescent probe DAF-FM diacetate (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) (Invitrogen Molecular Probes). For the nitric oxide fluorometric assay, M21 and SCC VII cells were incubated in growth medium containing 0–10 mM GLYN in 24-well cell culture plates at a cell density of 1×10^5 in 1 ml medium for 24 h. The nitric oxide production in the medium was assayed following the manufacturer's instructions. NO concentration was calculated from a nitrite/nitrate standard curve.

For measurement of NO production using the fluorescent probe DAF-FM, cells were grown in a black 96-well plate overnight. DAF-FM probe at 10 uM was added, incubated for 1 h, and then washed out with PBS. GLYN at 0.1–10 mM was added to the cells. In the combination studies with irradiation, cells were immediately irradiated after addition of GLYN. Following addition of GLYN and/or irradiation, the green fluorescence was observed under a fluorescence microscope (Leica Microsystems), and quantitated using a fluorescence microplate reader (Molecular Devices). The green fluorescence was measured again at 10, 30 min, and 1–24 h after addition of GLYN.

2.4. In vitro clonogenic assay

Radiation survival curves were generated using an in vitro clonogenic assay as previously described [2]. Briefly, HT29 and SCC VII cells were plated in triplicate in 60-mm Petri dishes and irradiated with 0–15 Gy in the presence or absence of GLYN using a ^{137}Cs source with a dose rate of 3 Gy/min. After irradiation, cells were incubated for 14 days and stained with 0.25% crystal violet. Colonies containing $\geqslant 50$ cells were counted for calculation of radiation survival.

Fig. 1. Chemical structure of GLYN (Glycidyl nitrate). Molecular formula: $C_3H_5NO_4$. Molar mass: 119.08.

2.5. Tumor model and therapy

C3H mice (male, 7–8 weeks old and 20–25 g in body weight) were purchased from Charles River Laboratories. Mice were normally bred and maintained under specific pathogen-free conditions, and sterilized food and water were available *ad libitum*. Mice were injected subcutaneously in the right flank with 5×10^5 SCC VII tumor cells in 0.05 ml Hank's solution. When tumors reached an average size of 150 mm^3 (100–300 mm^3), mice were randomly assigned to treatment groups. GLYN was injected i.p. at doses as specified in each experiment. For irradiation, the unanesthetized tumor-bearing mice were placed in individual lead boxes with tumors protruding through a cut-out window at the rear of each box. Radiation was delivered using a Philips RT-250 200 kVp X-ray unit (12.5 mA; Half Value Layer, 1.0-mm Cu) at a dose rate of 140 cGy/min. The length and width of the tumors were measured with calipers before treatment, and three times a week thereafter until the tumor volume reached at least 4 times (4×) the pre-treatment volume. The tumor volume was calculated using the formula: tumor volume = $\pi/6 \times$ length $\times$ width^2. The tumor volume quadrupling time (TVQT) was determined by a best-fit regression analysis. The tumor growth delay (TGD) time is the difference between the TVQT of treated tumors compared to that of untreated control tumors. Both the TVQT and TGD time were calculated for each individual animal, and then averaged for each group. The data are presented as percent (%) of the pretreatment volume on Day 0. Body weight of tumor-bearing animals was measured three times a week. The animal experiments described herein were approved by the Stanford University Administrative Panel for Laboratory Animal Care.

2.6. Microbubble-enhanced ultrasound image

Blood flow and blood volume in tumors and surrounding normal tissues were assessed by microbubble-enhanced ultrasound imaging as previously reported [2]. Briefly, tumor-bearing mice were treated with GLYN, and then imaged at various time points on a Vevo770 system (VisualSonics Inc.). Mice were anesthetized with a 3% isoflurane-oxygen and imaged with a 6 × 6 mm view field. A baseline image was acquired before microbubble injection. The contrast enhanced image was acquired 3–5 s after a bolus injection of the non-targeted microbubble contrast agent (1.0×10^8 microbubbles/0.1 ml) via a tail vein catheter. Images were acquired at a frame rate of 18 frames per second for a total of 800 frames. The image data were analyzed using Vevo770 Contrast Mode software by comparing the contrast cine loop with the baseline cine loop to generate a contrast overlay to identify the differences in image intensity between these two loops. The blood flow rate was calculated as contrast signals per second in tumors.

