UNIVERSITY OF CALIFORNIA, SAN DIEGO

Cobinamide as a Radioprotective Agent

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in

Biology

by

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Professor Douglass Forbes

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Co-Chair

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ABSTRACT OF THE THESIS

COBINAMIDE AS A RADIOPROTECTIVE AGENT

by

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Master of Science in Biology

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Professor Gerry Boss, Chair

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Ionizing radiation is classified as a potent carcinogen and its injury to cells is, to a large extent, due to oxidative damage generated by reactive oxidative species (ROS). The biomolecules most often reported to be damaged by ionizing radiation are lipids and DNA. Dr. Boss has shown that cobinamide, a structural analogue of cobalamin (vitamin B12), binds to superoxide anion *in vitro*. We now show that in cultured cells cobinamide can work as a possible therapeutic agent for radiation-induced injury. First, in HeLa cells cobinamide improved increased DNA synthesis by an average of 19 percent when given prior to irradiation, and 7 percent when given 24 hours post-irradiation. Secondly, in irradiated MDA MB-231 cells, the doubling rate was 19 percent higher in cobinamide-treated cells than in non-treated cells. And finally, cells treated with cobinamide showed less fragmentation and blebbing following irradiation than non-treated cells. These data suggest that cobinamide might be beneficial in treating the effects of ionizing radiation.

Introduction

Adverse Biological Effects of Ionizing Radiation is due to the Generation of Free Radicals

Ionizing radiation can be defined as any type of electromagnetic (such as X- or gamma rays) or particle radiation (such as neutron or alpha particles) with sufficient energy to ionize atoms or molecules by ejecting electrons from their outer orbitals. Ionizing radiation passing through living tissues generates reactive free radicals that interact with critical macromolecules-- such as nucleic acids, proteins or lipids-- damaging them directly and indirectly. For its direct action, ionizing radiation must "hit" the macromolecule; because the volume of the biomolecules is very small compared with the total volume of the cell, the probability of this occurring is relatively low[1]. The indirect actions of radiation occur when it interacts with water molecules in the cell, resulting in the production of highly reactive free radicals, such as $^{\cdot}OH$ and O_2^- (Figure 1). The half-life of these free radicals is extremely short, on the order of 10^{-6}–10^{-10} seconds; however, they immediately react with any biomolecules in the vicinity and produce oxidative damage. Irradiated cells can have deleterious effects on nearby cells such as induction of mutations, cell-killing, changes in signal transduction, and genomic instability [2].

Damage to DNA may be the most important factor in cell death from irradiation. Free radicals attack the sugar-phosphate backbone of DNA, resulting in the production of single- and double-strand breaks, as well as DNA–DNA and DNA–protein cross-links [3]. Whereas single-strand breaks can be repaired quickly using the undamaged DNA strand as a template, double-strand breaks are not easily repairable. When DNA is damaged, it can lead to altered cell division, altered metabolism, organ system

dysfunction and, if the radiation dose is sufficiently high, to cellular and organismal death.

Besides DNA, free radicals also interact with unsaturated sites in lipids, resulting in the production of hydroperoxides. Hydroperoxide changes the interactions between adjacent chains of phospholipids in the lipid bilayer altering the electric constant across the bilayer, which in turn leads to changes in membrane permeability [4]. Free radicals generated by exposure to ionizing radiation also induce detectable changes in the structure and function of proteins. Although cells and tissues are equipped with endogenous enzymes capable of detoxifying and removing products of water radiolysis (e.g. superoxide dismutase), irradiation produces an excess amount of reactive oxygen species that overwhelms the endogenous system making it incapable of protecting cells from the hazardous effects of free radicals.

