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Bouvardin is a Radiation Modulator with a Novel Mechanism of Action

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Abstract

Protein synthesis is essential for growth, proliferation and survival of cells. Translation factors are overexpressed in many cancers and in preclinical models, their experimental inhibition has been shown to inhibit cancer growth. Differential regulation of translation also occurs upon exposure to cancer-relevant stressors such as hypoxia and ionizing radiation. The failure to regulate translation has been shown to interfere with recovery after genotoxic stress. These findings suggest that modulation of translation, alone or in conjunction with genotoxins, may be therapeutic in oncology. Yet, only two drugs that directly inhibit translation are FDA-approved for oncology therapies used today. We have previously identified the protein synthesis inhibitor, bouvardin in a screen for small molecule enhancers of ionizing radiation in *Drosophila melanogaster*. Bouvardin was independently identified in a screen for selective inhibitors of engineered human breast cancer stem cells. Here we report the effect of bouvardin treatment in preclinical models of head and neck cancer (HNC) and glioma, two cancer types for which radiation therapy is the most common treatment. Our data show that bouvardin treatment blocked translation elongation on human ribosomes and suggest that it did so by blocking the dissociation of elongation factor 2 from the ribosome. Bouvardin and radiation enhanced the induction of clonogenic death in HNC and glioma cells, although by different mechanisms. Bouvardin treatment enhanced the radiation-induced antitumor effects in HNC tumor xenografts in mice. These data suggest that inhibition of

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SUPPLEMENTARY INFORMATION

Fig. S1. Protein levels and translation inhibition.

Fig. S2. IC₅₀ of bouvardin on NCI-60 cancer cell lines.

Fig. S3. Greater growth inhibition at higher bouvardin concentrations.

Fig. S4. The effect of radiation and bouvardin on PARP cleavage in SNB-19 cells and on growth and proliferation in U251 cells.

Fig. S5. DNA damage in Det562 and FaDu cells.

Fig. S6. The effect of radiation and bouvardin on Det562 and FaDu cells.

translation elongation, particularly in combination with radiation treatment, may be a promising treatment option for cancer.

INTRODUCTION

Protein synthesis is required for growth, yet it remains underutilized as a drug target in diseases of unregulated growth, such as cancer. In recent years, the concept of inhibiting the ribosome to target cancers has become more appealing as the evidence that tumors utilize protein synthesis for oncogenesis has increased (1, 2). For example, several translation initiation and elongation factors are found to transform normal cells when overexpressed (3–6). Translation initiation and elongation factors are also overexpressed in many solid tumors (7, 8). Importantly, downregulation of translation factors has been shown to inhibit cancer cell growth in preclinical models (8–10).

Regulation of protein synthesis also plays a critical role under stress conditions that are relevant to tumor cells such as hypoxia, nutrient deprivation and exposure to ionizing radiation (11–14). For example, irradiated glioblastoma cells show changes in polysome-associated mRNAs that outnumber changes in the transcriptome by 10 to 1 (15). Thus, altered translation may be more profound than altered transcription after irradiation. A potential mechanism is the switch from cap-dependent to cap-independent initiation that occurs under stress (12, 13). About 10% of cellular mRNAs include an internal ribosome entry site (IRES) motif for cap-independent recruitment of the ribosome and encode survival and proliferation factors such as cyclin D1 (13, 16). Translation of such factors after irradiation via cap-independent translation may be critical for cell survival after irradiation. Consistent with this idea, a recent study shows that stimulation of translation elongation, by inhibiting EF2 kinase that phosphorylates and inhibits elongation factor 2, is required for osteosarcoma cells to recover from genotoxic stress (17).

Given that protein synthesis is critical for growth, many growth factor signaling pathways including PI3K/TOR and RAS/MAPK stimulate translation (18–20). Therefore, drugs that inhibit these pathways interfere indirectly with translation. But growth signaling has many targets in addition to translation. Given the role of translation in cancer, drugs that modulate translation directly may be therapeutic in oncology treatments. There are only two such drugs approved for cancer: Denileukin diftitox (Ontak®), a fusion between interleukin-2 and diphtheria toxin, which inhibits translation elongation factor EF2, and is approved for cutaneous T-cell lymphoma (21); and Omacetaxine mepesuccinate (Synribo®), another inhibitor of translation elongation, is approved for chronic myelogenous leukemia. The identification of new translation inhibitors with anticancer activity would increase therapeutic options.

We have identified three chemically distinct inhibitors of translation in an unbiased screen for radiation enhancers in *Drosophila melanogaster* (22). In this screen, we administered small molecules to larvae after irradiation to inhibit cellular processes that operate postirradiation to facilitate survival. Such molecules differ from radiation sensitizers that act during irradiation. The identification of translation inhibitors is consistent with the above-mentioned findings that translation of key mRNAs may be critical for survival after

irradiation. One important question is whether radiation enhancers in *Drosophila* would act similarly in mammalian cells. One of the hits from the *Drosophila* screen, bouvardin, was identified independently in a screen for selective inhibitors of engineered breast cancer stem cells [(23) PTC application no. WO2011/130677]. Given the proposed role of cancer stem cells in regeneration after therapy, bouvardin treatment may interfere with the regrowth of tumors after irradiation.