2.7. Statistics

Data were statistically analyzed using a two-tailed Student's *t*-test.

3. Results

3.1. GLYN induces nitric oxide production in tumor cells

To test our hypothesis that GLYN results in NO generation, we first measured NO production using a nitric oxide fluorometric

assay at 24 h following incubation of SCC VII and M21 cells with GLYN at concentrations of 0–10 mM. As can be seen in Fig. 2A, there was a marked generation of NO that peaked at concentrations of 1 mM GLYN for M21 cells and 2 mM for SCC VII cells, respectively, followed by a relative plateau at higher doses. To verify the GLYN-induced NO generation, we pre-treated SCC VII cells with NO scavengers, carboxy-PTIO (cPTIO) and N-acetyl-L-cysteine (NAC) and then exposed cells to 2 mM GLYN for up to 24 h. Results showed that the NO signal was significantly reduced by 60–80% in cells treated with cPTIO (Fig. 2B) or NAC (Fig. 2C) compared to that in GLYN treated cells without NO scavengers ($P < 0.01$).

Next, the dose response and time course experiments of GLYN in combination with gamma irradiation were performed in which SCC VII cells were treated with GLYN at concentrations of 0, 0.1, 1, and 10 mM with or without subsequent irradiation with 0, 2 or 10 Gy and the NO generation was measured at 10, 30 min, and 1–6 h after GLYN/irradiation. Results showed that NO was rapidly generated in the cells 10 min after exposure to GLYN and irradiation, with high levels of NO maintained for up to 6 h (Fig. 2D). There was a dose response relationship between NO levels and GLYN concentration at all time points. Radiation alone also induced the NO production in tumor cells. Interestingly, 10 Gy irradiation alone did not induce more NO production compared to 2 Gy irradiation.

3.2. GLYN is a potent chemosensitizer

We hypothesized that GLYN would be an efficient NO donor, result in increased tumor blood perfusion and therefore have the potential to increase the delivery of chemotherapeutic agents to tumors. To test this hypothesis, we studied the effect of GLYN in combination with cisplatin (CDDP) in mice bearing SCC VII tumors. We first estimated the maximum tolerated dose (MTD) of GLYN and found that the MTD for daily administration of GLYN is 400 mg/kg. Then, mice were treated with 300 mg/kg GLYN alone, 5 mg/kg CDDP alone, or both CDDP and GLYN at either 100 or 300 mg/kg. Results are shown in Fig. 3A and Table 1. The combined treatments were significantly more efficacious ($P < 0.01$) than either CDDP alone or GLYN alone with tumor growth delay time of 0.5 ± 0.5 days for GLYN alone, 1.1 ± 0.4 days for CDDP alone and 2.8 ± 0.7 days for 100 mg/kg GLYN plus CDDP, and 7.2 ± 3.1 days for 300 mg/kg GLYN plus CDDP ($P < 0.01$ vs. GLYN or CDDP alone). The body weight of tumor-bearing mice was monitored as an indicator of systemic toxicities. There were no significant differences in body weight of mice for the different treatment regimens, except mice treated with GLYN at 300 mg/kg plus CDDP resulting in the greatest weight loss (15% decrease compared to pre-treatment body weight) (Supplemental data Fig. 1).

3.3. GLYN has radiosensitizing effects in vitro and in vivo

We then studied the effect of GLYN on the radiation response of tumor cells in vitro and in vivo in tumor-bearing mice. Initially, SCC VII cells and HT29 cells were irradiated in the presence or absence of GLYN and cell survival was assayed using an in vitro clonogenic survival assay. GLYN alone reduced the survival of HT29 cells in a dose-dependent manner, with 98% survival at a concentration of 0.01 mM GLYN and 70% survival at 0.1 mM of GLYN. When combined with radiation, GLYN had a modest effect on the radiation survival and shifted the radiation survival curves downward significantly at high doses of radiation. However, GLYN has no significant radiosensitization effect on SCC VII cells in vitro (Supplemental data Fig. 2).

