THESIS

# EVALUATING THE GENOTOXIC AND CYTOTOXIC EFFECTS OF SYNTHETIC NUCLEOSIDES IN VITRO

Submitted by

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ABSTRACT

EVALUATING THE GENOTOXIC AND CYTOTOXIC EFFECTS OF SYNTHETIC NUCLEOSIDES IN VITRO

The convoluted interplay between various cellular organelles has been a prominent area of study since humans have had the ability to research and explore the microscopic cellular world. Particularly, significant attention has been exercised in the effect that various compounds, pharmaceuticals, drugs, and therapies have on cellular division; particularly cancer cell division. Although documentation is scant, monitoring cell division has been of great interest for years. The utilization and administration of tritiated thymidine, to visualize cellular replication, was unarguably the first strategy to monitor cellular division. However, this method was deemed toxic and cumbersome. 5-bromo-2'-deoxyuridine (BrdU) soon took high notoriety. BrdU, a halogenated pyrimidine, and its structurally related analogs are known to mimic the deoxynucleoside, thymidine, during S-phase of cellular replication. BrdU is incorporated in place of thymidine during S-phase, and its rate of incorporation can be monitored via immunohistochemical antibody detection. However, current literature has demonstrated that BrdU presents a number of complications regarding long-term labeling, cell cycle progression, cellular mutagenicity and cytotoxicity, and unwanted photosensitivity. BrdU's shortcomings were bypassed by the advent of 5-ethynyl-2'-deoxyuridine (EdU) (25). EdU, an additional synthetic analog of thymidine, having a terminal 5'-ethynyl- substituent, instead of thymidine's or BrdU's terminal 5'-methyl- or 5'-bromo- substituent, respectively. EdU has gained popularity as the preferred method in detecting cellular division due to its inherent ability to readily incorporate into newly synthesized DNA. In order to detect and incorporate BrdU into DNA, the

process requires the expenditure of an antibody. Binding of the BrdU-antibody tandem to DNA necessitates denaturation of DNA via volatile acid or heat treatment, which presents complications as unqualified and unfaithful base-paring and reannealing. Conversely, EdU incorporation and detection is a fast, simple, and effective method in labeling actively dividing cells. By way of "click" chemistry, EdU is readily introduced and synthesized into new DNA. The latter is accomplished, in part by a small-sized fluorescent azide, qualifying easy access to DNA without considerable steric hindrance. It is expected that successful incorporation of EdU, via "click" chemistry will result in high resolution microscopy analysis. However, current research suggests that implementation of EdU may result in unwanted biological effects. Using an *in vitro* system, the experimental basis described herein sought to determine the effects that BrdU or EdU had on cell cytotoxicity and genotoxicity when incorporated in DNA.

Whilst a vast majority of research experiments use concentrations of said nucleosides' in the range of 10-50 μM, these conditions may induce strong genotoxic and cytotoxic effects inherently higher than the expected background frequency. By treating various DNA repair deficient cells with BrdU or EdU, at concentrations ranging from 1-100 μM, there was a significant increase in the induction of sister chromatid exchanges. Also, with identical concentrations as the latter, the doubling time of particular DNA repair deficient cell lines increased dramatically. To examine the effects of BrdU and EdU on DNA repair, a poly (ADP ribose) polymerase (PARP) ELISA assay was carried out. The PARP assay concluded that BrdU possessed the highest degree of PARP inhibition, with thymidine second, and EdU with the least PARP inhibition. One suggested mechanism by which BrdU is thought to implicate or hinder DNA repair is through its incorporation and modification of DNA repair thus, slower repair kinetics.

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) mutation analysis suggests that manufacturers recommended EdU concentration (10μM), result in a significantly higher HPRT mutation frequency, compared to control. In addition to BrdU's SCE-induction capability and HPRT-induction incidence, clinical and radiotherapeutic properties have been examined. CHO cells exposed to 2 and 8 μM BrdU and 4 or 15 Gy X-rays, increase DNA repair duration, increased chromosomal fragmentation, and induce radiosensitization. However, little or no evidence is available in regard to EdU's propensity to affect cell viability. To assess the induction of cellular radiosensitivity and chromosomal aberrations, we investigated CHO and A549 (human lung cancer) cells replicative ability in the presence of three external radiation. An in vitro clonogenic and chromosomal aberration assay, in the presence of UVC-, photon (fluorescent)-, and γ-irradiation and BrdU or EdU, was implemented. Our results support BrdU's ability to decrease cell viability. Although each synthetic analogue presented their own biological contribution, their mechanism is still not fully understood. This study aims to discern any cytotoxic and/or genotoxic effects that EdU or BrdU pose on cell cycle progression, clonogenicity and viability, mutation-induction, chromosomal aberrations, and induction of radiosensitization.

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CHAPTER ONE

BACKGROUND

## 1.1 Introduction

The very existence of humans depends on our cells' ability to replicate, divide, and flourish. As humans obtained greater knowledge into the cellular world, their innovative techniques to explore it improved exponentially. So, how exactly do cells replicate, divide, and pass on their genetic information? Originally, the process of detecting cellular replication was accomplished by using tritiated thymidine. However, the process was extremely time consuming, fickle, and required undesirable radiation exposure.

Robert Leif, an expert in flow cytometry, postulated the idea of an anti-BrdU antibody; "why not make an antibody against BrdU and use it to detect incorporated nucleosides?" (1). Thus, the birth of a novel means of detecting and monitoring DNA replication. The utilization of 5-bromo-2'-deoxyuridine (BrdU) has since been a staple in research for examining actively dividing cells in S-phase. BrdU is a halogenated synthetic nucleoside analogue of thymidine. However, BrdU has presented a plethora of complications regarding cell culture techniques, reproducibility, and fidelity.

To counterbalance BrdU's potential complications researchers devised a unique and effective halogenated synthetic thymidine analog: 5-Ethynyl-2'-deoxyuridine (EdU). The effects of EdU incorporation on cellular division, clonogenicity, induced-radiosensitivity, and chromosomal fidelity, subject to an array of natural or manmade radiation sources, remain yet to be fully understood. Incorporation of BrdU into DNA has exhibited several cellular consequences, besides inherent user-technique errors. Such as, observable mutations of genes,

induction of interstrand crosslinks, DNA-protein crosslinks, DNA double-strand breaks, nucleobase lesions that resemble UV-induced (6-4) photoproducts (2), increased sister chromatid exchange frequency (3), cell-cycle checkpoint accumulation(s) (4), and X-, UV-, and fluorescent light-radiosensitization (5). Likewise, EdU has presented ability to promote cell necrosis, increase sister chromatid exchange frequency, cell-cycle arrest, inhibition of metabolic enzymes, and inhibition of viral replication (6). Though BrdU-induced radiosensitization has been described and witnessed explicitly, implications of EdU, regarding its ability to result radiosensitization and subsequent decrease/altered cell viability, have yet to be completely discerned. This research aims to identify various effects that BrdU and EdU have on cellular cytotoxicity and mutagenicity when cells are exposed to dissimilar radiation types and qualities, particularly, gamma-, UV-, and fluorescent light-irradiation. The succeeding passages illustrate and review background information appropriate for this study.

## 1.2 Structures, composition, and mechanisms of BrdU, EdU, Thymidine

### *1.2.1. Thymidine*

Thymidine (T), not to be confused with thymine, is one of four pyrimidine deoxynucleosides found in DNA (fig.1). Thymidine is an essential nucleobase and pairs with deoxyadenosine (A) in DNA. Thymidine is a DNA nucleoside that, when incorporated during DNA transcription, pairs with the deoxynucleoside deoxyadenosine (A). Thymidine composition is fairly simple and includes a deoxyribose entity, joined covalently to a pyrimidine base, thymine. Cell cycle and cell division lie at the heart of cellular biology (7). Therefore, a method of cell cycle progression and analysis was necessary in order to better identify and examine cell cycle checkpoints. As there are many different methods and reagents to investigate cellular

dynamics, thymidine was of the first chemicals administered to examine such. Cell cycle synchronization is a particularly trivial and effective method for investigating a cell cycle regulated event (7). Further, in order to investigate the $G_1$/S boundary, thymidine can be administered to cell cultures, resulting in inhibition of DNA synthesis.

Nucleosides and nucleotides differ, however. The bases and sugars in DNA and RNA are joined together into units called nucleosides. Because thymine is not usually found in RNA, the "deoxy" alias for its nucleoside is commonly assumed, and the deoxynucleoside is simply called thymidine (8). Essentially, a nucleoside comprises of a sugar (ribose or deoxyribose) and a base (adenine, guanine, cytosine, uracil). These bases are attached to the 1'-position of the ribose entity. On-the-other-hand, nucleotides are the subunits of DNA or RNA, which are basically nucleosides with an additional phosphate group attached through a phosphoester bond. When three nucleotides are covalently bonded to each other, via phosphodiester bonds, a trinucleotide is formed. Lastly, it is this trinucleotide (3 bases) that encodes a codon; a codon constitutes for individual unique amino acids which are eventually transcribed and translated into genes, proteins, and other essential, vital cellular constructs.