In this study, we addressed the mechanism of translation inhibition by bouvardin treatment and investigated whether it can enhance radiation treatment in models of human cancers for which radiotherapy is a common therapeutic option. Our data suggest that bouvardin blocks translation elongation on the human ribosome by interfering with the cyclic association-dissociation of EF2 and the 80S ribosome. Bouvardin enhanced the effect of radiation treatment in head and neck cancer (HNC) and glioma cells *in vitro*. Molecular correlates that can explain this effect were identified as Cdk1, Ki67 and cyclin D1. Finally, we were able to validate the enhancement of radiation treatment with bouvardin in a mouse xenograft model of HNC.

MATERIALS AND METHODS

Cell Culture

Glioma cells were acquired from the National Cancer Institute (Bethesda, MD). HNC, HeLa (S3), HCT1116 (p53^{+/+}) and fibroblast cells were authenticated by DNA finger printing during the course of or at the end of the experiments. Cells were grown in 5% CO₂ in either DMEM (Gibco®; Life Technologies, Grand Island, NY; fibroblasts, HNC and HeLa), RPMI (Gibco; glioma cells) or McCoy's 5A (Sigma-Aldrich® LLC, St. Louis, MO; HCT116 cells) media supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA) and 1× antibiotic/antimycotic (100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, also known as Fungizone®; Invitrogen™, Carlsbad, CA).

Translation Assays

Luciferase mRNA (1 µg/µl final) was translated for 15 min at 37°C in rabbit reticulocyte lysates according to the manufacturer's instructions (Promega Inc., Madison, WI), following published protocols (22). Luciferase activity was measured immediately after substrate addition in a plate reader (Synergy 2; BioTek®, Winooski, VT). L-azidohomoalanine incorporation was performed according to the manufacturer's instructions (Click-iT™ Protein Reaction Buffer Kit; Invitrogen). Cells were incubated with methionine-free DMEM (Invitrogen) for 30 min before L-azidohomoalanine was added to a final concentration of 50 µM, along with dimethyl sulfoxide (DMSO), cycloheximide (Sigma-Aldrich) or bouvardin (NCI) and processed after an additional 2.5 h. Gels were imaged using GVM20 (Syngene, Cambridge, UK).

Fractionation of Ribosomes

HeLa cells were grown in 15 cm (diameter) culture dishes in log-phase and treated with DMSO or drugs, and the extracts fractionated on 10–60% sucrose gradients as described

previously (24). rRNA was extracted using an RNeasy Mini Kit (QIAGEN®, Valencia, CA) and analyzed on 1% agarose gels.

Cell-Based Assays

Cell growth (Table 1) was assayed as described previously (22), using CellTiter-Glo® (Promega). The IC₅₀ values were calculated using Prism's nonlinear regression dialog (GraphPad Software Inc., LaJolla, CA).

For clonogenic assays, cells were seeded in 6-well plates at a density of 300 cells/well (U251, Det562), 400 cells/well (SNB-19) and 800 cells/well (FaDu) and then grown overnight. Cells were irradiated and treated with fresh media containing DMSO (control) or bouvardin. After 24 h, the media in all wells was replaced with fresh media without DMSO/drug and cells were incubated for 8–9 additional days. Cells were fixed with TCA, stained with sulforhodamine B (SRB) and imaged on an Olympus SZX12 Stereo Microscope (Olympus Imaging America Inc., Center Valley, PA). SRB was solubilized and quantified in a plate reader (Synergy 2, BioTek).

For an additive interaction, the product of two normally distributed variables with means m_1 and m_2 and standard deviations (SD) of s_1 and s_2 , are computed as: mean = $m_1 m_2$ and SD = square root of $(m_1^2 s_2^2 + m_2^2 s_1^2 + s_1^2 s_2^2)$ [see page 140 from ref. (25)].

For cell doublings, cells were seeded in 6-well plates at a density of 300 cells/well and treated as in clonogenic assays. Individual cell/colonies were “marked” with circles drawn on the bottom of the plate and live cells were counted every 24 h on a GeneMate inverted compound microscope (BioExpress, Kaysville, UT).

Cytology

For caspase 3, cells were seeded on cover slips in 12-well plates at a density of 20,000 cells/well. Cells were fixed in 4% formaldehyde in PBS and stained with rabbit anti-caspase-3 antibody (1:100; Cell Signaling Technology®, Danvers, MA), followed by rhodamine secondary antibody (1:500; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Cells were stained for DNA with 10 µg/ml Hoechst 33258 (Sigma-Aldrich).

For phospho-histone H3 (pH3), Ki67 and β-galactosidase staining, cells were seeded on cover slips in 6-well plates at the clonogenic seeding densities as described previously. Cells were fixed in 10% formaldehyde in PBS with 0.2% Tween® and stained with rabbit anti-phospho-S10 histone H3 (1:1,000; Millipore, Billerica, MA) or anti-Ki67 (1:500; Abcam®, Cambridge, MA) primary antibodies. Cells were stained with secondary antibody and for DNA as described for caspase staining. Beta-galactosidase staining was performed as previously described (26).

