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Antisense Inhibition of Cyclin D1 Expression is Equivalent to Flavopiridol for Radiosensitization of Zebrafish Embryos

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Running Title: Radiosensitization by cyclin D1 knockdown

CONFLICT OF INTEREST

The authors deny the existence of any actual or potential conflicts of interest.

ABSTRACT

Purpose: Flavopiridol, a small molecule pan-cyclin inhibitor, has been shown to enhance the radiation response of tumor cells both *in vitro* and *in vivo*. The clinical utility of flavopiridol, however, is limited by toxicity, previously attributed to pleiotropic inhibitory effects on several targets affecting multiple signal transduction pathways. Here we utilized zebrafish embryos to investigate radiosensitizing effects of flavopiridol in normal tissues. Methods and Materials: Zebrafish embryos at the 1-4 cell stage were treated with 500 nM flavopiridol or injected with 0.5 pmol antisense hydroxylprolyl-phosphono nucleic acid oligomers to reduce cyclin D1 expression, then subjected to ionizing radiation (IR) or no radiation. Results: Flavopiridol-treated embryos demonstrated a 2-fold increase in mortality following exposure to 40 Gy by 96 hours post fertilization (hpf) and developed distinct radiation-induced defects in midline development (curly-up phenotype) at higher rates when compared to embryos receiving IR only. Cyclin D1-deficient embryos had virtually identical IR sensitivity profiles when compared to embryos treated with flavopiridol. This was particularly evident for the IR-induced curly-up phenotype, which was greatly exacerbated by both flavopriridol and cyclin D1 downregulation. Conclusions: Treatment of zebrafish embryos with flavopiridol enhanced radiation sensitivity of zebrafish embryos to a degree that was very similar to that associated with downregulation of cyclin D1 expression. These results are consistent with the hypothesis that inhibition of cyclin D1 is sufficient to account for the radiosensitizing action of flavopiridol in the zebrafish embryo vertebrate model.

Keywords: flavopiridol; cyclin D; hydroxyprolyl-phosphono peptide nucleic acids; ionizing radiation; radiosensitizer

INTRODUCTION

Flavopiridol, a small molecule semi-synthetic flavonoid inhibitor of cyclin dependent kinases [1], has been shown to enhance the radiation response of tumor cells both *in vitro* [2] and *in vivo* [3] model systems. However, the clinical utility of this synthetic flavone as an anti-cancer therapeutic has been disappointing, in part, due to dose-limiting toxicity. In addition to the potent inhibition of cyclin-dependent kinases (CDKs), and consequently cyclin function, numerous "off-target" activities have been ascribed to the synthetic flavone, including inhibition of numerous signal transduction cascades involving the EGFR, PKA, PKC, HIF-1 and NF-κB, to name but a few [4, 5]. To investigate the radiosensitizing effects of flavopiridol on normal tissues, we examined the response of zebrafish embryos to ionizing radiation (IR) with and without flavopiridol pretreatment.

In order to elucidate whether the radiosensitizing action of flavopiridol is due to inhibition of its primary pharmacologic target, we compared flavopiridol effects on the radiation response of zebrafish embryos to those associated with cyclin D1 (*ccnd1*) downregulation achieved by antisense hydroxylprolyl-phosphono peptide nucleic acid (HypNA-pPNA) oligomers. Previously, we demonstrated the viability and phenotypic characteristics of zebrafish embryos microinjected with the antisense *ccnd1* HypNA-pPNA 16-mer described below [6].

Since cyclin D1 is involved in G1/S checkpoint control, abrogation of its function by antisense downregulation should block the progression of cells into S phase, a known radioresistant part of the cell cycle [7]. The cyclin D1-inhibited cells should, therefore, be more sensitive to the effects of IR than their uninhibited controls. Flavopiridol has previously been shown to lower the level of cyclin D1 in human ovarian cancer cells as one its pleiotropic mechanisms of radiosensitization [2].

In this work, we describe the effect of IR exposure on *ccnd1* knockdown embryos as compared to the radiation response of wild-type embryos treated with flavopiridol prior to IR exposure. Thus we hereby test the hypothesis that the radiosensitization phenotype exhibited by flavopiridol is due to its cyclin inhibitory effect.