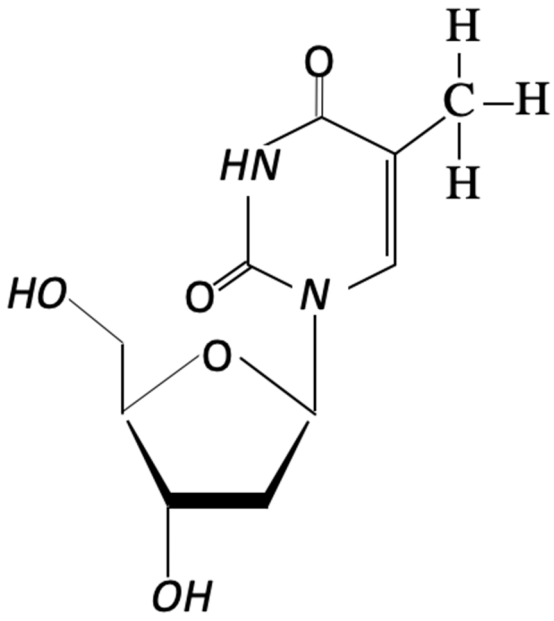


**Figure 1. Structure of thymidine**

### *1.2.2. Tritiated thymidine*

Tritiated thymidine or, $^{3}$H-TdR (methyl-$^{3}$H), is a radiolabeled deoxynucleoside (fig. 2). Of the first method(s) for monitoring cellular division, $^{3}$H-TdR is the most notable and original. $^{3}$H-TdR was first synthesized at Brookhaven National laboratory in 1956, led by Walter Hughes (9). $^{3}$H-TdR is a low-energy β-emitting compound, and historically a popular method for monitoring cellular dynamics. $^{3}$H-TdR has a maximum β energy of 18 KeV and an average range in nuclear emulsions of 1 to 2 μm (10), permitting high resolution autoradiograph constructs. Because of its bioavailability, it can readily be injected into excised tissue specimens or intravenously. In addition, samples obtained by $^{3}$H-TdR -labeling produced clear, black-and-white autoradiographs fit for visual analysis. The basis of the technique, is to feed a population of cells a label ($^{3}$H-TdR) that is taken up into cells processing through S-phase, then to observe the appearance of that label in mitotic cells as they move through around the cell cycle from S-

to-M phase (11). In essence, cells actively proliferating in medium enriched with $^{3}$H-TdR will uptake and incorporate $^{3}$H-TdR into their DNA, in preference of naturally exogenous thymidine.

However, this technique is laborious, and requires a large number of serial samples. First, the cell population (*in vitro/vivo*) must be flash-labeled (exposure time~20 minutes), enabling incorporation into DNA. After flash labeling, media is removed and replenished with fresh unenriched-media; permitting cells progression through their normal cell cycle. In predetermined regular intervals, usually 1 hour, a specimen of the desired cell population is removed, fixed, and stained, thus, providing further processing of an autoradiograph. This incremental process is carried out for a total time longer than the cells' innate cell cycle duration. Sequential samples are extracted until cell cycle division is completed

Nevertheless, analysis of cellular kinetics, through $^{3}$H-TdR assay, presents unfavorable consequences. As mentioned, this technique is not only arduous but cumbersome. Although the specific activity of $^{3}$H-TdR is relatively low (5 mCi), studies of patients with leukemia and solid tumors were given i.v. doses of 10 to 40 mCi; concentrations compulsory for adequate autoradiographs. Samples extracted from patient blood, urine, stomach lumen, and cerebrospinal fluid were performed and $^{3}$H-TdR activity measurements were employed. It was reported that 50% of the injected $^{3}$H-TdR was incorporated into DNA (12). Presumably, deleterious effects to surrounding tissue and DNA is plausible; culminating undesirable exposure to normal, healthy tissue. Unsurprisingly, widely accepted experimental concentrations of $^{3}$H-TdR have exhibited adverse effects on DNA-cellular kinetics: inhibition of DNA synthesis, increased cell doubling time, apoptosis, mutations, chromosome aberrations, and apoptosis (13). Even flash-pulse (15 minutes) labeling with $^{3}$H-TdR presented the latter. Therefore, alternative methods of analyzing

cellular division and kinetics were derived to circumnavigate the $^{3}$H-TdR impasse; explained in the following sections.

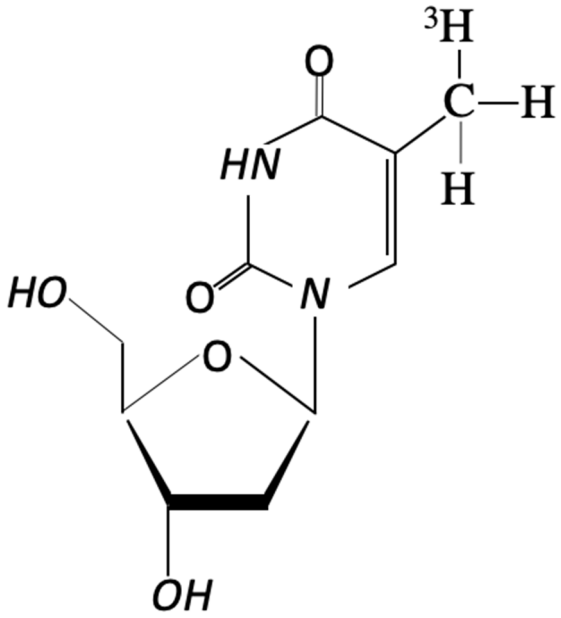


**Figure 2. Structure of tritiated thymidine-(thymidine + tritium)**

### *1.2.3. Bromodeoxyuridine and its mechanism*

The fruition of 5-bromo-2'-deoxyuridine as a powerful scientific tool occurred in 1972 while Dr. Robert Leif was sitting at home reading a journal (1). Where he excitingly realized that utilization of an antibody could be tagged to a fluorescent nucleoside. Bromodeoxyuridine (BrdU) is a synthetic analogue of the endogenously existing deoxynucleoside thymidine, and is one of the many 5-halogenated pyrimidine nucleosides' (14). The mere structural difference between BrdU and thymidine is the bromo- and methyl- terminal substituents, respectively (fig. 3). BrdU was the first synthesized analogue of thymidine, providing a means of detecting actively dividing cells and DNA replication. By injecting BrdU into a subject of interest (i.e. serum, blood, or medium-containing cells), cells will incorporate BrdU into newly synthesized DNA.

In order for successful detection of BrdU, one must use an antibody. Moreover, to successfully incorporate and visualize BrdU in newly synthesized DNA, samples must undergo volatile denaturing conditions such as, acid or high temperature treatment. Such volatile conditions may result in a low BrdU detection level, which may be insufficient for the detection of slowly cycling cells (15). In addition, BrdU visualization requires many steps and is cumbersome. Too this day, researchers alike, ubiquitously use BrdU as an aid in cellular research.

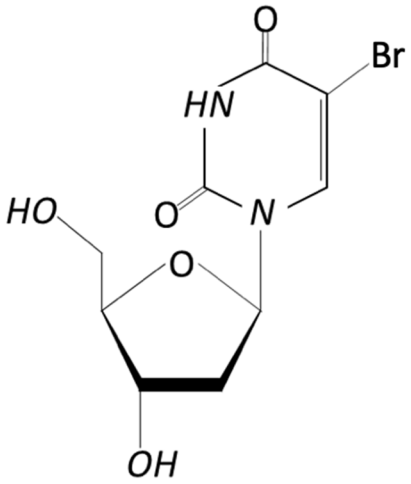


**Figure 3. Structure of 5-bromo-2'-deoxyuridine**

### *1.2.4. BrdU radiosensitization, cytotoxicity, and antineoplastic paradigms*

BrdU has been coveted as one of the first thymidine analogues to label actively dividing and replicating cells and DNA. But, its application has been transposed to other aspects of cellular biology to inspect items such as, radiosensitization, cell-cycle doubling time, and antiproliferative activity. A previous study reported that BrdU (10μM-50 μM) increases cell doubling time by a factor of 1.37-2.21 compared to that in a BrdU-free medium (16). An additional study reported that under the influence of BrdU, cells established unbalanced growth, which ultimately led to the inability to divide, and inevitable cell death (17). As previously

stated, BrdU has been used to monitor cell division; neurogenesis has rightly utilized BrdU to observe development of the adult nervous system. Moreover, however, BrdU is a mutagenic and toxic agent. A neurogenetic study proclaims that BrdU promotes the formation of teratomas, lengthens the cell cycle, triggers cell death, and effects transcriptional and translational machinery (18), therefore, conferring false results if necessary controls are not adequate. In respect to the nervous system, specifically, the brain, glioma cells and cancer stem cells display a cell density cumulative growth curve that decreases with BrdU concentration and prolonged exposure (19). Although BrdU has been subject to numerous studies, the mechanism by which BrdU induces its altered cellular effects has yet to be agreed upon (19, 20).