Stained cells were mounted in Fluoromount G® (SouthernBiotech, Birmingham, AL). Antibody-stained cells were imaged using a Leica DMR compound fluorescence microscope, a SensiCam CCD camera and Slidebook software (Intelligent Imaging Innovations Inc., Denver, CO). Images were processed in Adobe Photoshop. Beta-galactosidase-stained cells were imaged using a Nikon E600 with a SPOT Insight QE

(model no. 4.2; SPOT Imaging Solutions™, Sterling Heights, MI) camera and a 10× objective.

Western Blot Analysis

To generate enough material for Western blotting, cells were seeded at 175,000/15 cm plates (for samples collected up to 6 days after treatment) or at a density of 300 cells/well in 6-well plates (for samples at 9 days after treatment). Cell extracts were prepared in RIPA buffer and analyzed by 10% SDS-PAGE. Western blots were probed with anti-PARP (1:750; cat. no. 9542, Cell Signaling Technology); anti- γ -tubulin (1:1,000; cat. no. T-5192, Sigma-Aldrich); anti-eEF1A, eEF2 or RPL13a (rabbit, 1:1,000; Cell Signaling Technology); anti-cyclin D1 (rabbit, 1:250–500; cat. no. SC-753, Santa Cruz Biotechnology® Inc., Dallas, TX); anti-nucleolin (mouse, 1:5,000; cat. no. SC8031, Santa Cruz Biotechnology); anti-Cdk1 (1:1,000; cat. no. 9116, Cell Signaling Technology), anti-phospho-Y15 Cdk1 (1:1,000; cat. no. 9111, Cell Signaling Technology); anti-p21 (1:500; Cell Signaling Technology); anti- γ -H2Ax (1:250; Abcam); HRP-conjugated secondary antibodies and SuperSignal West Pico Solutions (Thermo Fisher Scientific Inc., Waltham, MA).

Xenografts

Published protocols were followed using athymic nude mice (22), with the following changes. Frozen stock (5 μ l; 40 mg/ml bouvardin in DMSO) was diluted with 45 μ l 0.5% carboxymethylcellulose (Sigma-Aldrich) to produce a working stock. The working stock (3 μ l) was diluted into 100 μ l saline to generate the injection solution. Mice were anesthetized with ketamine/xylazine before irradiation and shielded with lead to expose only the tumor-bearing leg. Tumor volume was calculated using the formula $V = (a^2 \times b)/2$, where a and b are the smallest and largest tumor diameters determined using calipers. Animals were euthanized when tumor volume exceeded 2 cm³. Animal procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Colorado.

Comet Assays

Cells were seeded in 6-well plates at a density of 50,000 cells/well and allowed to grow overnight before irradiation. Bouvardin was added immediately after irradiation and removed 24 h later by media replacement. At 24 and 48 h after irradiation, cells were processed for comet assays as described previously (27). The tail DNA content (%) was quantified from pseudo-colored images such as those shown using CometScore v1.5 software (TriTek Corp., Sumerduck, VA).

Irradiation

For comet assays and xenografts, irradiations were performed in a RS2000 Biological Irradiator (Rad Source Technologies Inc., Alpharetta, GA) delivering 1 Gy/min. For all others, irradiations were performed in a Torrex X-ray generator (Torrex Equipment Corp., Livermore, CA), set at 115 kV and 5 mA, delivering 1.44 Gy/min.

RESULTS

Bouvardin Inhibits Translation in Human Cells

In previous studies, bouvardin treatment inhibited eukaryotic but not bacterial protein synthesis (28–30). We also found that bouvardin inhibited the translation of luciferase mRNA in rabbit reticulocyte lysates with an IC_{50} in the low nM range (22). Here, we reproduced this result (Fig. 1A) and extended it to human cells and ribosomes (Fig. 1B–D). In Detroit 562 (Det562) HNC cells, bouvardin inhibited new protein synthesis, detectable as incorporation of an amino acid analog, in a dose-dependent manner with an IC_{50} in the nM range (Fig. 1B). To the best of our knowledge, these data indicate, for the first time, that bouvardin treatment inhibits translation in human cells.

Previous studies using yeast and rabbit ribosomes showed that bouvardin inhibits the elongation step of translation (30). To investigate whether this applies to human ribosomes, we fractionated ribosomes from HeLa cells, a cell line commonly used for this assay. Inhibition of elongation typically increases the abundance of 80S ribosomes relative to 60S and 40S subunits, as seen in cycloheximide-treated samples (Fig. 1C). Cycloheximide does not alter the relative abundance of polysomes (31). Bouvardin also increased the relative amount of 80S ribosomes without altering polysomes. We conclude that bouvardin, like cycloheximide, inhibits elongation on human ribosomes.