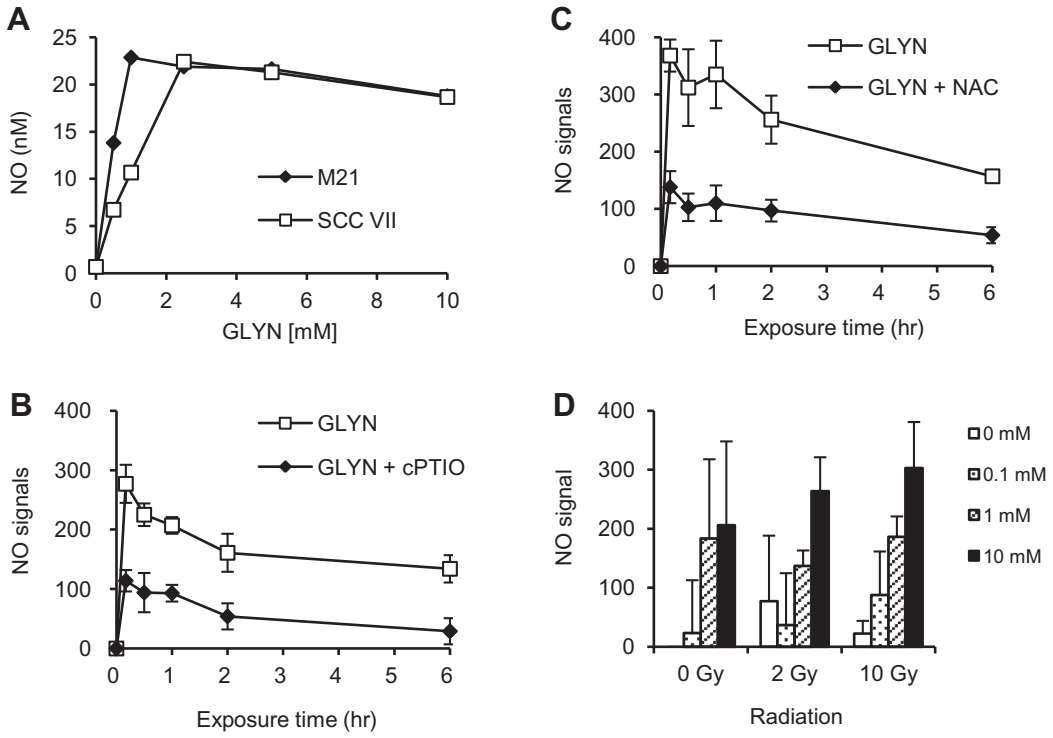

Fig. 2. GLYN induced NO generation in tumor cells. (A) SCC VII and M21 cells were incubated in GLYN-containing medium for 24 h and the NO concentration was measured in the culture supernatant by using a nitric oxide fluorometric assay kit. (B–C) SCC VII cells were treated with either 10 uM cPTIO or 10 mM NAC for 1 h and then incubated in medium containing 2 mM GLYN for up to 24 h and the NO signal was measured using a fluorescent probe DAF-FM. (D) NO generation after exposure to GLYN and gamma irradiation in SCC VII cells. The NO signal was measured 1 h after addition of GLYN/irradiation using a fluorescent probe DAF-FM. Data are the mean ± S.D. from four to five samples.