MATERIALS AND METHODS

Embryo harvesting and maintenance

Zebrafish husbandry, embryo collection, dechorionation, and embryo maintenance were performed according to accepted standard operating procedures [8] and with approval by the Institutional Animal Care and Use Committee at Thomas Jefferson University. Zebrafish were maintained in the Zebrafish Core Facility of the Kimmel Cancer Center at Thomas Jefferson University at 28.5°C on a 14-h light/10-h dark cycle, and embryos were staged as described [9].

Flavopiridol treatment

Groups of 15 newly fertilized zebrafish embryos were treated with either 0 or 500 nM flavopiridol, generously provided by Aventis (Bridgewater, NJ), in 10 mL of zebrafish embryo medium (EM) [8] immediately after fertilization. Fresh flavopiridol was added again every 24 h up to 120 h. Statistical analysis was performed using a one-tailed student's *t*-test.

Antisense HypNA-pPNA treatment

Antisense (N-GTGCTCCATATCTTCA-C) and single mismatch (N-GTGCTCCAaATCTTCA-C) 16-mer HypNA-pPNAs targeting the zebrafish *ccnd1* mRNA initiation region were obtained from Active Motif (Carlsbad, CA). Melting

temperature (Tm) analysis revealed a 6°C lower Tm in the single mismatch sequence relative to that for the targeted *ccnd1* antisense sequence [6]. For HypNA-pPNA microinjection into zebrafish, a final concentration of 0.5 mM of each oligonucleotide was prepared in 1:9 (v:v) phenol red dye and phosphate-buffered saline, and ~1 nL injected into groups of 15 zebrafish embryos at the 1–4 cell stage using a nitrogen gas pressure injector (Harvard Apparatus, Cambridge, MA). Statistical analysis was performed using a one-tailed student's *t*-test.

Radiation exposure

All protocols using radiation were approved by the Radiation Safety Committee of Thomas Jefferson University. Embryos 24 hours postfertilization (hpf) were exposed to doses varying from 0 to 40 Gy using a Gammacell 40 low-dose ^{137}Cs laboratory irradiator (AECL, Kanata, Canada) or 250 kVp X-ray machine (PanTak, East Haven, CT). After irradiation, embryos were incubated at 28.5°C for 24 h, dechorionated, and maintained at 28.5°C for up to 144 h to evaluate morphology and survival. Photomicrography of representative embryos was performed using a Leica M2FLIII microscope (Leica, Wetzlar, Germany) at 50× magnification.

RESULTS

Control and flavopiridol-treated zebrafish embryos were exposed to IR at doses of 0, 10, 20, or 40 Gy at 24 h post-fertilization (hpf). IR produced dose-dependent alterations of normal embryo morphogenesis, as we reported previously [10], with 100% of control embryos demonstrating a characteristic aberrant dorsal tail curvature, designated "curly-up," by 48 hpf after treatment with 40 Gy (Fig. 1). In contrast, pretreatment with 500 nM flavopiridol alone produced no gross developmental defects in the embryos, but resulted in the curly-up phenotype following exposure to a 50% lower dose of IR (20 Gy) than required to produce the same radiation-induced teratogenic effects in corresponding control embryos (Fig. 1).

The survival of irradiated embryos pretreated with flavopiridol was adversely affected to a greater extent than embryos solely exposed to IR (Fig. 2). Specifically, exposure to 20 Gy IR produced 100% mortality by 144 hpf in the pretreated embryos, whereas 97.0 ± 4.2% of similarly irradiated control embryos remained viable (P=0.010). Following 40 Gy IR, none of the flavopiridol treated embryos survived, while 55 ± 7.1% of similarly irradiated control embryos were still alive at 120 hpf (P=0.028).

The effect of *ccnd1*-specific HypNA-pPNA oligomer antisense knockdown of *ccnd1* transcripts on the radiation response of zebrafish embryos was similarly examined. Embryos were microinjected with phenol red, *ccnd1* antisense or single mismatch 16-mer HypNA-pPNAs by 1 hpf and then exposed to IR at doses of 0, 10, 20, or 40 Gy at 24 hpf. A dose-dependent alteration of normal embryo development was again observed 24 hours following irradiation, with 100% of phenol red-injected and mismatch-injected control embryos demonstrating the curly-up phenotype at the highest dose of IR tested (Fig. 3 and data not shown). Perturbation of normal cyclin function with *ccnd1* HypNA-pPNA produced multiple morphologic abnormalities in the zebrafish embryos at baseline, including microcephaly, microophthalmia, micrognathia, and pericardial edema, in agreement with our previous observations [6]. These phenotypic alterations were exacerbated by radiation treatment. Of note, cyclin D1 reduction by *ccnd1* HypNA-pPNA oligomers resulted in the curly-up phenotype 24 h following irradiation with only 20 Gy (Fig. 3), as was seen following flavopiridol pretreatment (Fig. 1).