During each elongation cycle, elongation factors EF1a and EF2 associate transiently with the ribosome. Consistent with this, Western blot analysis of 80S fractions from control cells, normalized using the ribosomal protein RPL13a, showed few or no elongation factors (Fig. 1D). In contrast, bouvardin treatment dramatically increased EF2 in the 80S fractions. The effect was specific for EF2; EF1a did not accumulate in the same fractions. The effect was also specific for bouvardin; cycloheximide did not have this effect, which is consistent with the literature (32). Western blots of extracts loaded onto gradients show the presence of EF2 and RPL13a in all samples (Supplementary Fig. S1A; <http://dx.doi.org/10.1667/RR14068.1.S1>). These data suggest that bouvardin stabilized the association of EF2 and the 80S ribosome. EF2 must dissociate and re-associate with the 80S ribosome to add the next amino acid. Therefore, stabilization of the EF2-80S complex by bouvardin could explain how it blocks elongation.

We confirmed that bouvardin treatment inhibited new protein synthesis in two additional human cell lines (Supplementary Fig. S1B; <http://dx.doi.org/10.1667/RR14068.1.S1>), at doses that produced about 50% growth inhibition (IC_{50}) in each cell line, as described later.

Bouvardin Enhanced the Effect of Ionizing Radiation in HNC and Glioma Cells

Our interest in bouvardin stems from its ability to enhance the effect of ionizing radiation in *Drosophila*. Human cancers for which radiation therapy is a main treatment option include gliomas and HNC. Therefore, we investigated whether bouvardin could enhance the effect of radiation on glioma and HNC cells. Prior data from the NCI show that bouvardin inhibited the growth of NCI-60 human cancer cell lines with an average IC_{50} of ~10 nM. Glioma and melanoma lines show consistent sensitivity to bouvardin (Supplementary Fig. S2; <http://dx.doi.org/10.1667/RR14068.1.S1>). We confirmed the efficacy of bouvardin on

glioma cells as predicted by the NCI data, and extended it to HNC cells, which are not among the NCI-60 (Table 1). In contrast, in two immortalized but non-transformed fibroblast cell lines, the IC_{50} for bouvardin was higher. For instance, the IC_{50} in the human fibroblast line Detroit 551 was tenfold higher than the average IC_{50} in the NCI-60 panel (Table 1 and Supplementary Fig. S2; <http://dx.doi.org/10.1667/RR14068.1.S1>). We note that two of seven glioma cell lines we studied, SF-295 and T98G, exhibited higher IC_{50} values than other glioma lines and nontransformed fibroblasts. Based on the publicly available data from the NCI, SF-295 is consistently more resistant than other CNS cell lines to several chemotherapeutic agents that include paclitaxel and translation inhibitors baccharinol and harringtonine (<http://dtp.cancer.gov/>). T98G is not among the NCI-60. The selective resistance of a fraction of glioma cell lines to bouvardin treatment suggests that some gliomas may be refractory to the therapeutic effects of this molecule.

Next, we used clonogenic assays to investigate whether treatment with bouvardin could enhance the effect of radiation (Fig. 2). Exposure to 10 nM or higher of bouvardin for 24 h can inhibit clonogenicity completely in all four cell lines used here (glioma cells shown in Supplementary Fig. S3; <http://dx.doi.org/10.1667/RR14068.1.S1>). However, to detect drug–radiation treatment interactions, we combined doses of bouvardin and radiation that produced partial (~15–30%) effects on their own in the respective cell line. On HNC cells, the combined effect of bouvardin and radiation on HNC cells exceeded the simple sum expected if the treatments were additive. For example, on Det562 cells, clonogenic growth of cells treated with 6 nM bouvardin and 6 Gy radiation was $0.64 \pm 0.04\%$ and $0.84 \pm 0.04\%$ of controls, respectively (Fig. 2C). If additive, the combination treated cells should grow to $0.53 \pm 0.042\%$ (Fig. 2C dashed line; see Materials and Methods). Instead, the combination reduced growth to $0.30 \pm 0.06\%$. On SNB-19 and U251 glioma cells the differences between what was observed and what was expected for additive combinations were smaller. Regardless, on all four cell lines, bouvardin treatment enhanced radiation-induced effects. A range of radiation doses with a single active dose of bouvardin enhanced the radiation-induced effects seen for both SNB-19 and Det562 cells (Fig. 2E and F). In contrast, under the same conditions, bouvardin did not enhance the effect of radiation on a nontransformed fibroblast cell line, Detroit 551 (Fig. 2G). The dose-modifying factors at 50% growth inhibition were 2.5, 1.7 and 1.0 for SNB-19, Det562 and Detroit 551 cells, respectively.

Bouvardin and Radiation Combined Treatment Compromised the Mitotic Activity of SNB-19 Cells

To understand the cellular basis for growth inhibition by bouvardin, we examined its effect on cell proliferation and death in SNB-19 cells. Cells were treated using the dose and the schedule used for this cell line, as shown in Fig. 2, and stained 48 h after drug addition for cleaved PARP, a marker for apoptosis. PARP cleavage was detectable in positive controls (HCT116 + 5-FU) but not in SNB-19 cells in any treatment group (Supplementary Fig. S4; <http://dx.doi.org/10.1667/RR14068.1.S1>). We also observed no cellular debris indicative of apoptosis at all time points examined (not shown). The failure of radiation-induced apoptosis in SNB-19 cells was expected; in a previous study (33) it was found that similar radiation doses did not induce caspase activation in these cells unless exogenous caspases were

transfected. We conclude that the level of growth inhibition seen in clonogenic assays (e.g., 70% by bouvardin and radiation combined treatment) cannot be explained by apoptosis.