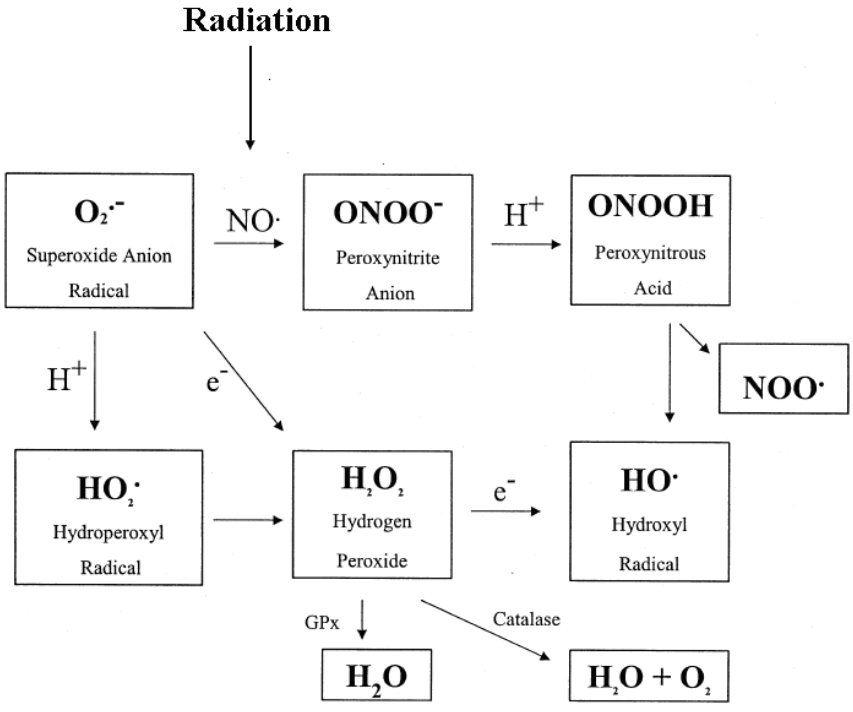


Figure 1: Interrelationship of free radicals generated after irradiation

Radiation in Medicine and the World

Radiation therapy injures or destroys cells in the area being treated by damaging DNA, making it impossible for these cells to continue to grow and divide. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The nature and degree of such unwanted side effects depends upon the dose of ionizing radiation and the sensitivity of the organs that are irradiated. With respect to the potential application of ionizing radiation in medical practice (e.g. radiotherapy and nuclear medicine) and also potential exposure to radiation (e.g. industrial nuclear accident and dirty bombs), the development of effective radioprotectants is of great medical importance.

Physiological Defense

Defense and repair mechanisms exist in living cells to protect against oxidant species. The antioxidative defense system is composed of methods to (i) transfer sensitive material to compartments better protected from the action of reactive species, (ii) complex with transition metals, a potential source of electrons, thereby rendering them unreactive, (iii) inhibit vulnerable processes such as DNA replication, (iv) repair damaged molecules, (v) activate antioxidant enzymes, (vi) use a variety of direct free radical scavengers, and (vii) initiate apoptosis, possibly the most important factor considering the liability to internal and external modifying factors. Of the enzymes involved in antioxidative defense, particularly well documented are the antioxidative activities of the superoxide dismutases , glutathione peroxidases, and catalase [1].

Radioprotectants.

Because radiation-induced cellular damage is attributed primarily to the harmful effects of free radicals, molecules with direct free radical scavenging properties are particularly promising as radioprotectants. Initial attempts were focused on synthetic thiol compounds. These agents are effective at reducing lethality induced by irradiation. Of this class, amifostine is the only radioprotectant that has been clinically approved by the Food and Drug Administration (FDA) for mitigating side effects in patients undergoing radiotherapy [4]. Although amifostine was reported to be tolerated well in radiotherapeutic clinical trials, it was later found to have some undesirable side effects. These include hypotension, nausea, vomiting, sneezing, hot flashes, mild somnolence, and hypocalcemia [5]. These side effects are severe enough to limit the amount of the drug administered to levels lower than necessary to achieve maximal radioprotection. Over the years, a number of compounds have been tested for their radioprotective efficacy with generally limited success. Thus, there is still an urgent need to identify novel, nontoxic compounds to protect humans from the damaging effects of ionizing radiation.

Cobinamide

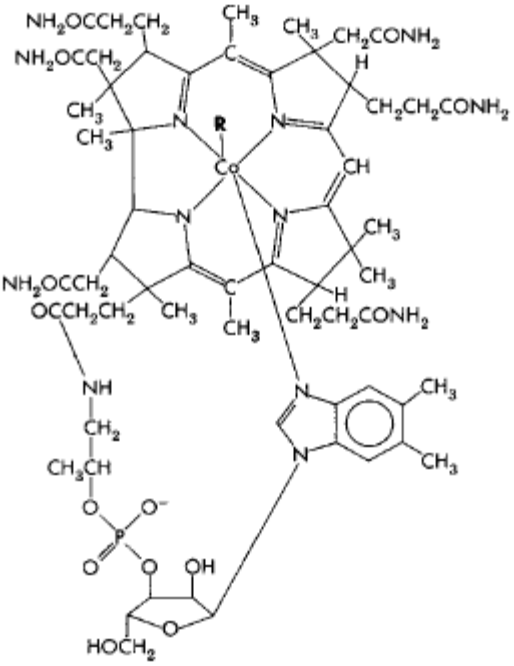
Cobinamide (Cbi) is the penultimate compound in cobalamin (Cbl) biosynthesis. Cobalamin— vitamin B_{12}—is an essential micronutrient required for the health and well-being of all higher animals, including man. Cobalamin is highly polar and has a cobalt atom coordinated to four nitrogens in a corrin ring, which is roughly planar, with a 5,6-dimethylbenzimidazoyl moiety (Bzm) occupying a fifth coordination site and attached by a nucleotide tether to the D ring of the corrin (Figure 2). Cobinamide, lacks the Bzm, and

the tether is abbreviated to an aminopropanol side chain of the corrin [7]. In each molecule the metal is found in either an oxidized (+3) or reduced (+2) oxidation state. The reduced forms of both can be oxidized by oxygen, and hence are unstable in air. The oxidized form of cobinamide binds cyanide and reacts with nitric oxide; the oxidized form of cobalamin binds cyanide, but reacts with nitric oxide only at a low pH [7-9].