It is now clear that BrdU has much potential (and detriment) within the realm of human medicine and research. In addition to the latter capabilities that BrdU has, BrdU-induced radiosensization has yet to be fully understood. A clonogenic survival assay reports that in vivo bifilar-BrdU incorporated DNA has 3.57 fold decrease in survival compared to BrdU-negative control, when exposed to 4 Gy low-LET X-rays (20). In addition, the same bifilar-BrdU incorporated DNA has a 4.4 fold decrease in survival compared to BrdU-negative control, when exposed to 4 Gy high-LET Carbon ions (20).

### *1.2.5. BrdU-induced UV sensitization*

As explained in a following section, UVA, UVB, and UVC have wavelengths corresponding to 320nm-400nm, 280-320nm, and 100nm-280nm, respectively. Interestingly, canonical DNA absorbs light optimally around 260nm (2) (fig.12). Moreover, BrdU, and respective 5-substituted halogenated pyrimidines, also absorb light at around 280 nm (2). Therefore, it is appropriate to suggest that cells encounter probable adverse DNA-cellular effects when exposed to incident UV photons within this wavelength; with adjuvant BrdU

administration. Moreover, it has been hypothesized that an increased incorporation of halogenated pyrimidines into DNA is correlated with an increased UV-Induced sensitization effect (21). However, the mechanism(s) by which BrdU-induced UV sensitization occurs has yet to be fully and completely elucidated. As mentioned, the similarity between BrdU and thymidine is so close that BrdU is incorporated in place of thymidine. Substituting BrdU for thymidine "weakens" the DNA, and subsequently cells are more susceptible to damage by UV-radiation (11). Moreover, it has been reported that UV-induced single-strand breaks are not critical lesions responsible for sensitization or cell killing (22, 23). On the other hand, double-strand breaks may be the culprit behind sensitizing and killing. It is speculated that when BrdU-incorporated DNA is subject to UV, the nucleobase within BrdU is oxidized. Thus, allowing it to interact with nearby (adjacent) halogenated nucleobases; ultimately, leading to DNA interstrand crosslinks, DNA-protein crosslinks, and lethal DNA strand-breaks (2)-inducing sensitization and/or cell death.

Indeed, exposure to UV-radiation may incur DNA interstrand crosslinks, DNA-protein crosslinks, and DSBs, however, therapeutic treatments have been derived from photo(chemo)therapy. However, effective, yet indiscriminate, protocols implementing therapeutic UV-exposure has been developed. Intriguingly, phototherapy has been commonly prescribed as a viable treatment option for hyperproliferative skin disorders such as psoriasis, atopic dermatitis, vitiligo, chronic idiopathic urticaria, and other various forms of topical/subdermal cancers (2, 24). Although UVC/B exposure alone does present pathological and therapeutic effects, however, these treatments have proven to be optimally effective when adjuvant sensitizer (BrdU) is administered. The latter is effective because BrdU-UV-exposure creates a synergistic cytotoxic effect. Aggregation and accumulation of DNA-protein crosslinks,

DSBs, and other photolesions within the DNA can, in turn, result in potentially lethal effects to the cell. Therefore, UV-radiation modalities necessitate pertinent therapeutic values when correct protocols are observed.

At the protein level, when a cell is ready to divide, a protein called retinoblastoma (Rb) is (hyper)-phosphorylated (inactive Rb). However, there is indication that cells exposed to BrdU and 10 J/$m^2$ UV- induces a hypophosphorylated Rb (active) protein; resulting in cell cycle arrest, even in the absence of UV exposure (14). Therefore, a causative UV-BrdU-mediated Rb hypophosphorylation may play partial in the BrdU sensitizing effects (14).

### *1.2.6. 5-Ethynyl-2'-deoxyuridine*

EdU is a synthetic analogue of endogenous thymidine. While thymidine is the naturally occurring pyrimidine deoxynucleoside that is incorporated during DNA synthesis, EdU can be readily substituted for thymidine and incorporated during DNA synthesis. The only structural difference between EdU and thymidine is the ethynyl- and methyl- substituents, respectively (fig. 4). EdU is a biologically active compound which promptly incorporates into the DNA of actively dividing cells in S-phase, which, in turn, provides a quantitative and qualitative assay to assess the rate of DNA synthesis in cell culture. Initially, EdU had been administered to treat certain viruses, and beholds anticancer/tumor therapeutic properties (25). EdU has been utilized as a means to identify bacteriophage (26).

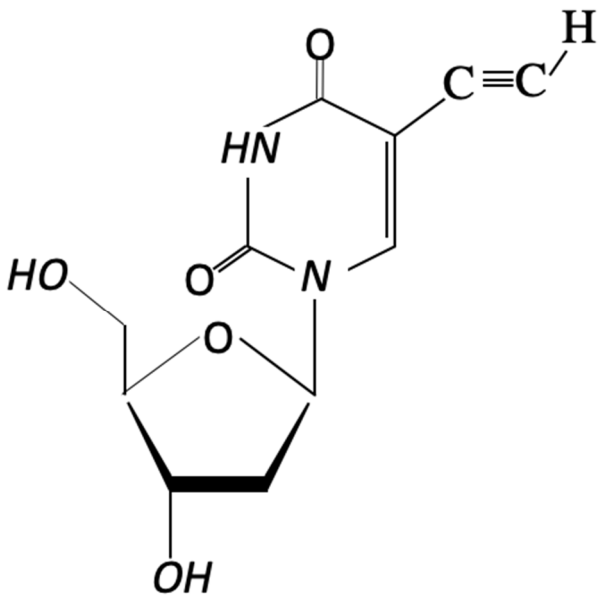


**Figure 4. Structure of 5-ethynyl-2'-deoxyuridine**

### *1.2.7. Antiviral and anticancer/tumor properties of EdU*

Indeed, EdU has demonstrated significant inhibitory effects against herpes simplex virus-2 (virus rating=2.3; >1 indicates definite and significant antiviral activity), and slightly reduced inhibitory effects against herpes simplex virus-1 (25). Moreover, with the addition of a (synthetically) heterocyclic ring (e.g. formycin), C-EdU has demonstrated comparable antiviral effects similar to its precursor, EdU (25).

### *1.2.8. Dissecting the chemistry and benefits of "click" (EdU)- reaction*

"Click" chemistry, otherwise known as tagging, is a biochemical reaction that achieves the fast and efficient binding of a substrate to a desired biomolecule. In addition, "click" chemistry is vastly implemented among the scientific community in order to achieve bioconjugation, drug discovery, materials science, and radiochemistry (27). Researchers claim that "click" chemistry is very rapid, highly selective and regiospecific, and delivers relatively high yields of desired components (28). "Click" reaction avoids the use of bifunctional chelating agents, multiple error prone steps, cross-reactivity or nonspecific interactions with additional

functional groups present. There are two predominate “click” reactions presently available; Azide-alkyne Huisgen cycloaddition and Staudinger ligation. However, this report will focus solely on the former reaction. The Azide-alkyne Huisgen reaction had not gained notoriety until recently, though discovered in 1963, due to the requirements of high temperatures and pressures. However, through ingenious discovery (29), use of a Cu(I) as a catalyst permits the bypassing of several steps rendering the reaction extremely useful. The reaction is coined the “Cu-Catalyzed Huisgen 1-3 dipolar cycloaddition of Azides and Terminal alkynes” (28). Through experimental kinetic data and modeling, the latter reaction is thought to proceed in a short and simple stepwise fashion. The first step involves the marriage of the alkyne and azide, resulting in a triazide complex. The following step involves Cu(I) rapidly inserting itself into the π complex of the alkyne entity producing Cu-acetylide (30). Finally, after stabilization of the Cu-acetylide complex, this complex can tag itself to an array of desired targets such as, a fluorophore, peptide/protein, biotin, additional proteins, antibodies and oligonucleotides (28).