Next, we examined mitotic activity by staining with an antibody to phospho S10 of histone H3 (pH3). Cells were treated with bouvardin, radiation or the combination using the dose and the schedule shown in Fig. 2 and stained 9 days after irradiation. Bouvardin or radiation treatment alone had little effect but the combination reduced the mitotic index (Fig. 3A–E). We also stained for Ki67, a marker present in proliferating cells but absent in cells in G0; the fraction of Ki67-positive cells in a tumor correlates with clinical outcome (34). We found that the effect of bouvardin and radiation treatment on Ki67 paralleled their effect on pH3 (Fig. 3F–J). To account for reduced proliferation, we stained for senescence-induced β -galactosidase (Fig. 3K–O). While radiation or bouvardin treatment alone did not have an effect, the combination increased the fraction of cells with β -galactosidase staining, suggesting a senescence-like state in the latter. These findings with pH3, Ki67 and β -galactosidase activity 9 days after completion of treatment indicate long-lasting effects. We examined p21, a Cdk1 inhibitor whose levels have been correlated with senescence in some but not all contexts [e.g., see refs. (35, 36)]. We did see a small increase in p21 in irradiated cells at earlier time points, perhaps reflecting the activation of a DNA damage checkpoint. This increase was attenuated by bouvardin, which is consistent with the published findings that blocking new protein synthesis by cycloheximide also attenuated the induction of p21 by exposing to radiation (37). More relevant, we did not see elevated p21 levels at times when we saw increased β -galactosidase staining. Instead, we saw reduced Cdk1 from day 2–10 and increased inhibitory phosphorylation on Cdk1-Y15 on day 10 in combination treated SNB-19 cells, providing molecular correlates that could explain the reduction in proliferation (Fig. 3P).

Similar results were obtained in U251 glioma cells; we saw no sign of apoptosis (not shown), reduced pH3 and Ki67 staining and increased β -galactosidase activity after the combine treatment of radiation and bouvardin (Supplementary Fig. S4B–D; <http://dx.doi.org/10.1667/RR14068.1.S1>).

Given that radiation-induced DNA damage responses include increased expression of DNA repair proteins, translation inhibition by bouvardin treatment could impact repair. Comet assays and antibody staining for γ -H2AX showed that the combination of drug and radiation treatment resulted in a greater level of damage than each single agent in SNB-19 cells. This effect, however, was small and transient; by 48 h after bouvardin removal (72 h after irradiation), single and combination treated cells were not different from controls (Supplementary Fig. S5; <http://dx.doi.org/10.1667/RR14068.1.S1>). We conclude that persistent DNA damage is not the explanation for growth inhibition by the combination of bouvardin and radiation treatment seen at later time points.

Bouvardin and Ionizing Radiation Treatment Inhibit Det562 Cell Doubling

Det562 HNC cells showed a low level of cell debris after a combined treatment of radiation and bouvardin (not shown), suggesting a low level of apoptosis. This was confirmed by staining for cleaved caspase 3 (Fig. 4A–E) and by Western blotting for PARP cleavage (Fig. 4F). To our surprise, we saw no differences in pH3 and senescence-induced β -galactosidase

among control and treated Det562 cells (Fig. 4G–K and Supplementary Fig. S6A–D; <http://dx.doi.org/10.1667/RR14068.1.S1>), unlike in SNB-19 cells. Yet, reduction of clonogenicity in Det562 cells and SNB-19 cells by these treatments was similar (~70% of controls; Fig. 2). We conclude that different growth inhibitory mechanisms must operate in HNC cells. Interestingly, FaDu cells behaved similarly to Det562 cells, with similar pH3 staining among the treatment groups and no indication of β -galactosidase (Supplementary Fig. S6E–M; <http://dx.doi.org/10.1667/RR14068.1.S1>).

To identify mechanisms for growth inhibition of Det562 cells, we monitored cells viability each day after treatment (Fig. 4L and M). As in clonogenic assays, cells were irradiated on day 0 and exposed to bouvardin for 24 h from day 0 to day 1. For up to 4 days after irradiation, bouvardin or radiation alone inhibited cell doubling. The combination, however, had a greater effect such that cell numbers increased only twofold in a 96 h period. In some experiments, cells were fixed before counting but produced similar results (not shown). The inhibitory effect on cell doubling was transient; from day 7 to day 9, cells in control and all treatment groups doubled to a similar extent (Fig. 4M). This is consistent with the finding that by these times, pH3 staining was similar among all groups (Fig. 4K). We conclude that Det562 cells show a transient inhibition of cell doubling, followed by full recovery, with initial effects producing a measurable difference in clonogenic growth quantified 8–9 days after drug treatment (Fig. 2).

