Biochemical and Biophysical Research Communications 447 (2014) 537–542

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Shoucheng Ning [a], Mark Bednarski [b], Bryan Oronsky [c], Jan Scicinski [c], Susan J. Knox [a,*]

[a] Department of Radiation Oncology, Stanford University Medical Center, Stanford, CA 94305, USA
[b] Department of Radiology, Stanford University Medical Center, Stanford, CA 94305, USA
[c] RadioRx, Inc., Mountain View, CA 94040, USA

ARTICLE INFO

Article history:
Received 28 March 2014
Available online 13 April 2014

Keywords:
Nitric oxide
Blood flow
Cancer
Chemotherapy
Radiotherapy

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.

* Corresponding author. Address: Stanford University Medical Center, Department of Radiation Oncology, 1050 Arastradero Road, A248, Palo Alto, CA 94304, USA.
E-mail address: sknox@stanford.edu (S.J. Knox).

http://dx.doi.org/10.1016/j.bbrc.2014.04.032
0006-291X/

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