Historically, utilizing BrdU to detect cell proliferation, DNA synthesis, and DNA content was a long, toxic, laundry-list of reagents, and cumbersome process. However, impromptu innovation led to improved protocols and synthesis. The EdU assay kits have nearly bypassed all the pitfalls that BrdU based-detection presented. Edu “click” reaction does not require an antibody detection, nor DNA denaturation for detection of nucleoside incorporation into newly synthesized DNA (fig. 5). The click reaction utilizes an azide-linked-labeled dye, which is of small size abiding access for EdU to be incorporated in the DNA. EdU is a synthetic halogenated nucleoside analogue of thymidine, the naturally occurring pyrimidine nucleoside, which contains an alkyne group. Moreover, since EdU is nearly molecularly indistinguishable from thymidine, cells (of one’s liking) incorporate EdU into its DNA efficiently as if it were thymidine. DNA

denaturation for incorporation during S-phase is not necessary. Administration of a small azide-linked-labeled dye, locates and tags DNA-incorporated EdU and forms a covalent bond between it. Thus, detection is achieved fast, efficiently, specific and accurately signaling.

In comparison to other labelling techniques, click chemistry with alkyne-modified nucleobases has many advantages. First, incorporation of EdU leads to specific labelling only of newly synthesized DNA or RNA. Second, BrdU requires immunostaining and antigen retrieval steps; unlike EdU click reaction (31). As noted, BrdU labeling requires harsh acid denaturing conditions, whereas EdU- "click" chemistry labeling is pH-insensitive. Also, the "click" reaction is significantly selective in that the reaction only occurs between an azide (a fluorophore in this present study) and alkyne components (terminal 5'-ethynyl group on EdU). Importantly, detection of EdU is highly sensitive and can be accomplished in minutes (32). The high selectivity diminishes the possibility of interacting with organic groups or proteins present in DNA. Notably, the reaction takes place in water, therefore the reaction is readily biocompatible. To reiterate, the reaction is exceedingly quick, quantitative, and simple.

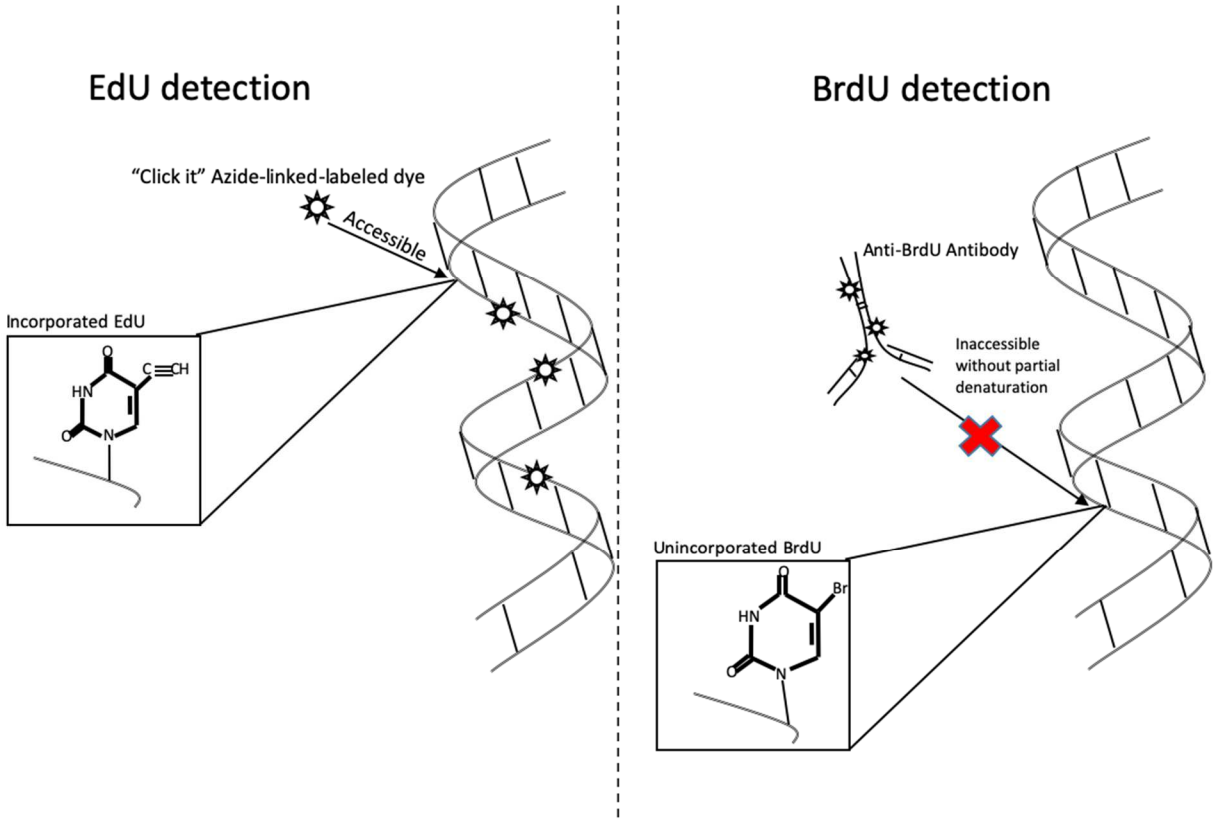


**Figure 5. Detection of EdU or BrdU fluorescent labeling**

## 1.3 Ionizing radiation qualities, physics, chemistry, and cell death

### *1.3.1. Ionizing radiation*

Radiation is a broad term. However, radiation is defined as varying ranges of energy that traverses through matter or space (33). When this ionizing radiation, or energy, travels through space and embarks on matter it can induce a variety of biological, molecular, or structural effects. Ionizing radiation, in every shape, form, and entity, has proven a great vast array of power. Fascinatingly, radiation, as mentioned, presents itself in many forms; be it naturally occurring or manmade. All soils and rock contain naturally occurring radioactive material (NORM) (34). Uranium, in particular, is a heavily sought-after NORM-rare-earth element. While uranium presents several isotopic variations, $^{238}U$ is the most prevalent and abundant isoform found on earth. Uranium radioactively decays in a multiplicity of methods; however, the primary route of decay is through alpha and beta decay and spontaneous fission. There are countless

applications of uranium, however, many are far beyond the scope of this report, such as its utilization in production of military grade weapons, tank armor, shielding material used in devices to store and transfer radioactive material, and most notably fuel for nuclear power plants and thermonuclear bombs. Interestingly, many geographical regions of the earth contain very high background levels of NORM including, but not limited to, Brazil, Penna Franca, SW coast of India, Yangjiang, China, Ramsar, Iran, and certain regions of the United States and Canada (35). The three most pertinent forms of NORM and its related substituents are cosmic radiation, terrestrial radioactivity, and indoor radon (36). The average dose, received by us through background radiation, is approximately 2.4 mSv/year but, can be as high as 50 mSv/year depending on geography and latitude of where one lives (ref?). Notably, a majority of background radiation exposure originates from the rocks that comprise the earth's crust; radon. Moreover, 16% comes from artificial sources (medical) and 1% coming from the nuclear industry (37).

When harnessed and coordinated properly, ionizing radiation presents a plethora of beneficial and therapeutic properties. Whether it be a friend, colleague, or relative, we all know someone who has/had cancer. Several forms of cancer therapy and treatment exist such as, chemotherapy or surgical resection of cancerous region. However, when surgical resection fails to completely remove a cancerous region, often treatment is combined with radiotherapy. Though, contingent on the location of the tumorous node, certain forms of radiotherapy are utilized: External Beam Radiation Therapy (EBRT) and Internal Radiation Therapy (IRT); of which, this report will not elaborate in detail. EBRT is a beam of ionizing radiation focused on region of interest from a machine **outside** the body. Moreover, IRT, just like EBRT-but opposite, is simply defined as a radioactive "seed" or pellet placed **internally** in or adjacent to a tumorous

node. With all things considered, EBRT is the most common and effective approach for treating prostate cancer. However, with the addition of chemotherapy, EBRT resulted in significant reduction of tumor expansion, and complete remission rate from 85% to 96% (39). Although, the all-around encompassing goal is to deposit and focus largely the beam of radiation on tumor region. Thus, selectively depositing energy into a relatively small volume of tumor, reducing damage to nearby normal, healthy tissue.

At any rate, radiation therapy-internal or external-affords an approach toward regulating and eradicating diseases and tumors at a promising and beneficial rate. To illustrate the application(s) of ionizing radiation would require several passages far beyond the scope of this report. However, ionizing radiation plays an integral function in our surroundings and everyday life.