To address the molecular mechanisms for the effect of radiation and bouvardin on Det562 cells, we monitored Cdk1 and cyclin D1, which is known to be important for cell growth and survival after irradiation (38). In contrast to SNB-19 cells, Det562 cells did not show a drop in overall Cdk1 protein levels. Instead, inhibitory Y15 phosphorylation on Cdk1 increased transiently in irradiated cells, with or without bouvardin, as expected for checkpoint activation. Cyclin D1 was present in control cells but became downregulated in the presence of bouvardin (Fig. 4N, 12–24 h). Cyclin D1 has a short half-life of about 20–30 min (39). Inhibition of elongation by bouvardin and rapid depletion of the existing pool by protein turnover would reduce cyclin D1. Importantly, by day 2 after irradiation (1 day after bouvardin removal), the levels of cyclin D1 had recovered in cells treated with radiation or drug alone. In contrast, cells treated with the combination had not fully recovered at this time, showing reduced levels of cyclin D1 (Fig. 4N, day 2). All cells did eventually recover, as in the day 10 samples (9 days after bouvardin removal), in agreement with full recovery of proliferation (Fig. 4G–J). The stronger/prolonged effect of the radiation and bouvardin combined treatment on cyclin D1 could explain the greater inhibition of cell doubling seen in the first 4 days after treatment (Fig. 4L).

We also monitored DNA damage in Det562 cells. We were unable to detect γ -H2AX in these cells even under conditions that are known to induce DNA double-strand breaks (DSBs), using two different antibodies [(27) and data not shown]. In comet assays, the results in Det562 cells were similar to those in SNB-19 cells; although the combination of bouvardin and radiation treatment induced a higher level of damage than each single agent, this effect was transient and had disappeared within 48 h after the end of treatment (Supplementary Fig. S5; <http://dx.doi.org/10.1667/RR14068.1.S1>). Repair of DNA DSBs can occur through G₁, S and G₂ phases of the cell cycle (40), but is compromised during

mitosis (41). A delay in progression through mitosis could in principle cause a transient defect on repair in Det562 cells. But mitotic indices were not significantly different in irradiated cells with and without bouvardin (Supplementary Fig. S5F; <http://dx.doi.org/10.1667/RR14068.1.S1>), helping to rule out this possibility.

Bouvardin and Ionizing Radiation Inhibit Tumor Growth in Mouse Xenografts

To investigate whether radiation enhancement by bouvardin observed in cells was conserved *in vivo*, we used Det562 xenografts in mice (Fig. 5A). Radiation doses used here (2 fractions/week of 2 Gy each) represent clinically relevant conditions. In the first leg of the experiment, which consisted of two weeks of treatment, bouvardin or radiation alone showed partial inhibition of tumor growth. The combination further decreased the tumor volume relative to drug or radiation alone, with statistical significance at higher drug dose (Fig. 5B). We concluded that bouvardin has activity as a single agent and enhanced the effect of radiation in mouse xenografts, which is similar to what we observed in clonogenic assays. Similar results were observed in a second independent xenograft experiment (data not shown).

After the first leg, the tumors resumed growth such that mice in vehicle, drug or radiation treatment alone groups needed to be euthanized soon after. Tumors in combination treated mice also resumed growth but more slowly (day 20–30, bottom two lines in Fig. 5). The initial delay and resumption of growth in xenografts parallel what we saw in clonogenic assays of Det562. One reason for regrowth could be the replenishment of unstable proteins as the tumors recovered from radiation exposure and bouvardin became cleared. Another possible reason is the emergence of resistant cells. To distinguish between these possibilities, the mice in the combination treatment groups were randomized into two groups. One group received no further treatment (solid lines) while the other received an additional week of the same combination of drug and radiation treatment as in the first leg (dashed lines). The final tumor volumes in the retreatment group were significantly lower, suggesting that the first lag of treatment did not select for cells resistant to treatment and that the survivors remained responsive to retreatment.

DISCUSSION

Previous studies demonstrated that bouvardin inhibits translation elongation and protein synthesis in yeast and rabbit cell extracts *in vitro* (30). We report here that bouvardin can also inhibit translation elongation in human cells and provide data to suggest that it does so by stabilizing 80S-EF2 interaction. This mechanism of action has been described for only two other drugs: fusidic acid and sordarin lock bacterial and yeast EF2s onto their respective ribosomes (32, 42). To our knowledge, bouvardin is the first agent capable of locking human EF2 on the human ribosome.

EF2 is essential for protein synthesis and its complete depletion, by diphtheria toxin for example, can kill the cell (21). Yet modulation of EF2 activity, without complete inhibition, may be therapeutic. Indeed, EF2 kinase (EF2K), which phosphorylates and inhibits EF2, provides an important regulatory mechanism. Cells under starvation activate EF2K to inhibit EF2 (17). This may be to curb protein synthesis, the most energy-consuming cellular

process, during lean times. EF2K is also deployed in osteosarcoma cells immediately after exposure to doxorubicin and radiation, but is inactivated later for successful recovery from genotoxic stress (17). These results suggest that activation of EF2 is required for recovery from radiation exposure and that blocking its function may enhance the effect of radiation. These results in human cancer cells may explain why we identified three chemically distinct inhibitors of translation elongation in the *Drosophila* screen: didemnin B (43), streptovitacin A (NSC 39147, a natural analog of cycloheximide) and bouvardin (we show the data in this current study). These findings are also consistent with our published data that compromising translation capacity in *Drosophila* by halving ribosomal protein gene dosage did not affect survival of nonirradiated larvae but reduced the survival of irradiated larvae (22).