The enhanced radiosensitivity of zebrafish embryos with deficient cyclin D1 expression also translated into reduced survival following IR exposure, as was observed in the flavopiridol-treated fish. Control phenol red and single mismatch microinjected embryos were resistant to 20 Gy IR exposure, with 100% survival at 144 hpf, whereas only 24 ± 5.7% of the embryos with reduced cyclin D1 activity remained viable at 144 hpf (p=0.017) (Fig. 4 and data not shown). This is comparable to the effect on survival observed following irradiation of embryos pretreated with flavopiridol (Fig. 2). As was also observed in the flavopiridol treated embryos, none of the embryos with antisense-reduced cyclin D1 were alive following 40 Gy IR exposure by 120 hpf, while 43.5 ± 5.0% of similarly irradiated control embryos remained viable (p=0.026) (Fig. 4).

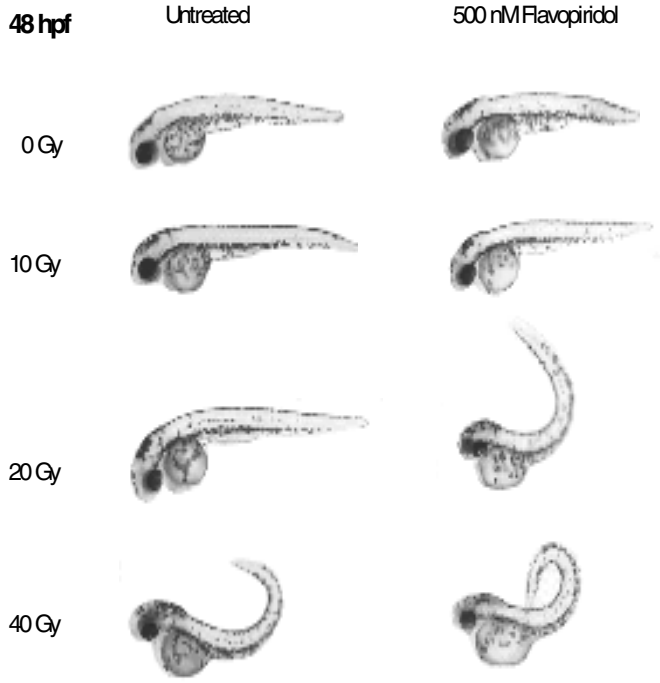

Figure 1: Flavopiridol-mediated radiosensitization of zebrafish embryos. Embryos were treated with 0 (untreated) or 500 nM flavopiridol. Embryos were then exposed to 0-40 Gy γ-radiation at 24 hpf. Morphology at 48 hpf of representative embryos from replicate experiments is shown at 50× magnification. Note the aberrant dorsal tail curvature ("curly-up" phenotype), as well as reduced head and eye size in irradiated control embryos receiving a dose of 40 Gy, and in treated embryos receiving doses of 20 and 40 Gy.

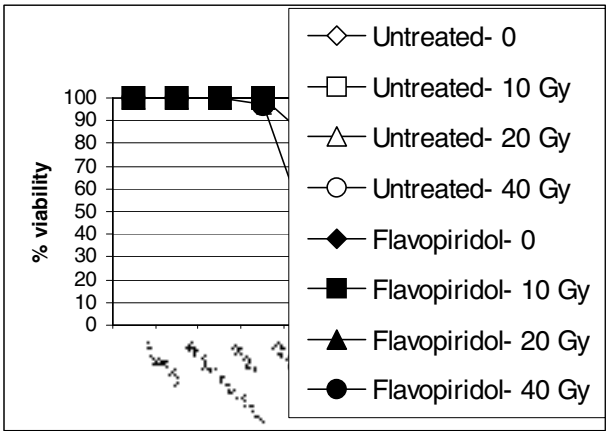

Figure 2: Effect of flavopiridol treatment on zebrafish viability following ionizing radiation exposure. Embryos were either treated daily with 500 nM flavopiridol or left untreated, exposed to various doses of IR, and monitored for survival up to 144 hpf. Data points show the mean and standard deviation of replicate experiments using 15 embryos per group. Survival of embryos following exposure to 40 Gy IR was significantly better in the untreated controls than in the flavopiridol pre-treated embryos at 120 hpf (p=0.029).