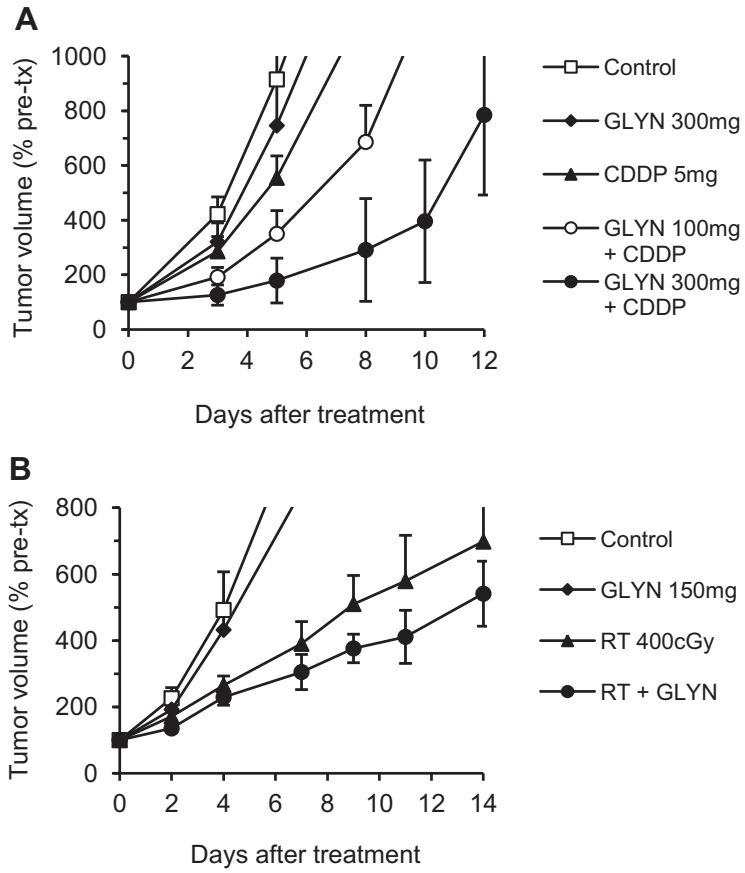

Fig. 3. Antitumor efficacy of combination therapy of GLYN and (A) cisplatin or (B) gamma irradiation in SCC VII tumor model in mice. Tumor growth curves are presented as the average tumor volume of each group (n = 5–7, mean ± S.D).

Table 1
Antitumor efficacy of GLYN in combination with either CDDP or gamma irradiation in mice.

	Number of mice	4X TGT (day)	TGD (day)	P value*
GLYN + CDDP				
Control	5	3.0 ± 0.4		
GLYN 300 mg/kg	5	3.6 ± 0.5	0.5 ± 0.5	
CDDP 5 mg/kg	5	4.1 ± 0.4	1.1 ± 0.4	
GLYN100 + CDDP	6	5.8 ± 0.7	2.8 ± 0.7	<0.01
GLYN300 + CDDP	7	10.2 ± 3.1	7.2 ± 3.1	<0.01
GLYN + 250 cGy radiation				
Control	6	2.9 ± 0.3		
GLYN 100 mg/kg	6	3.8 ± 0.4	0.9 ± 0.4	
250 cGy	6	4.2 ± 0.9	1.3 ± 0.9	
GLYN + 250 cGy	6	6.5 ± 1.5	3.6 ± 1.5	0.01
GLYN + 400 cGy radiation				
Control	6	3.6 ± 0.7		
GLYN 150 mg/kg	6	3.9 ± 0.5	0.3 ± 0.5	
400 cGy	6	7.9 ± 2.4	4.3 ± 2.4	
GLYN + 400 cGy	6	10.4 ± 2.3	6.8 ± 2.3	0.09

* P values: combination vs. CDDP alone or radiation alone.