Cobinamide as a Free Radical Scavenger

The potential use of cobinamide as a free radical scavenger is related to the findings that cobalamin scavenges superoxide under *in vitro* conditions and that cobinamide can scavenge nitric oxide [1]. Electron paramagnetic response (EPR) spectroscopy revealed that cobinamide scavenges toxic agents by binding to them via its metal ion, rendering them inert [5]. Each molecule of cobinamide can bind to two cyanide ions, with at least 100 times more affinity than cobalamin ($K_A = 10^{14}\ M^{-1}$). Cobinamide also binds nitric oxide, but with less affinity than cyanide ($K_A = 10^{2}\ M^{-1}$). The potential ability of cobinamide to scavenge free radicals by binding superoxide anion radicals and peroxynitrite anion radicals could be an important property in its protection against oxidative stress. These data led us to hypothesize that cobinamide, at appropriate concentrations, may detoxify radiation-induced free radicals. We now provide early data that this hypothesis may be correct, and thus cobinamide might be a good agent to ameliorate radiation toxicity.

Cobalamin



Cobinamide

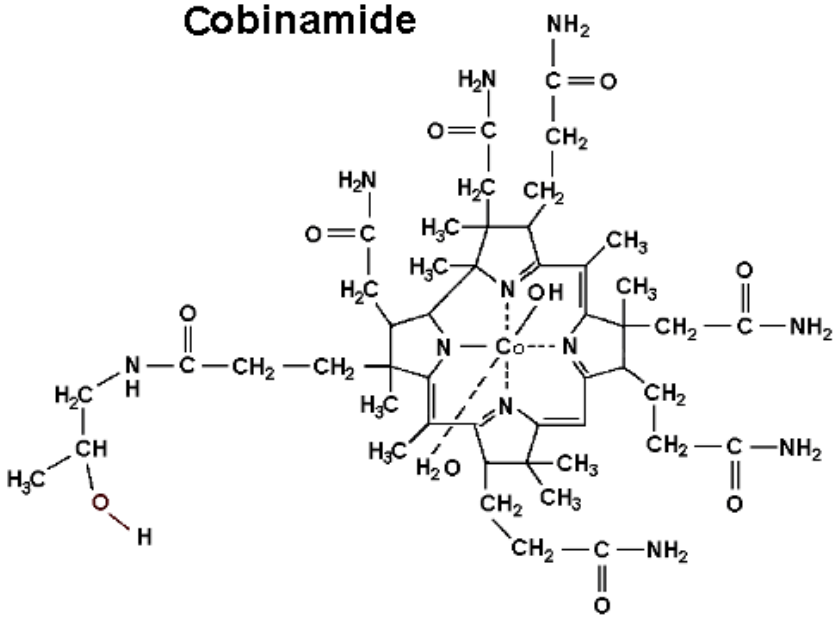


Figure 2: The chemical structures of cobalamin and cobinamide. Cobinamide lacks the dimethylbenzimidazole ribonucleotide tail coordinated to the cobalt atom in the lower axial position.
The "R" is an OH group in hydroxocobalamin and a cyanide ion in cyanocobalamin (vitamin B_{12}).

Materials and Methods

Cell Lines. Human adenocarcinoma HeLa cells and human breast cancer MDA MB 231 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum at 37º C and 5% CO_2.

Irradiation. Cells were irradiated with 10 or 15 Gy doses from a Cobalt-60 radiation source.

Synthesis of Cobinamide. Cobinamide was prepared from hydroxocobalamin by base hydrolysis at pH 9.5 using cerium hydroxide. The cobinamide product was separated from unreacted hydroxocobalamin on a weak cation exchange column eluted with a NaCl gradient. The eluted cobinamide was desalted on a C18 reverse phase resin column, concentrated on a rotary evaporator, and lyophilized to a solid state.

Dose and Administration of Cobinamide and Cobalamin. Stock solutions of cobinamide and cobalamin were made at 100 x and kept at 4ºC. The effects of cobinamide and cobalamin were assessed following irradiation at drug concentrations of 10, 20, 50, and 100 µM in cells.

DNA Synthesis Assay. Cells were plated in 12 well dishes (150,000/well) 24 hours prior to irradiation. One hour before irradiation, the medium was replaced with 0.5 mL DMEM with 10% fetal bovine serum dialyzed against saline. After irradiation at 10 Gy, the cells were placed back in the incubator and the drugs added at time points 2 and 24 hours. Forty-seven hours post irradiation, cells were labeled with tritiated thymidine (1.5µl/well) for 1 hour. At the end of the incubation period, cells were washed three times with phosphate-buffered saline (PBS), extracted in 10% trichloroacetic acid (TCA), and acid-precipitated material was collected on Microfiber glass discs (Whatman GF/C). The

filters were washed three times with 10% TCA, one time with 70% ethanol, and dried in an oven. Radioactivity was measured by liquid scintillation counting. Each condition was performed in duplicate.

Prior to extracting cells directly in 10% TCA, 0.1% SDS was used to lyse cells. This method gave poor reproducibility of results. In addition, we found that changing media after irradiation yielded poor results because some of the cells were only loosely attached and were removed during the media change. The protocol was therefore changed to that stated above.