### *1.3.2. Physics of ionizing radiation*

When an atom has gained or lost one more electron due to incident radiation, it is termed "ion". However, only specific energies can provoke ionization. If the incident radiation has necessary energy to eject an electron from the atoms orbital, it is termed ionizing radiation (IR) (11, 33). IR is an abundant and ubiquitous environmental stressor capable of ensuing genotoxic and epigenotoxic conditions (40). Particles of ionizing radiation include heavy charged particles (HCP), such as alpha particles ($\alpha^{2+}$), protons, beta particles ($\beta^{-}/\beta^{+}$), and lighter charge particles, such as positrons, and energetic extranuclear electrons (33). Although the behavior of alpha particles and protons differ greatly from that of electrons and positrons. Such that, a heavy charged particles path through matter is dense and linearly ionizing, while an electrons path is tortuous (haphazard) due to multiple scattering events-depositing ionizing energy at each atomic collision-caused by coulombic deflections. Thus, creating an ion pair. One must discern the *path*

*length* and *range* of a particle: *path length*-total distance the particle travels in matter, *range*-the absolute depth of penetration of the particle in matter (fig. 6). In regard to electrons, the path length almost always exceeds its range. Whereas, HCP path length and range are almost equal.

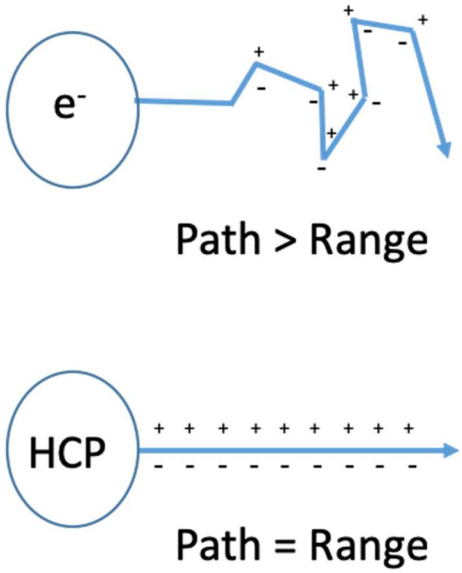


**Figure 6. Particle tracks: Path vs. Length**

When IR traverses through matter such as air or tissue, it interacts with atoms within the substance and creates ion pairs; thus, a linear energy transfer (LET). However, deposition and absorbed energy by materials differs greatly. A large HCP deposits it's energy spanning a shorter range and, as a result of greater coulombic forces, producing considerable damage to cells compared to lighter charged particles.

Just as we measure distance in units of inches, centimeters, kilometers, or miles, radiation is none too much different. In that, as a whole-international or domestic-committees and scientists have come to an agreeance upon International System of units (SI) and standards for radiation. Historically, the unit of radiation was expressed as Roentgen (R), 1 R=2.58 x $10^{-4}$ C $kg^{-1}$. Although Roentgen is a valid unit of exposure, however, it is only applied and limited to

exposure of photons in air, solely. Specifically, the application of X-rays and γ-rays. A more viable and useful unit radiation is imparted energy or joule; total amount of energy deposited per unit mass in any matter:

$$E=(J/kg) \times kg = J$$

*Kerma* (K) is an acronym for *ki*netic *e*nergy released in *ma*tter (33). Kerma is classically defined as the kinetic energy transferred to charged particles by indirectly ionizing radiation per unit mass or, absorption energy in 1 Kg of air. As beam of indirectly ionizing radiation (X-rays and γ-rays) traverses through a medium, deposition of its energy is a two-step process: 1. Energy carried by the incident radiation is transformed into kinetic energy of charged particles (electrons). 2. The charged particles deposit their energy in the medium by way of excitation or ionization. The common SI unit of Kerma is joules per kilogram with a special name of gray (Gy) or milligray (mGy), where 1 Gy = 1 J $Kg^{-1}$. Unlike Kerma, where it is defined for only indirectly ionizing radiation, *absorbed dose* (D) is defined for all types of ionizing radiation (direct or indirect). Although Kerma and *absorbed dose* have the same SI unit (Gy), they differentiate appreciably and accordingly. Measurements of Kerma take into account extraneous ionization events occurring such as bremsstrahlung (X-rays) or highly energetic electrons, or other radiative losses. Absorbed dose is the total amount of energy deposited in material, not taking into account radiative losses. Though, Gy is commonly applied as measurement of dose, rad (radiation absorbed dose) is the older unit of *absorbed dose*. Where one rad is equivalent to 0.01 J $Kg^{-1}$, and 100 rads equals one Gy, 1 rad = 10 mGy. Therefore, Gy is widely accepted as the primary unit of radiation dose.

It is important to note that not all types of ionizing radiation result the same biological damage. For sake of simplicity, in order to reflect the relative effectiveness of a type of radiation

in producing biological damage, a new term is introduced: radiation weighting factor ($W_R$). $W_R$ was introduced because high LET radiation (alpha particles, protons, HCP) yields considerably more densely ionized radiation tracks in material, consequently resulting in more biological damage than low LET radiation (X-rays, γ-rays, energetic electrons) types. Essentially, high LET radiation increases the probability of stochastic effects like diseases or cancers, thus they are appointed higher radiation factors. Such that, the product of *absorbed dose* and radiation weighting factor equals *equivalent dose* (H):

$$H = D\ W_R$$

Although *equivalent dose* is similar to *absorbed dose,* the SI unit for *equivalent dose* is joules per kilogram with the special name of Sievert (Sv), 1 Sv = 1 J $Kg^{-1}$. Nearly identical, but qualitatively different than gray. Another mode of distinction between Sievert and gray is simply that, gray is a physical quantity, where Sievert is biological effect of exposed dose; Sievert is a measure of health effects of receiving ionizing radiation.

As mentioned in previous section(s), distinctive radiation modalities and types account for a colorful array of applications and functions. Such as cancer radiation therapy, integrity of concrete and steel assemblies, energy production, and nuclear physic research. With a promising and bright future, radiation has boundless facets, orientations, and applications. In fact, through dynamic engineering, advancement of technology, and remarkable innovation, radiation therapy is prescribed to approximately 50% of all patients with localized malignant or inoperable tumors (41).

### *1.3.3. Electrons*

The electron is primary fundamental particle. Its role in science, technology, and worldly matters is boundless. An electron possesses an elementary negative charge (-1) and is a

subatomic particle. Moreover, electrons have the ability to radiate or absorb energy by way of photons when they are decelerated or accelerated. Also, electrons have mass approximately 1/1836 that of a proton ($1.672x10^{-27}$ kg); specifically, $9.109382x10^{-31}$kg (42). In addition, electrons have a charge of $e=1.602x10^{-19}$C (43). Electrons also play an essential role in certain radiotherapies such as electron beam therapy. As one can see, electrons have many aspects and play a critical role in our everyday lives.

### *1.3.4. Photons*

The fundamental differences between photons and, the previously discussed electrons, are that electrons have a negative charge, while photons possess neither a positive nor negative charge. Moreover, photons are "packets" of energy of an electromagnetic wave; not bits of matter (33). Meaning, photons are "massless" particles. In addition, photons travel at the speed of light ($2.99x10^{8}$m/s)(c) when placed in a vacuum, such as outer space (33). Photons, which include x-rays, UV radiation, and fluorescent light, reside within the electromagnetic spectrum (33). Lastly, photons that we experience every day, all the time, are the photons that are within the visible light spectrum.

### *1.3.5. X-rays*

In 1895 the German physicist Wilhelm Conrad Rontgen discovered "a new kind of ray", in which a ray was emitted by a gas discharge tube (11). He coined this ray "x-ray"; "x" stands for unknown. These x-rays have since been used diagnostically and therapeutically. X-rays are profoundly complex, yet simple in the same respect. They are utilized diagnostically (radiography), prognostically, therapeutically, industrially, and scientifically. In regards to diagnostics, x-ray energies range between 25kV and 150kV, whereas, therapeutically, the energies vary between 10kV and 300kV (43). X-rays may be produced by an electrical device

that accelerates electrons to high energies and then subjects them to a metal target (33). X-rays may be thought of as a wave of electrical and magnetic energy. As we will see, in following passages, x-rays can be produced in a multitude of processes. Moreover, x-rays are considered to be electromagnetic radiation. X-rays can be thought of as a stream of photons, or "packets" of energy. Where each packet contains a certain amount of energy that can be deposited into a target. Most x-rays retain wavelengths ranging from 0.01 to 10nm; corresponding to energies 100eV to 100keV, respectively. However, x-rays are considered indirectly ionizing. Meaning, they do not produce chemical and biological damage themselves, but when they are absorbed in the target through which they pass, x-rays relinquish all or portions of their energy to produce secondary fast-moving charged particles. Consequently, it is these fast-moving charged particles that induce damage to the fundamental biological building blocks, DNA. There are, however, two kinds of induced-damage; indirect action and direct action.

### *1.3.6. Direct and indirect effect of x-ray irradiation*

X-rays exert their powerful biological effects on cellular matter, and more importantly DNA, by two distinctly unique courses:

**Direct effect:** Direct action radiation of an x-ray occurs when an incident (photon) particle interacts with the target (other atoms or molecules in a cell) and creates a secondary electron. Concurrently, the secondary electron produced directly interacts with DNA to produce an effect (11) (fig. 7 & 8).