Since NO might have its important biological effects on the tumor microenvironment, e.g. microvasculature that could affect radiosensitivity of tumor cells, we then studied the effect of GLYN combined with local tumor irradiation in mice bearing SCC VII tumors. In the first studies of combined therapy with radiation, tumor-bearing mice were treated with either 100 mg/kg GLYN i.p., daily for 10 days, or local tumor radiation of 250 cGy daily for 10 days, or both GLYN and radiation. Results showed that GLYN and radiation alone had a very small effect on tumor growth, and produced TGD times of 0.9 ± 0.3 and 1.3 ± 0.9 days, respectively (P < 0.01 and P = 0.4, respectively compared to control). The combination treatment of radiation and GLYN resulted in a TGD time of 3.6 ± 1.5 days (P < 0.01 compared with control, GLYN or radiation alone treated mice) (Table 1). A second hypofractionated regimen was studied in which SCC VII tumor-bearing mice were treated with 150 mg/kg GLYN daily for 5 days immediately prior to daily local tumor irradiation with 400 cGy (Fig. 3B and Table 1). Using this dosing regimen, GLYN alone did not have a significant effect on tumor growth. The TGD for radiation alone was 4.3 ± 2.4 days (P < 0.01 compared to control) and 6.8 ± 2.3 days for the combined treatment of GLYN and radiation (P < 001 compared to GLYN; P = 0.09 compared to radiation alone). In the combination therapy, none of the treatment regimens caused a significant decrease in animal body weight compared with untreated control mice (supplemental data Fig. 1). These results demonstrate that GLYN had modest radiosensitizing effects on well established and rapidly growing SCC VII tumors.

3.4. GLYN increases blood flow in tumors

Because the metabolism of GLYN results in loss of nitro groups leading to the formation of NO, experiments were carried out to determine whether the observed radiation and chemosensitizing effects of GLYN could be due, at least in part, to changes in tumor blood flow secondary to the effect of NO on tumor blood vessels. We therefore measured the blood flow and blood perfusion rate