Cell Growth. Cells were plated in 12 well dishes (150,000/well) 24 hours prior to irradiation. One hour before irradiation, the medium was replaced with 0.5 mL DMEM with 10% dialyzed fetal bovine serum. After irradiation at 15 Gy, the cells were returned to the incubator and the drugs added at time points 2 and 24 hours. Forty-eight hours later cells were harvested by washing with PBS, trypsinized with 0.25% Trypsin/EDTA (TED), and counted using a Coulter Counter. Cell growth was quantified in terms of doubling using the formula:

$$\text{Doubling} = (n_f/n_0)/ \ln 2$$

where n_f is the final cell count and n_0 is the initial cell count. The doublings of untreated cells and treated cells were compared to one another.

Cell Morphology. Cells from the cell growth assay were washed with PBS. The photographs were taken with a 10.2 mega pixel Nikon D40X Outfit Camera and a 20 x objective using a contrast Nikon TMS microscope

Statistical Analysis. One-way ANOVA was used to compare means from treated and untreated cells. $P<0.005$ was considered to be considered statistically significant.

Results

Effect of cobinamide and cobalamin on non-irradiated cells

We chose cobalamin as a positive control because in addition to being the precursor to cobinamide, it has been shown to react with superoxide anion. To determine whether cobinamide or cobalamin affected thymidine incorporation, HeLa cells were treated with the drugs 30 minutes prior to irradiation as well as 2 and 24 hours post irradiation. The average thymidine uptake for non-treated cells was about 24,000 cpm (Figure 3). Treating HeLa cells with 50 µM cobinamide and 50 µM cobalamin for up to 48 hours had no statistically significant effect on thymidine incorporation.

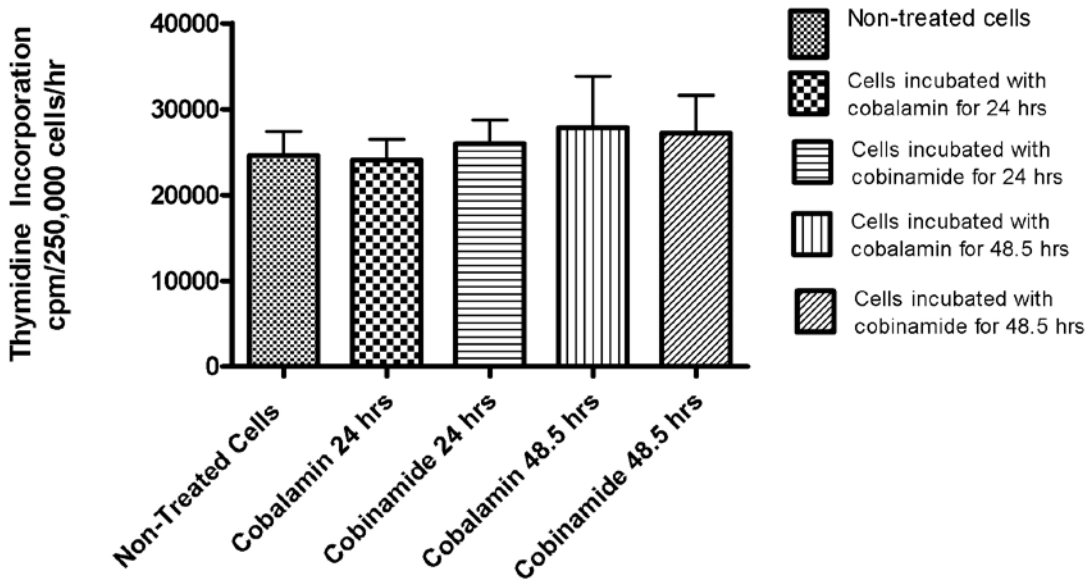


Figure 3: Effect of cobinamide and cobalamin on DNA synthesis in non-irradiated HeLa cells. Both drugs were administered and incubated for 24 and 48.5 hours. The cells were incubated with tritiated thymidine at 47 h post irradiation and harvested at 48 h.

Cells treated with cobinamide following irradiation show an increase in DNA synthesis

Following inadvertent exposure to radiation (i.e. industrial accident or a dirty bomb), treatment with a radioprotectant would hopefully occur within a couple hours and certainly by 24 hours. We therefore decided to test both an early (2 hours post exposure) and a late time point (24 hours post exposure).

After HeLa cells were exposed to 10 Gy radiation and incubated for 48 hours, there was a significant decline in thymidine uptake (Figure 4). Irradiated cells incorporated thymidine at an average rate of about 6,600 counts per minute (cpm), about 69% less than control cells (Figure 5). In addition to showing less thymidine incorporation, the irradiated cells demonstrated typical apoptotic behavior of fragmentation and blebbing (Figure 6A and 6B).