**Indirect effect:** Indirect action radiation of an x-ray occurs when an incident (photon) particle interacts with the target (other atoms or molecules in a cell), producing a secondary electron, ionizing it. Production and ionization of this secondary electron then acquires the ability to interact with surrounding biological material. For example, the secondary electron

interacts with a water molecule to produce a hydroxyl radical, which in turn interacts with DNA, ultimately depositing its energy and damaging the DNA. Indirect action is the predominant method by which x-rays induce damage in cellular components (11) (fig. 7 & 8).

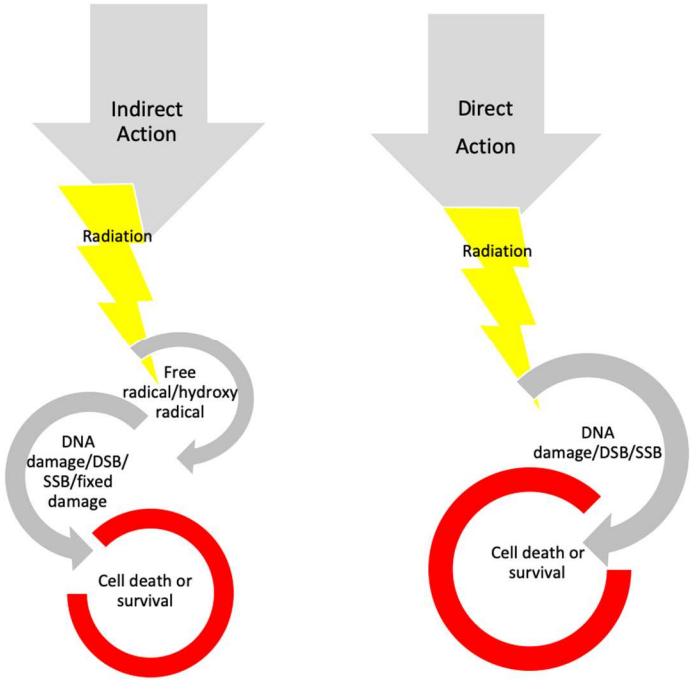


**Figure 7. Direct and indirect action of radiation**

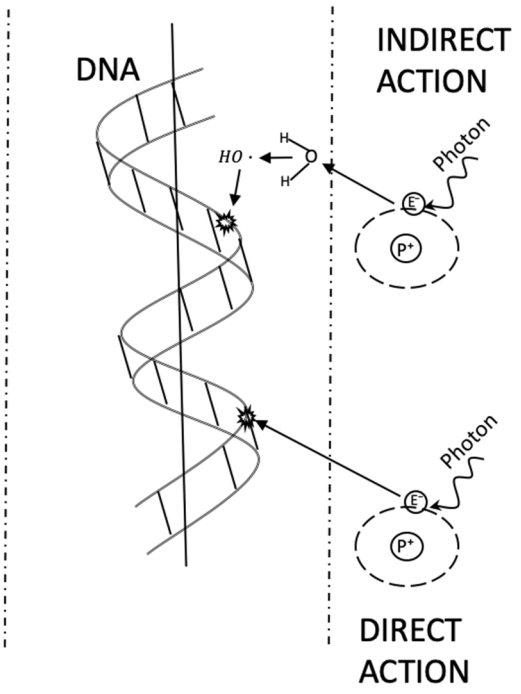


**Figure 8. Direct and indirect effect of X-irradiation**

### *1.3.7. X-ray photon absorption process*

The process by which x-ray photons are absorbed in a given material is dictated by the energy of the photons of subject and molecular composition of absorber material.

**Photoelectric process:** The photoelectric process (fig. 9) occurs at small values of gamma ray or x-ray photon energies. As previously stated, a gamma ray is a photon. It is this incident photon that interacts with an electron bound in an atomic orbital (i.e M, L, or K) in absorber material (33). It must be noted that the absorber material can be composed of a plethora of substances; soft tissue, metals, dirt, water etc. Moreover, the ejected electron ($E_{pe}$) contains an energy equal to the incident x-ray photon energy ($E_o$) minus the binding energy of the orbital electron ($E_b$): $E_{pe}= E_o- E_b$. Importantly, the binding energy of the ejected electron must be equal to or less than that of the incident photon energy in order for the photoelectric emission to occur (33). The probability of a photoelectric emission is nearly dependent upon the previously mentioned atomic orbital shell-binding energy; a photon that has greater energy than k-shell

binding energy, for example, a photoelectric interaction is most probable within this shell (33). After such interaction, the atom within the absorber material is now ionized and results in a shell electron vacancy. The vacant shell cannot remain as is, therefore, an electron from a shell with lower binding energy will "jump" down into the vacant orbital, creating an electron cascade from lower to higher shell binding energies (outer to inner shells). Thus, resulting in a characteristic x-ray or auger emission (33).

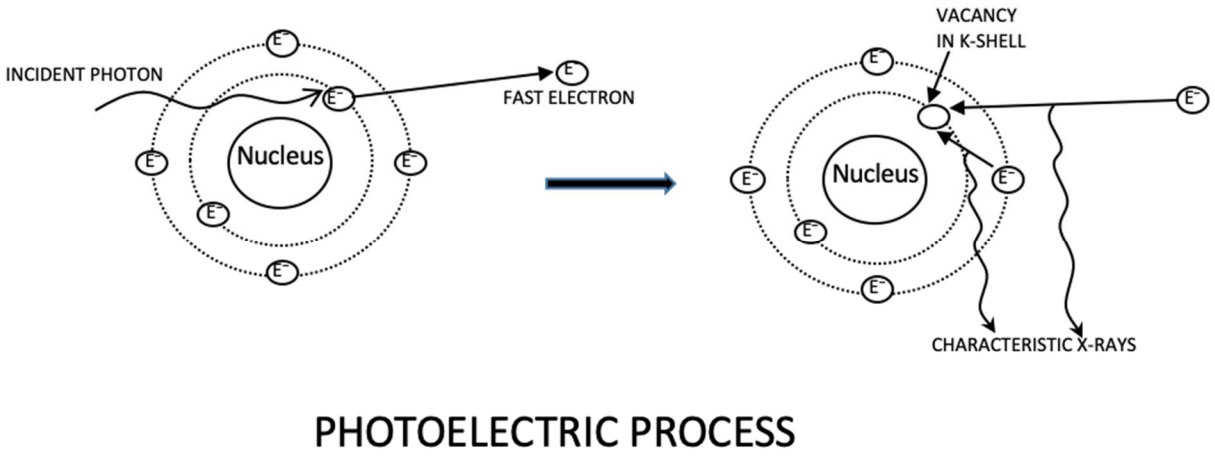


**Figure 9. Photoelectric absorption and emission of an X-ray**

**Compton Scattering:** Compton scattering (fig. 10) (also referred to as inelastic or nonclassical scattering) predominates the x-ray/gamma-ray photon interaction in the diagnostic energy range with soft tissue. Compton scattering is a probabilistic physical reaction, predominating at energies near 26 keV (diagnostic) to well beyond 30 MeV. Although Compton scattering can occur within the photoelectric energy range and pair production energy range, the probability decreases (145). In addition, the latter energy range(s) are greatly dictated by the medium of which the photon interacts with. Also, Compton scattering probability depends on the electron density of the absorber material (#e-/g "x" density). In essence, the incident photon must have significantly greater energy than the electron binding energy of the electron(s) in the

absorber material for it to occur (145). The Compton interaction is most probable between an incident photon and an outer valence-shell electron (outermost shell in an atom). This interaction results in the ionization of an absorber atom and separation of the photons energy between scattered photon and ejected electron. The overall energy of the incident photon ($E_o$) is equal to the sum of the energy of the scattered photon ($E_{sc}$) and the kinetic energy of the ejected electron ($E_{e-}$): $E_o=E_{sc}+E_{e-}$ (33). Importantly, $E_o$ must be largely greater than the electron binding energy ($E_b$) for Compton scattering to occur, however, $E_b$ is negligible in the Compton energy ranges. Provided sufficient incident photon energy, the Compton process results in an ejected electron and scattered x-ray or gamma-ray. After such an interaction, the scattered photon has the ability to peregrinate the absorber medium without interaction or pursue further Compton scattering. However, unlike the photoelectric process, the probability of a Compton scattering increases with increasing incident photon energies (33).