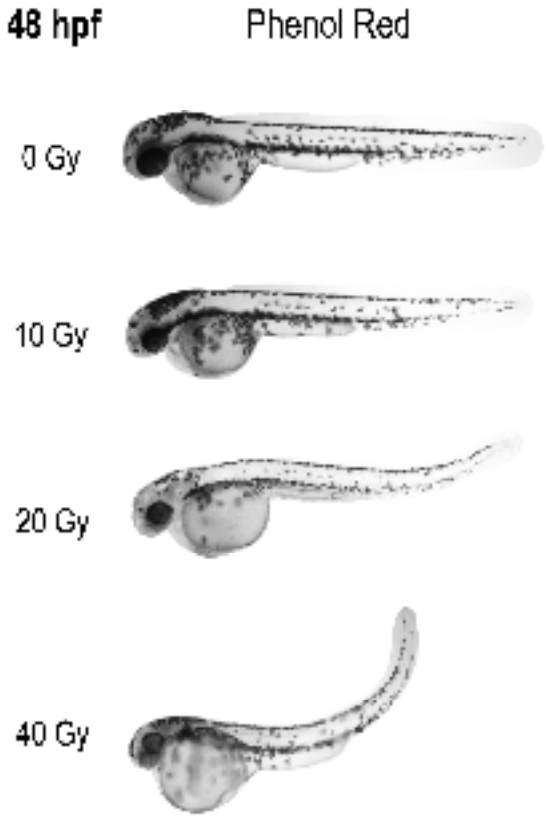

Figure 3: *ccnd1* antisense-mediated radiosensitization of zebrafish embryos. Embryos were injected with either vehicle (phenol red) or *ccnd1* antisense HypNA-pPNA and exposed to 0-40 Gy γ-radiation at 24 hpf. Morphology at 48 hpf of representative embryos from replicate experiments is shown at 50× magnification. Note the aberrant dorsal tail curvature ("curly-up" phenotype), as well as reduced head and eye size in irradiated control embryos receiving a dose of 40 Gy, and in treated embryos receiving doses of 20 and 40 Gy.

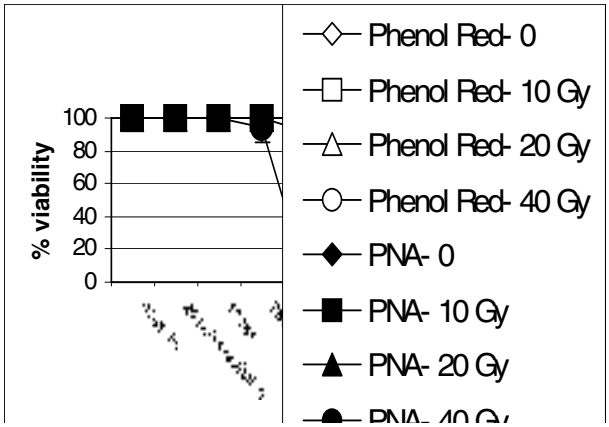

Figure 4: Effect of *ccnd1* antisense HypNA-pPNA treatment on zebrafish viability following ionizing radiation exposure. Embryos were injected with either vehicle (phenol red) or *ccnd1*-targeted HypNA-pPNA and monitored for survival up to 144 hpf. Data points show the mean and standard deviation of replicate experiments using 15 embryos in each group. Survival of embryos following exposure to 40 Gy IR was significantly better in the controls than in the *ccnd1*-downregulated embryos at 120 hpf (p=0.026).