Irradiated cells treated with cobinamide showed increased resilience to irradiation, reflected by an increase in thymidine uptake and morphology compared to untreated cells (Figures 4- 6). Cobinamide treated cells incorporated thymidine at an average rate of about 11,400 cpm when given 30 minutes prior to irradiation, and 7,900 cpm and 8,600 cpm when given 2 and 24 hours post irradiation respectively. The effect when cobinamide was given prior to irradiation was statistically significant from untreated cells (Figure 4). There was much less debris and fragmentation in the cells treated with cobinamide and as further seen in Figure 6C-D, the effect of cobinamide could be seen already at 24 hours.

Cells treated with cobalamin showed similar improvement to that of cobinamide. When given prior to irradiation, cells treated with cobalamin had an average thymidine uptake rate of about 11,200 cpm. When administered 2 and 24 hours post irradiation, the rate of uptake was about 7,500 and 9,300 cpm respectively. The treatment of cobalamin prior to irradiation, like cobinamide, was statistically significant from untreated cells.

Although neither cobinamide nor cobalamin when administered 2 and 24 hours post-irradiation caused an improvement, there was a suggested trend. We therefore averaged the data and calculated it in terms of percent recovery, setting control cells at 100 percent, and found statistical significance (Figure 5). When cobinamide was administered 2 and 24 hours after irradiation, thymidine uptake increased by 5 and 7 percent respectively. Cobalamin was equally effective, increasing DNA synthesis by 6 percent when given 2 hours post-irradiation and 9 percent when given 24 hours post-irradiation.

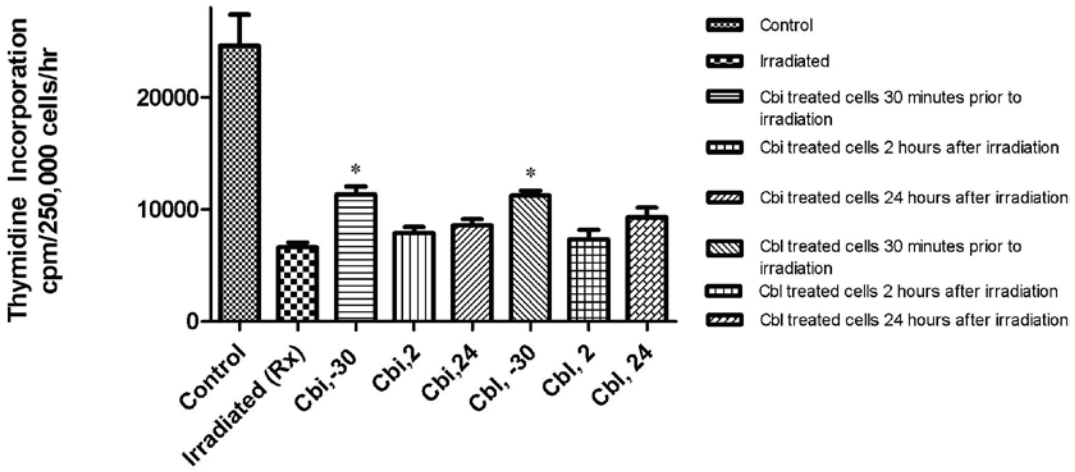


Figure 4: Effect of cobinamide and cobalamin on DNA synthesis in irradiated HeLa cells. Both drugs were administered 30 minutes prior or 2 and 24 hours post irradiation. The cells were incubated with tritiated thymidine at 47 h post irradiation and harvested at 48 h. * Denotes statistical significance when compared to irradiated cells ($P<0.005$)

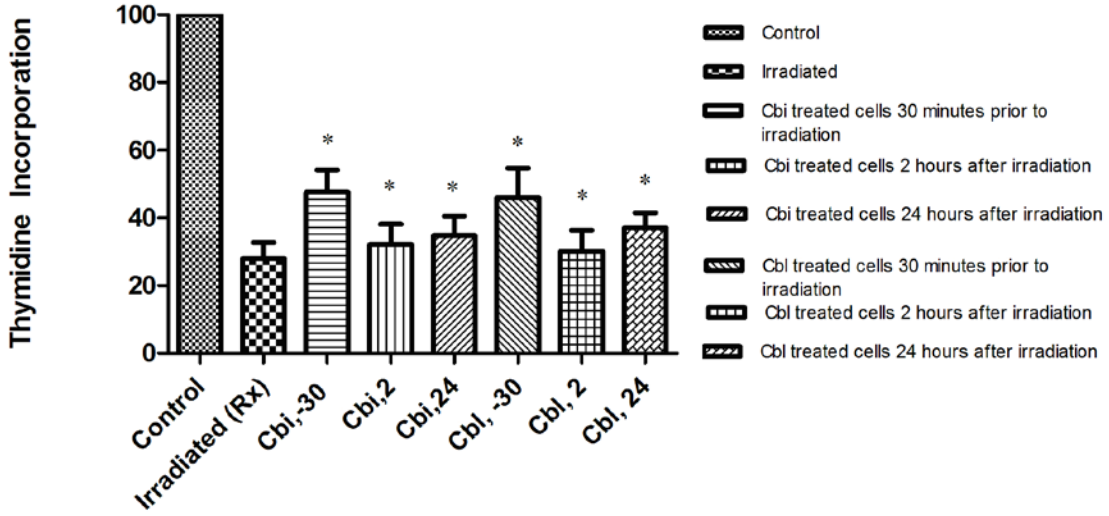


Figure 5: Thymidine incorporation data from Figure 4 were averaged and compared to control cells which were set at 100 percent. * Denotes statistical significance when compared to irradiated cells (P<0.005)