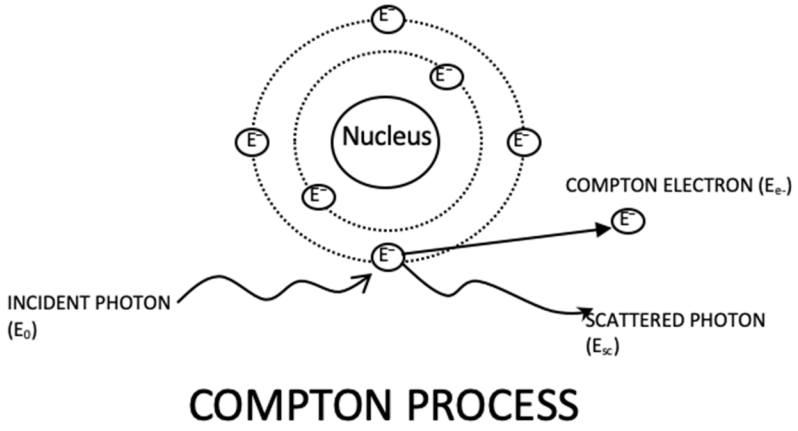


**Figure 10. Compton scattering process**

**Pair Production:** Pair production (fig. 11) will proceed only if the x-ray or gamma-ray energy surpasses the 1.02 MeV threshold. Pair production is markedly distinct from Compton scattering and photoelectric emission in that, pair production occurs when a photon interacts with the electric field of an absorber material's atomic nucleus. An incident photon eclipsing the

1.022 MeV threshold results in an interaction with the atomic nucleus and, significantly, an electron (Negatron or beta minus particle)-positron (positively charged) pair (33). The photon imparts its energy on the electron-positron pair, while simultaneously, the electron and positron lose their kinetic energy via excitation and ionization. The reduction in energy of the positively charged positron permits its attraction to a surrounding negatively charged electron. Thus, colliding with a subjected electron; producing two (pair) distinct and oppositely ($180^{O}$) directed 511 keV annihilation photons (33). Altogether, it is these photons that impose their destruction on surrounding material.

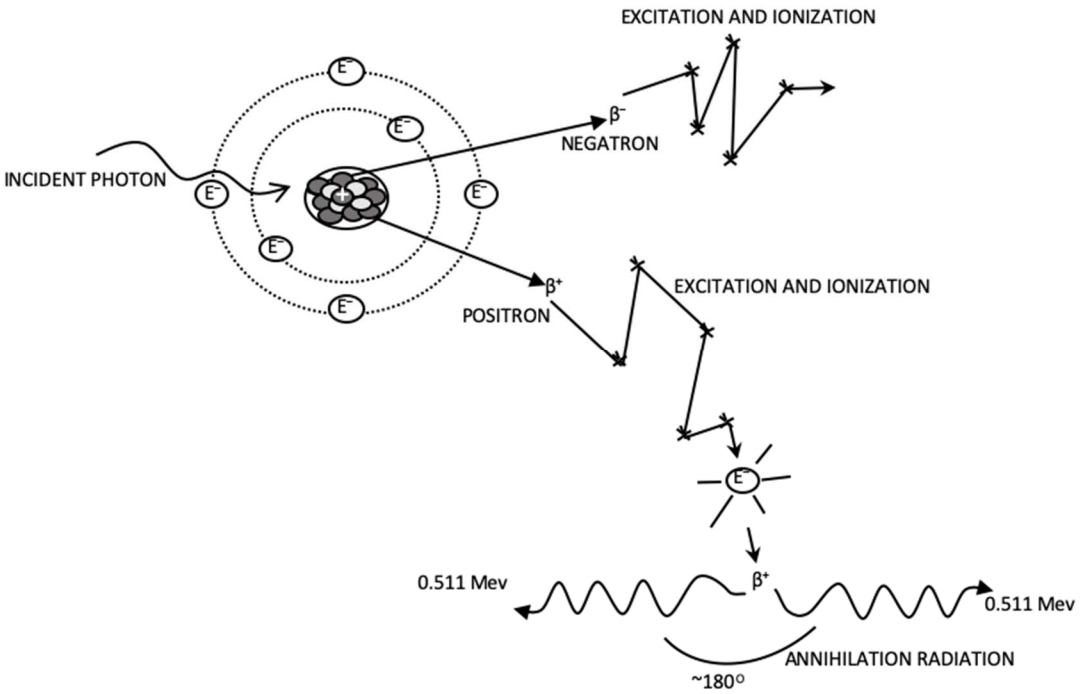


**Figure 11. Pair production process of annihilation photons**

### *1.3.8. γ-irradiation/ γ-rays*

One way γ-rays are produced during γ-decay is when a heavy nucleus disintegrates into a α-particle or a β-particle, the daughter nucleus has the ability to stay in an excited state (45). However, if the excited nucleus does not emit another particle or break apart, it has the chance to "fall" back to the ground state, emitting a γ-ray or high energy photon (45). It must be stressed

that γ-rays and x-rays only differ by way of production; intranuclearly and extranuclearly, respectively.

### *1.3.9. Intrinsic mitotic catastrophe implicates radiation*

There is overwhelming evidence that cells whom are subject to high or low-energy radiation will fail to retain viability, and eventually die. Particularly, radiation implicates great consequences on encompassing cellular integrity, division, and replication. Through direct and indirect action of radiation, it imparts its lethal cellular effects. However, radiation, alone, does not conclude cell death-it promotes it. The most distinguished route by which radiation results in sequential cell death is through inducing mitotic catastrophe (MC). Mitosis is assimilated into 6 general subtypes: prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis, which altogether regulate proper cellular division. However, radiation can and will drastically manipulate the latter.

MC, as defined in 2012 by the International Nomenclature Committee on Cell Death, is a *bona fide* intrinsic oncosuppressive mechanism that sense mitotic failure and responds by driving a cell to irreversible antiproliferative fate of death or senescence. Although MC does not represent a direct *bona fide* cell death mechanism in itself, MC forgoes and uses antiproliferative measures such as apoptosis, necrosis, and senescence to prevent the proliferation of defective cells (46). MC is consequence of alteration of mitosis and/or irreparable chromosome damage (47). Morphological-global features of MC include, but not limited to multinucleation, binucleation, polyploidy, aneuploidy, multicentricity, irregular kinetochores, and abnormal cell size (giant) (46). Cells that endure such morphological alterations, as aforementioned, generally cannot and will not survive, thus, eventual cell death. So, by way of high or low energy incident radiation, cell viability and proliferative capacity is abrogated. Cyclin B1 accumulation, an

important regulator of $S/G_2$ and M-phase entry, has been reported after 10 Gy gamma-irradiation. Cyclin B1 is tightly regulated by P53 protein. Inhibition of P53 function by incident radiation, consequently deregulates cyclin B1 levels, accumulation and premature entry into mitosis resulting in MC (48). Paradoxically, MC can precipitate tumor malignancy and cancer progression if malignant/cancer cells can (im)properly transcend MC event. Ultimately, the fate of a cell relies on its proliferative capacity, and whether it's subject to MC. Though, this ability can be greatly hindered through radiation.

### *1.3.10. Involvement of radiation-induced cell death*

The eventual desired sequel of treating malignancies through radiation therapy is to effectively kill and eradicate cancer/tumor cells by way of depriving them of their reproductive capabilities (49). How this is achieved may be through inhibition of diverse molecular pathways, signals, transcription factors, and transduction relays, or minute changes we are completely naïve to. Altogether, this is a severely complicated process, of which, is presently still not fully understood. Hence, cancer research is one of the primary funded fields of human health. Modes of cancer therapy vary, however, they all share a common objective: eradication of tumor/cancer cells. Biological damage and cell death procured through radiation exposure may be entirely determined by type of radiation, total dosage of exposure, dose rate, and the region of the body exposed (50). One effective approach to achieving cell death is by inducing tumor cell apoptosis (49). Though apoptosis is a particularly ambiguous term, it is both simple yet convoluted in its own respect. In past decades, it has become apparent and approved that inhibition of the proliferative capabilities of malevolent cells proceeding radiation therapy, can transpire alternative cell death modalities such as, induction of mitotic catastrophe, cell-cycle arrest, or senescence; the three principle cell deaths precipitated via radiation therapy (49, 50). The

fundamental basis for cellular selection for the latter modes of cell death hinges on an array of factors such as, cell type encountered and whether the cell is proliferating, malignant, metastasized, or transformed (50).