Why might stimulation of translation be important for recovery from genotoxic stress? mRNAs for cyclin D1 may be translated by either cap-dependent or cap-independent initiation and encode proteins with a short half-life that are particularly prone to depletion upon inhibition of elongation. Cyclin D1 is essential for cell growth and proliferation and we identified it as a molecular correlate that can explain the effect of bouvardin and radiation on Det562 cells. It is unlikely that it is the only critical player; additional proteins with similarly short half-lives are likely to contribute to the growth and recovery of these cells. Proteome-wide analyses to identify such proteins and study their role in recovery after irradiation would be a worthwhile goal.

The finding that modulation of EF2 (by EF2K) occurs naturally, opening the door to using this “essential” and “housekeeping” protein as a drug target. Many solid tumors overexpress EF2 (44–47). And in recent studies, EF2 and anti-EF2 antibodies have been found in the sera of cancer patients but not in healthy sera (44, 48). Knocking down EF2 with shRNA curbed the growth of cancer cell lines that overexpress EF2 but did not affect the growth of a cell line with low EF2 levels (48). These data suggest that partial inhibition of EF2 may inhibit the growth of EF2-overexpressing tumors while sparing cells with lower or normal levels. In this regard, it is interesting that proteomics analyses of the NCI-60 panel identified signatures of elevated PI3K signaling in glioma and melanoma lines (49), which show consistent sensitivity to bouvardin (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR14068.1.S1>). PI3K signaling stimulates translation (18–20). We speculate that cells with increased PI3K signaling are “addicted” to a high rate of protein synthesis, and that such cells are particularly sensitive to translation inhibitors, an idea that has been proposed before (2). Our finding that bouvardin was less potent (Table 1) and less able to enhance the effect of radiation on nontransformed cells (Fig. 2G) supports these ideas.

The ability of bouvardin to induce apoptosis, alone or combined with radiation treatment, was modest. Instead, the large reduction in clonogenic growth of Det562 cells may be attributed to a transient but significant decrease in cell doubling. Interestingly, while Det562 cells were able to recover and proliferate, a consistent response of SNB-19 cells similarly treated was to enter a senescence-like state. We speculate that these differences are due to differences in how cells normally respond to radiation exposure. Det562 cells may respond by transient growth inhibition through reduction of proteins such as cyclin D1. Further reduction of such proteins by incubation in bouvardin for 24 h would delay recovery but proliferation would eventually resume. SNB-19 cells on the other hand may enter

senescence as a default state after irradiation but this choice is prevented by as yet unknown factors that need to be synthesized in response to radiation. A transient presence of bouvardin may deplete such factors, resulting in a senescence-like state several days after drug removal. Again, proteome-wide analyses may help identify these factors.

In conclusion, we found that bouvardin inhibits translation on human ribosomes and in human cells. We propose that the mechanism of action is stabilization of 80S-EF2 complex, thereby interrupting the elongation cycle. We also found that bouvardin inhibited the growth of HNC and glioma cells. Bouvardin enhanced the effect of radiation on clonogenic survival of HNC and gliomas and the growth of HNC cells xenografted into mice. Finally, cellular mechanisms by which bouvardin enhanced the effect of radiation may be different for the two cell lines studied despite similar outcomes on clonogenic growth. Based on these results, we suggest that bouvardin and other translation inhibitors should be studied further as potential therapeutic options for cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Bouvardin inhibits translation elongation. Panel A: Bouvardin inhibited translation of luciferase *in vitro* in rabbit reticulocyte lysates. Panel B: Bouvardin inhibited new protein synthesis in Det562 cells. Cells were incubated with an amino acid analog L-azidohomoalanine for 2.5 h in 0.05% DMSO (CTRL), 50 μ M cycloheximide (CHX) or bouvardin at concentrations shown. Cell lysates were analyzed on 10% acrylamide gels imaged under 300 nm excitation. The mean value (Gy) was quantified using Image J software and shown below each lane. Coomassie stain showed that bulk protein levels were unaffected and indicated similar loading among the lanes. Panel C: Bouvardin increased the relative abundance of 80S ribosomes. Extracts of HeLa cells treated for 30 min with 0.1% DMSO, 100 μ g/ml cycloheximide or 10 μ M bouvardin (final concentrations) were analyzed

on sucrose gradients. The identity of the peaks corresponding to 40S (~fraction 5), 60S (~fraction 8) and 80S (~fraction 10) ribosomes and the polysomes (~fractions 14 and higher) were confirmed by the profile of 18S and 28S rRNAs. Panel D: Western blot analysis shows that bouvardin increased EF2 that co-fractionated with the 80S ribosome. Similar data were obtained in two independent experiments but only one set is shown.

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FIG. 2.