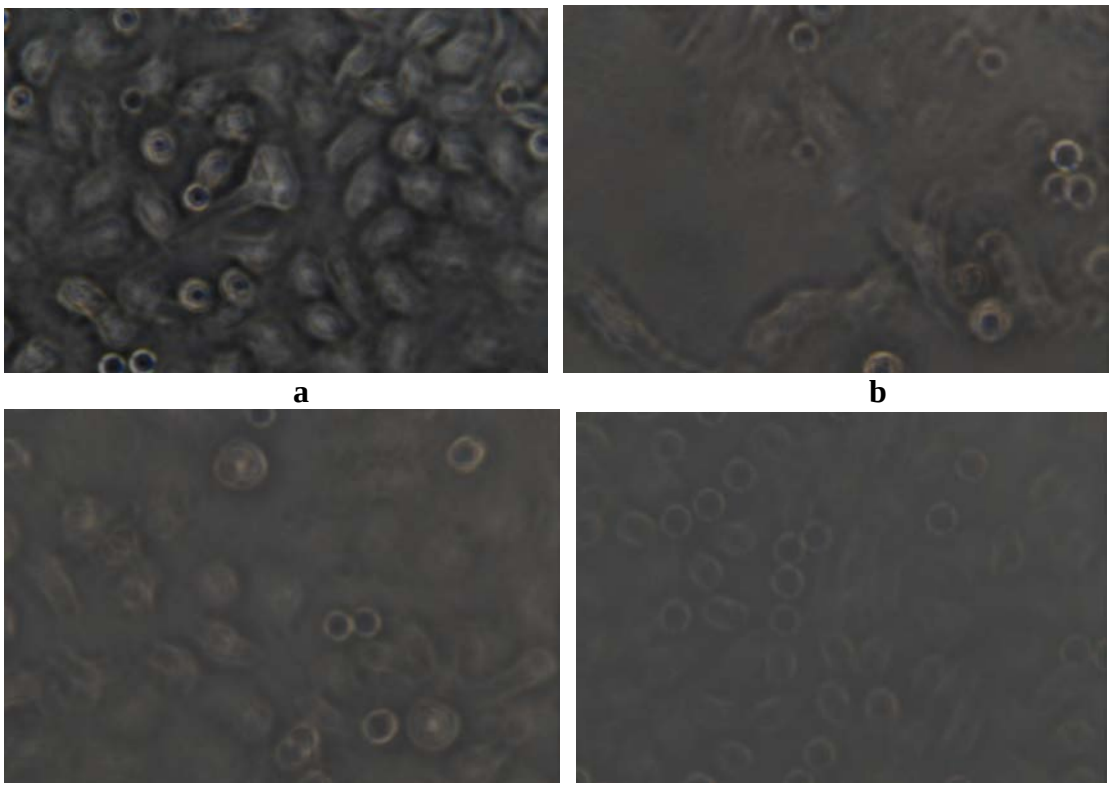


Figure 6A-D: Effect of cobinamide on HeLa cells. The cells were administered 50 uM of cobinamide at various times (a) Non-treated cells (b) Irradiated cells (c) Cobinamide added to cells 2 hrs after irradiation (d) Cobinamide added to cells 24 hrs after irradiation. The photographs were taken 48 h post irradiation.

Cobinamide was most effective at 50 uM

The effects of cobinamide and cobalamin were assessed at concentrations of 10 µM, 20 µM, 50 µM and 100 µM in HeLa cells 24 post administration and 48 hours post irradiation. As seen in Figure 7, at 10 µM and 20 µM, there was virtually no rescue by either cobinamide or cobalamin. Both drugs were most effective at 50 µM, without any further improvement at 100 µM.