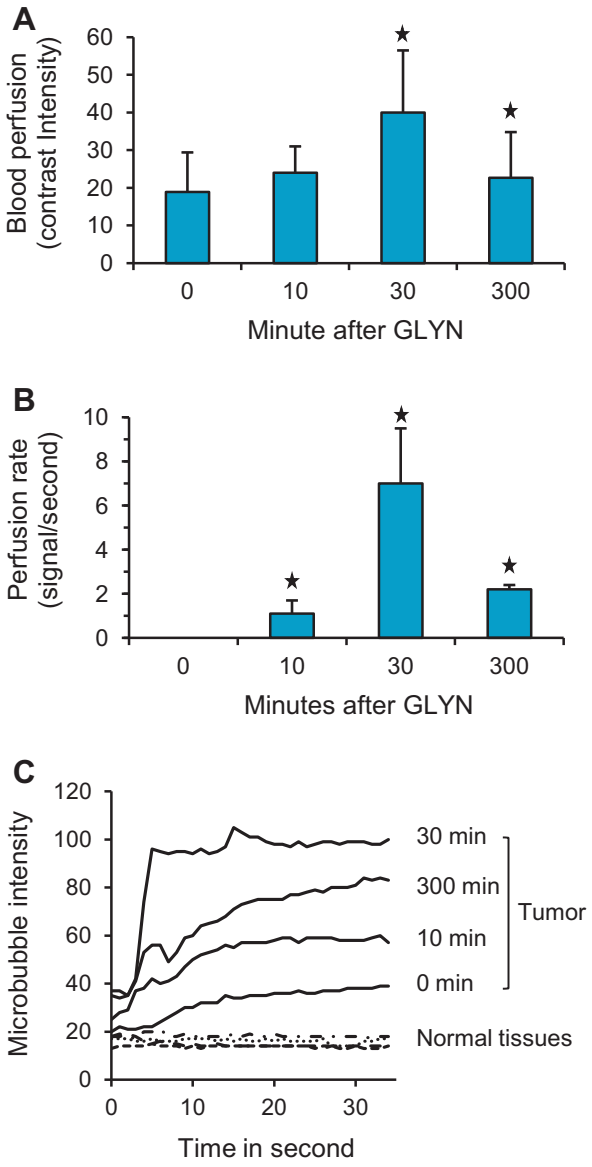

Fig. 4. Blood flow and perfusion of SCC VII tumors and normal tissues in GLYN-treated mice. Mice were i.v. injected with 300 mg/kg GLYN at 0 min and imaged at indicated times with a microbubble-enhanced ultrasound image system. The baseline imaging data (0 min time point) was collected before injection of GLYN. (A–B) Time course of blood perfusion and perfusion rate of tumors. (C) Microbubble signal curves from a representative tumor and the surrounding normal tissues during the course of imaging. The dashed lines show the microbubble signals of normal tissues that were imaged at 0, 10, 30 and 300 min after GLYN. The slope of the curve represents the relative rate of blood perfusion and the plateau representing the relative blood volume in the tissues. There were 3 mice per time point (mean ± S.D). $^{*}P < 0.05$ vs. baseline at 0 min.

in tumors and the surrounding normal tissues before and after treatment with a single dose of 300 mg/kg GLYN by microbubble-enhanced ultrasound imaging. As shown in Fig. 4, there was a significant increase in microbubble signals in tumors that peaked 30 min after administration of GLYN. The blood perfusion rate (i.e. microbubble signals per second) following administration of GLYN in tumors was increased by 2–7 folds from the baseline (0 min). Interestingly, the blood flow of the surrounding normal tissues did not change significantly after administration of GLYN. The microbubble signals remained in the baseline level that was significantly lower than that in tumors (Fig. 4C).

4. Discussion

GLYN is a small molecule originally synthesized as an energetic compound. It has not been studied to date for medical purposes. It is of particular interest as a potent NO donor, that may be especially useful in areas of restricted tumor blood flow and hypoxia. The preclinical studies reported here show that GLYN has sensitizing properties to both the chemotherapeutic agent cisplatin, and to gamma irradiation. GLYN is a modest radiosensitizer compared to another energetic dinitroazetidine compound, ABDNAZ (RRx-001) [2,3]. However, GLYN is a very effective chemosensitizer. This may be due to GLYN-induced NO production in tumor cells and the direct effect of NO on increased blood flow in tumors observed with microbubble-enhanced ultrasound imaging. Increased tumor blood flow could result in higher cisplatin concentrations in tumor and better diffusion within the tumor.

In addition, since NO is a radical with a free electron capable of interacting with reactive oxygen species, local NO generation in tumors could result in nitrosative stress secondary to the formation of NOx molecules such as peroxynitrite, nitrogen dioxide and dinitrogen trioxide, that are necessary for cytotoxic nitration and oxidation reactions [26]. The cytotoxic effect of NO may require relatively high concentrations of NO above a threshold dose of NOx and peroxynitrite. It has been hypothesized that tumor cells are more sensitive to these effects than normal cells because of impaired antioxidant defense systems in tumor cells [27]. Others have hypothesized that NO-mediated cytotoxicity may not require very high levels of NO, but rather "normalization" to physiologic levels [22].

There is conflicting evidence from preclinical studies of NO modulating drugs. Elevated levels of NO increased the responsiveness of prostate cancer tumors in mice to doxorubicin [22], while NOS inhibition radiosensitized glioma xenografts via effects on the tumor vasculature [28]. The latter effect is presumed to be due to effects on signal transduction pathways that result in loss of aberrant proangiogenic signaling networks [29,30].

Results from clinical studies support the concept that NO modulation using nitroglycerin can be of clinical benefit either alone [6] or in combination with chemotherapy [31]. GLYN is a novel NO generating compound, and was very well tolerated in mice at the effective doses at which it generates NO radicals and sensitizes tumor cells to radiation and cisplatin. Since GLYN had significant effects on tumor blood flow and response to cisplatin, it may be particularly useful for treating tumors that are relatively hypoxic and may have selective effects on tumor as compared to normal tissue, without the systemic effects of nitroglycerin and related drugs. The results presented here are very promising and provide a compelling rationale for future studies of GLYN and related compounds.

Conflict of interest

There were no conflicts of interest in the research data presented in this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.032.

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