Bouvardin and radiation treatment inhibits head and neck cancer and glioma cells in clonogenic assays. Panels A–D: Clonogenic assays of HNC and glioma cancer cell lines treated with bouvardin (BVD) and ionizing radiation. Cells were plated and allowed to adhere for 24 h (day –1 to day 0). On day 0, the drug was added to the cell media immediately after irradiation. The drug was removed 24 h later, on day 1. Clonogenic growth was quantified on day 9 or 10, with similar results. The data are from three biological replicates each of which consisted of at least three technical replicates. The data were normalized to the untreated controls. The expected outcome of bouvardin and radiation acting additively is shown as dashed lines. Error bar = 1 SD. Panels E–F: Radiation enhancement by bouvardin in clonogenic assays. Dotted lines = radiation alone. Dashed lines = the expected result for radiation and a single dose of bouvardin as indicated was

additive. Solid lines = the observed effects of radiation and bouvardin. The effect of bouvardin alone on SNB-19 and Det562 cells was lower in these experiments than in those shown in panels A and C, but the enhancement of radiation was apparent. Error bar = ± 1 SD.

FIG. 3.

The effect of bouvardin and radiation on SNB-19 glioma cells. The treatment schedule is the same as that described in the legend of Fig. 2. Panels A–J: Cells were treated with DMSO vehicle (CTRL), bouvardin (BVD), radiation or the combination and fixed and stained on day 10 to visualize DNA (blue), phospho-S10 histone H3 (pH3, red) and Ki67 (red) or processed for β-galactosidase activity (panels K–N). The data from at least two independent experiments, each of which consisted of at least three technical replicates per treatment, were quantified in panels E, J and O. Panel P: Cells were processed for Western blotting at various times after start of treatment, as in panels A–N. Nucleolin served as a loading control. Error bar=1 SD. The scale bar (50 μm) in panel A applies to panels A–D and the scale bar in panel F applies to panels F–N.

FIG. 4. The effect of bouvardin and radiation on Det562 HNC cells. The treatment schedule is the same as that described in the legend of Fig. 2 except where noted. Panels A–D: Cells were fixed 48 h after irradiation and stained for DNA (blue) and cleaved caspase 3 (red). Bouvardin was left on for the duration of the experiment. Panel E: Percentage of cells with cleaved caspase 3 is quantified. Panel F: Cells were processed for Western blotting 72 h after start of treatment with DMSO (CTRL), 6 nM bouvardin (BVD), 6 Gy radiation or the combination. Nucleolin serves as a loading control. Panels G–J: Cells were fixed on day 9

and stained for DNA (blue) and phospho-histone H3 (pH3, red). Panel K shows the percentage of cells with pH3 stain is quantified. Panels L–M: Cells were counted live and the fold change in number quantified. Panel N: Cells were processed for Western blotting at various times after start of treatment, as in panels G–J. Nucleolin served as a loading control. Error bar = 1 SD. Scale bar = 50 μ m.

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FIG. 5.

Bouvardin and radiation inhibit the growth of Det562 mouse xenografts. Panel A: Tumor volumes were plotted against treatment days. Bouvardin (BVD) was administered 24 h prior to irradiation (arrow). N=9 for the radiation only arm and N = 10 for each of the others. No clinical signs of toxicity (loss of > 10% body weight, lethargy and loss of appetite) were noted. Panel B: Tumor growth rates for up to day 23, for which the data were available for all groups. Except where otherwise noted, *P* values for pairwise comparisons are shown for up to day 23 for all groups. ^a*P* values for pairwise comparisons are shown for up to day 30,

the last day for the radiation only group (bold text indicates significance). A linear mixed model with mouse-specific intercepts and slopes were used. Between-group comparisons were based on contrasts in the mixed model. For re-treatments beyond day 23, the only significant difference was detected among $\pm$ re-treatment with the high-dose (0.6 mg/kg) drug at day 37 by Student's *t* test ($P < 0.05$), by nonparametric Wilcoxon-Mann-Whitney test due to non-normality ($P = 0.016$) and also with Bonferroni-corrected P values for multiple comparisons ($P = 0.048$).

TABLE 1Concentration of Bouvardin Required to Inhibit Growth by 50% (IC₅₀) in Human Cell Lines

Cancer cells	Concentration of bouvardin(nM)
Central nervous system cancer cells	
U87MG	2.9
U251	3.3
SF-268	4.7
SNB-19	6.6
SNB-78	11.4
SF-295	159.7
T98G	515.7
Head and neck cancer cells	
FaDu	4.6
CCL-30	4.9
Det562	7.1
SCC-25	13.9
MSK-921	15.2
PE/CA-PJ-34	21.9
Nontransformed fibroblast cells	
Detroit 551	97.4
J2T7	46.9

Notes. Drug concentration required to inhibit growth by 50% (IC₅₀) in head and neck cancer cells, central nervous system cancer cells and nontransformed immortalized human (Detroit 551) and mouse (J2T7) embryonic fibroblast cells, after five-day drug exposure. Averages of at least two biological replicates, consisting of six technical replicates per drug concentration, are shown.