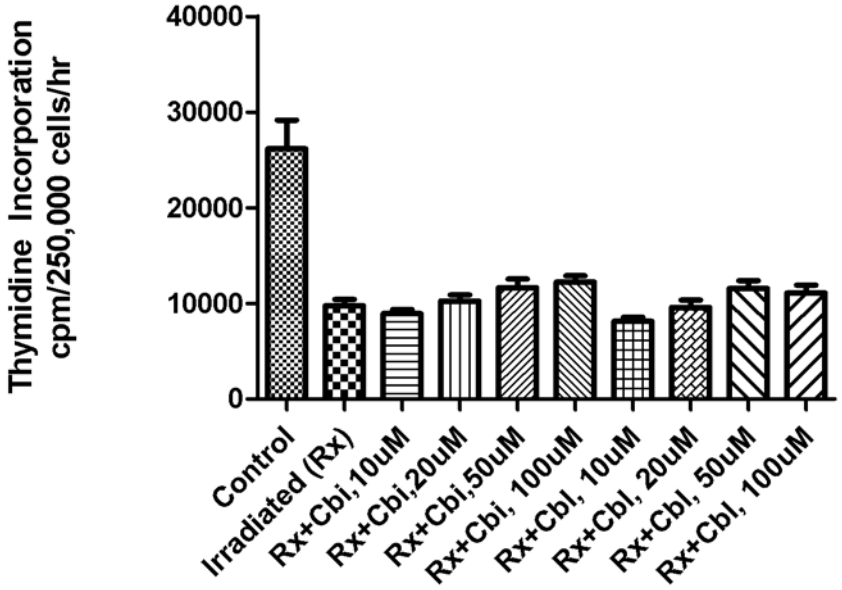


Figure 7: Effect of cobinamide and cobalamin on thymidine incorporation at varying concentrations when administered at 24 hours. The cells were harvested at 48 hours.

Cobinamide rescues cells from radiation damage as measured by cell growth

Further evidence of cell rescue was determined by measuring cell growth. MDA MB231 cells were used because they clumped less than HeLa cells and lend themselves more easily to counting. Control cells had an average of 2.13 doublings when harvested after 48 hours (Figure 8). After being irradiated at 20 Gy, the cells had an average of 1.39

doublings after 48 hours. Cells treated with cobinamide showed improved recovery with 1.84 doublings in 48 hours. Cells treated with cobalamin showed a similar doubling rate to that of cells treated with cobinamide.

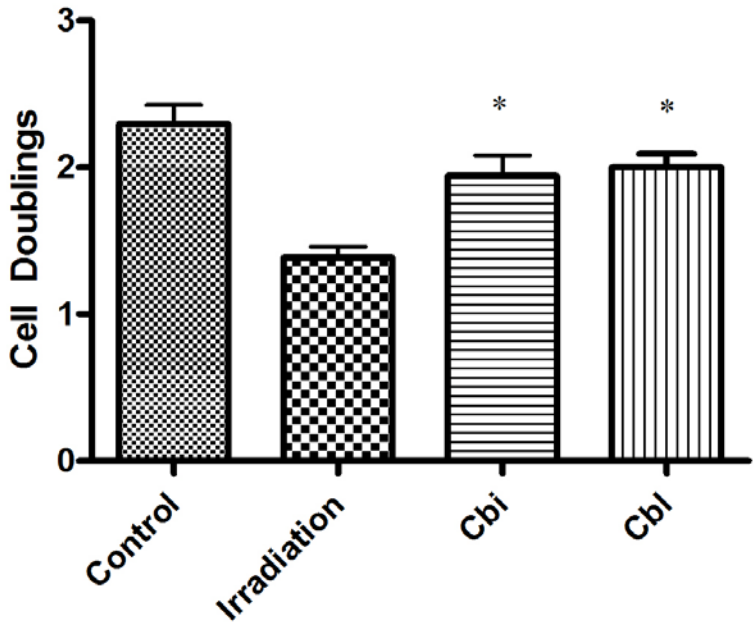


Figure 8: Effect of cobinamide and cobalamin on cell growth following irradiation at 20 Gy. Both drugs were administered at 24 hours post irradiation and average cell doublings were measured after 48 hours. Cell growth was measured in terms of doubling using the formula: Doubling= $(n_f/n_0)/ \ln 2$ where n_f is the final cell count and n_0 is the initial cell count. The doublings of control, untreated, and treated cells were compared to one another. * Denotes statistical significance when compared to irradiated cells ($P<0.005$)

Discussion

Apoptosis is a process that can be initiated by a number of different stimuli and radiation is one such initiator. Due to the very short life of free radicals generated by radiation, it is thought that most radioprotectants need to be present in the cell environment before production of free radicals in order to neutralize their destructive properties [2]. Therefore, in the past, most of the drugs developed--such as melatonin--were prophylactics. With tensions around the world higher than ever and nuclear industrial accidents occurring more often as the world transitions to nuclear power, there is a need for drugs that could be used as a therapeutic.

Viability of cells following radiation-induced oxidative stress was evaluated by three independent approaches: measurement of DNA synthesis, assessment of cellular morphology, and measurement of growth. The DNA synthesis assay measures uptake of thymidine. Proliferating cells incorporate nucleic acids to synthesize DNA. Therefore, measuring thymidine uptake is an appropriate means of measuring DNA synthesis.

Using the DNA synthesis assay, we found that cells treated with cobinamide 30 minutes before irradiation showed the greatest improvement, nearly 20 percent better than non-treated cells. This improvement in DNA synthesis was statistically significant. Although statistical significance was not achieved when cobinamide was administered 2 and 24 hours post-irradiation, there was a trend toward improvement. When cobinamide was given 2 hours following the irradiation, the results indicate that there was minimal rescue. Administration of cobinamide up to 24 hours post-irradiation increased DNA synthesis by an average of 7 percent over non-treated cells.