DISCUSSION

Flavopiridol, a semi-synthetic pan-cyclin inhibitor [1], has been reported to sensitize a variety of human cancer types, including esophageal [11], prostate [12], ovarian [2], colon and gastric [13] carcinomas as well as leukemia cells [14], to ionizing radiation. The radiosensitizing effect of the flavonoid has also been demonstrated *in vivo* using a murine model system with xenografted human tumors [15] and syngeneic mouse tumors [2, 3]. While flavopiridol was the first cyclin-dependent kinase inhibitor tested in clinical trials [16], the results of these trials have been discouraging due to the unexpected, significant toxicity of the agent, both when given as monotherapy [17] and when co-administered with various chemotherapeutic drugs [18, 19]. The sole trial examining the combination of flavopiridol with radiation therapy for unresectable pancreatic cancer has only recently been approved for patient accrual (ClinicalTrials.gov), and thus the toxicity of this combined modality approach in humans is still to be determined. Furthermore, because flavopiridol, in addition to being a very potent pan-cyclin inhibitor, has been shown to interfere with numerous other cellular processes involving diverse signal transduction pathways [4, 5], the exact mechanism of flavopiridol's normal tissue toxicity is not easily determined.

To begin to address this issue of normal tissue toxicity with flavopiridol, particularly in relation to IR exposure, we employed zebrafish embryos as a living, dynamic vertebrate model. Previously, we demonstrated the utility of this model system to characterize the radiation response and its modulation by different chemical agents [10]. In the present work, we investigated the effect of treating the embryos with 500 nM flavopiridol, determined to be the biologically active concentration [16], alone and in conjunction with IR exposure. At baseline, flavopiridol had no gross effect on normal embryonic morphology and survival, but when administered before IR, the embryos demonstrated dorsal tail curvature ("curly-up" phenotype) and significantly reduced viability compared with similarly irradiated control embryos.

To determine if the flavopiridol effect was due, in part, to cyclin inhibition, we transiently down-regulated the

expression of cyclin D1 using specific HypNA-pPNA antisense oligonucleotides. The previously reported[6] morphologic perturbations of normal embryonic development, identified as microophthalmia, microcephaly and dorsal tail curvature, with the affected sites corresponding to areas of high cyclin D1 expression in the developing embryos [6, 20].-were observed in the cyclin D1-reduced embryos, even without radiation treatment.

The antisense *ccnd1* HypNA-pPNA 16-mer lowered the Western blot level of cyclin D1 in treated embryos dramatically, compared to vehicle-injected controls[6]. Similarly, intratumoral injection of human peptide-*CCND1* PNA-peptide lowered the Western blot level of cyclin D1 in human breast cancer xenografts in immunocompromised mice by about half [21].

Peptide nucleic acid derivatives, like morpholino phosphorodiamidates, do not activate RNase H, and as a result do not lower the levels of their target mRNAs, but inhibit mRNA translation solely by hybridization arrest [22]. When we directly tested that point by incubating human MCF7 breast cancer cells in the presence of an antisense *MYC* PNA-peptide for 24 hr, no reduction in the level of *MYC* mRNA in the extracted RNA was observed by QRT-PCR [23].

Given the similarities between the phenotype of cyclin inhibition and radiation exposure alone, effectively interpreting and quantifying the additional effects of radiation is clearly recognized as a difficult task. This problem, however, was expected, particularly in light of modulating the expression of genes involved in homeostatic functions during development, and is not dissimilar to difficulties routinely encountered using knockout mouse technology.

Because cyclin D1 facilitates the G1/S transition in the cell cycle, *ccnd1* inhibition should theoretically block the cells at this checkpoint, known to be an exquisitely radiosensitive part of the cell cycle [7]. Following exposure to IR, the "curly-up" phenotype is more apparent in the antisense injected embryos than in the corresponding irradiated controls. Further, the survival curves for the *ccnd1*-inhibited zebrafish showed significantly increased sensitivity to IR. This finding is essentially indistinguishable from that observed in irradiated embryos pretreated with flavopiridol.

Collectively, these results support the hypothesis that the inhibition of cyclin D1 is sufficient to account for the radiosensitizing effects of flavopiridol in zebrafish embryos. This work additionally confirms the utility of zebrafish as a model system for studying the pharmacogenetics of radiation effects in vertebrates. Our results confirm and extend our previous observations that zebrafish embryos provide a powerful *in vivo* model to identify and characterize additional novel targets, namely those involved in the cell cycle and DNA damage response, for pharmacological modulation of radiation resistance in patients exposed to radiation. These results also suggest the feasibility of zebrafish embryos for high-throughput screening of radiosensitizers, given the readily scoreable phenotype following radiation exposure.

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