The growth assay allowed us to follow cell proliferation. The results indicate that normal healthy cells undergo 2.13 doublings every 48 hours, i.e. the cells double every 24 hours. Irradiated cells doubled 1.39 times in 48 hours, indicating that they were growing 65 percent slower than control cells. The addition of cobinamide after 24 hours of irradiation reached statistical significance. The doubling rate was 1.84, about 84 percent as well as non-irradiated cells.

A plausible explanation for the effectiveness of cobinamide as a therapeutic agent is that it reduced oxidative damage from peroxynitrate. Peroxynitrate is formed when NO reacts with O_2^-:

$$NO + O_2^- \rightarrow ONOO^-$$

Since cobinamide binds NO, it could be beneficial by preventing the formation of peroxynitrate. However, this does not seem likely because cobinamide and cobalamin had similar effects on irradiated cells and cobalamin does not bind to NO at physiological pH.

A more likely explanation for the effectiveness of cobinamide may involve it reducing the oxidative damage from superoxide anion. Radiation causes an initial wave of oxidative damage by generating free radicals through contact with the cells. However, a delayed, secondary wave of free radicals causes further oxidative damage. The initial wave of radiation can damage mitochondria, thus damaging the electron transport chain. Injured mitochondria can generate an inordinate amount of superoxide anion thereby causing much further damage to the cell. If cobinamide is added around the time of this

secondary wave of oxidative damage—around 24 hours post-irradiation--then it could reduce the harm done (Figure 9).

Since exposure to irradiation in radiotherapy, or accidental exposure to radiation, can produce significant unwanted side effects, it is important to ameliorate such effects by the use of a radioprotectant that can be administered before or after exposure. The ideal radioprotective agent should fulfill several criteria:

(a) It must provide protection against the effects of irradiation.

(b) It must have a general protective effect on cell.

(c) It must have an acceptable route of administration (preferably oral, or alternatively intramuscular).

(d) It must have an acceptable toxicity profile and protective time-window effect.

(e) It must have an acceptable stability profile (both of bulk active product and formulated compound).

(f) It must have compatibility with a wide range of other drugs that will be available to patients or personnel.

To this date, there is no radioprotectant that fulfills all this criteria [1]. In this paper, we show that cobinamide has the potential to be a radioprotectant.

In conclusion, we found that cobinamide plausibly provides some protection against the effects of irradiation. The present study was not able to show the mechanism behind the reduction of free radicals and other damage. However, it did suggest that cobinamide protects against oxidative damage by either directly scavenging free radicals or indirectly by some antioxidative means. In addition to providing a foundation for

further study, the identification of cobinamide as a prophylactic and therapeutic agent for radiation injuries opens a novel avenue into our understanding of radiobiology.

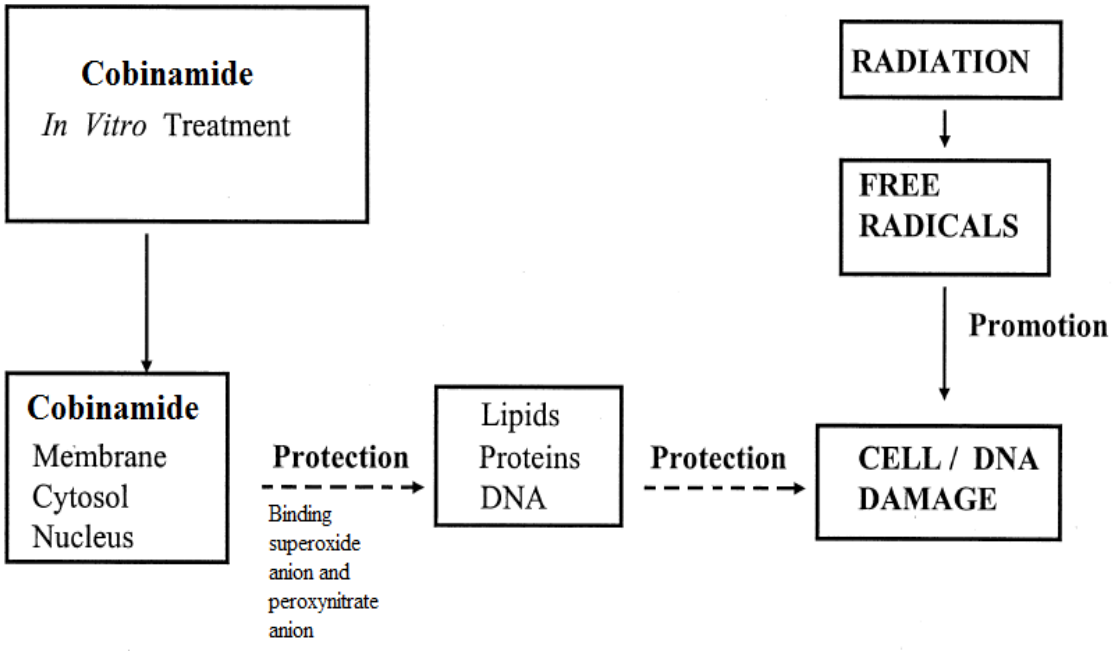


Figure 9: Potential mechanism of protection against radiation by cobinamide

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