Ionizing radiation unequivocally engenders direct and indirect biological damage. As previously explained, high LET radiation propagates a majority of cellular damage via direct ionization of cellular macromolecules such as, but not limited to, DNA, RNA, and proteins. Thus, ensuing an after-math of deregulated cellular networks and faulty regulatory proteins, enzymes, and organelles. Whereas, low LET radiation accumulates indirect biological damages via production of reactive oxygen species (ROS). Ionizing radiation produce ROS through interaction with water ($H_2O$) by way of radiolysis. What holds true for all radiotherapies, is that incident radiation primarily interacts with cellular $H_2O$ as a majority of the human body and cells constitute 70-80% water. Radiolysis is a series of cascade hydrolytic reactions producing DNA-damaging species (51).The primary ROS that retain a marked biological effect are superoxide radical ($O_2^{*-}$), $H_2O_2$ ($^*OH$), peroxyl radical ($ROO^*$), and alkyl hydroperoxides (ROOH) (52). Surmounting evidence suggests that these ROS are manufactured or generated by mitochondrial distress through ionization exposure (52). Even hours after initial radiation exposure, ROS abundance was clearly demonstrated. Consequently, these ROS have specific effects and roles pertaining to cellular integrity such as apoptotic signaling, protein denaturation, genomic instability, and climactically cell death. Previous studies demonstrate that ROS production and accumulation was obvious at $S/G_2$-phases of cell cycle (53); the most sensitive portion(s) of the cell-cycle. Therefore, it is transparent that applying radiotherapy, granted ROS production, is a beneficial means of cancer treatment if the desired biological outcome is attained.

Molecularly speaking, ionizing radiation implicates great predicaments at the protein-protein, protein-DNA, and DNA-matrix level. Alternations in the fabric of materialized DNA, through consequence radiation, invariably results a disconcerting array of molecular outcomes. It would seem practicable that radiation oncologists could prescribe a radiation protocol potent enough to sterilize all tumor cells within the treatment volume, however, this may in fact constitute a lethal dose to surrounding normal tissue. However, by focusing radiative energy at the protein level, a successful treatment can be obtained. In particular, P53, otherwise known as the "guardian of the genome", is a pinnacle protein responsible for genomic stability. Cellular P53 levels are rapidly increased in response to subjected exogenous and endogenous mutagens such as chemicals, stressors, viruses, and particularly, ionizing radiation. P53 acts as a tumor suppressor gene, and implicates a vital role in cellular proliferation, cycle-cycle progression, genomic stability, and all-around cellular wellbeing. Seemingly, P53 is of great interest in regard to cancer cell research. Although P53 is responsible for activation and repression of countless cellular proteins, in response to mutagens, we will only cover in brief a few. P53's counterpart, Murine double minute 2 (Mdm2), is a crucial constituent of the responses to both ionizing and UV radiation (54). Incident radiation DNA damage stimulates production of Mdm2, as a result, this activates P53; triggering a cascade of cellular responses. Of importance, is cell-cycle arrest and DNA repair. Mdm2 can inhibit P53 at many interfaces, but two are outstanding. One interaction of Mdm2 is directly binding to the amino terminus of P53, extensively blocking its ability to induce gene expression. Also, Mdm2 can also promote degradation of P53 by adding ubiquitin to it (54). By promoting p53's degradation, P53 is translocated from the nucleus to the cytoplasm. Deeming P53 functionless in regard to transcriptional activation of other various cell-cycle regulator proteins. In all, concentrations of Mdm2 and its ability to sterically bind to P53

are key in regulating radiation response. Susceptibility of Mdm2/P53-mutations induced by ionizing radiation are inevitable. When subsequent epigenetic mutations are acquired by P53, or it's repressors or activators, things can (and most likely will) go awry such as, erroneous DNA repair. The cells inability to halt cell-cycle succession, predictably results in haphazard accumulation of deleterious mutations and dysfunctions. Another key activator of P53 is Ataxia Telangiectasia Mutated (ATM) serine/threonine kinase. Production of double-strand breaks through ionizing radiation assembles ATM to the site of damage. Thus, propagating a torrent of cell DNA damage checkpoints, ultimately leading to proper DNA repair, cell-cycle arrest and apoptosis or accelerated senescence. With proper protein response to radiation induced damage, the cell is coerced into stimulating effective DNA repair such as homologous recombination or non-homologous end joining repair. If repair is not sufficient, cell will systematically reduce itself to self-destruction, necrosis, or apoptosis. But, as we have mentioned, this is not always the case. At long last, if gene function is thoroughly lost through acquired mutations, via assorted radiation qualities, the cell has but many choices and routes: improper succession of checkpoints, unregulated proliferation, necrosis, phenotypic variability, or apoptosis-cell death.

### *1.3.11. Ultraviolet light*

Ultraviolet light (UV), or ultraviolet radiation, is an example of electromagnetic radiation that society is exposed to daily. Anatomically, our eyes discriminately shield a majority of the harmful UV-spectrum. Quantities of UV radiation exposure are commonly expressed in terms of radiometric magnitudes: wavelength- $\lambda$, radiant energy-J (joules), radiant flux-W (watts), and radiant exposure-J/$m^2$. UV exposure quantities are similar to that of ionizing radiation (J/kg), however, UV exposure only takes into consideration the surface area, not overall dose absorbed. The biological and physical effects of UV radiation contrast greatly with wavelength, for this

reason the UV spectrum is further divided into three distinct subsets: UV-A, UV-B, and UV-C (55). Specifically, UV-A and UV-B light is found in the short-wavelength region of the electromagnetic spectrum (320nm-400nm & 280nm-320nm, respectively) (fig. 12). Largely, as a consequence of UV light absorption in clouds, the stratospheric temperatures fluctuate significantly (56, 57). On a bright summer's day, UV-B constitutes approximately 6% of terrestrial UV, and UV-A, ~93%. Although, since UV-B is considerably more much effective than UV-A at concluding biological damage, solar UV-B supplies 80% toward the harmful effects associated with sun exposure (55). Altogether, the stratosphere absorbs a considerable amount of UV-A and UV-B, though a portion of the latter still penetrates the earth's surface. If not for the stratospheric clouds, humans would be exposed to a relatively high dose of ultraviolet radiation. For example, research has shown that excessive exposure to UV-B and UV-A produces excess reactive oxygen and nitrogen species (58, 59). Moreover, these reactive oxygen species, when the balance between pro-oxidant and anti-oxidant stimuli is impaired, can initiate deleterious effects such as tumorigenesis, inflammation, and mitochondrial membrane alterations (59, 60).

While UV-A and UV-B as a whole, occupies the electromagnetic spectrum at wavelengths (λ) ranging from 280nm-400nm, short-wavelength UV-C spans 185nm to 280nm in wavelength (57). Because UV-Cs has a considerable physical short wavelength it inherently propagates high energy ($E = hc/\lambda$; shorter the wavelength, higher the energy) that results in destruction of the basic biological compounds found in earthly matter, deoxyribonucleic acids (DNA). In fact, DNA representatively optimally absorbs UV light within wavelengths less than 250 nm. Interestingly, ultraviolet germicidal irradiation is a highly effective practice that utilizes UV-C light to disinfect and/or inactivate bacteria, pathogens, viruses and other microorganisms

(61, 62). In addition, the effectiveness of UV to induce erythema (sunburn) declines more rapidly with longer wavelengths. To produce the same erthymatic response, 1000 times more UV-A is needed compared with UV-B. Because UV-B wavelength is shorter with respect to UV-A wavelength, UV-B penetrates skin to a less degree. Thus, a majority of tanning beds utilize UV-A tanning lamps (63).

Short-wave ultraviolet radiation produces two primary types of photoproducts, cyclobutane pyrimidine dimers (PD) and (6-4)-photoproducts, which are assembled between adjacent pyrimidine nucleotides (64). All of which are lethal photolesions that can result in abrogated cellular maintenance. UV-C induced radiation damage that produce said photoproduct result in a bulky DNA lesions that promptly halt RNA polymerases. If these RNA polymerases default to obsolete, incomplete DNA repair can implicate accumulation of cancer predisposing mutations.

It is reported that normal human fibroblast cells exposed to both a uniform and local UV radiation field, with energies ranging from 4.5 to 9.2 $J/m^2$, is sufficient for evoking a cellular cytotoxicity response (64). However, there were no significant differences in repair kinetics for either types of photolesions when exposed to a localized or uniform UV irradiation. One must discern the effects that UV irradiation of cells present on underlying molecular pathways. On-the-other-hand, a more recent study suggest that UV dose does in fact prompt a complex gene-specific activation when WS1 human skin fibroblasts were exposed to low (10 $J/m^2$) and high (50 $J/m^2$) dose UV radiation (65).

Now, let us briefly focus on molecular responses to UV radiation. Several DNA damage-induced signaling cascades, including ATR/Chk1, jun N-terminal kinase and p38 kinase pathways are activated following UV-C radiation. Conclusively leading to activation of NER

(explained in successive sections) and recovery mechanisms via genomic transcription factors (65). Succeeding 50 J/$m^2$ UV exposure, human fibroblast cell response contrasts from that of 10 J/$m^2$ UV exposure, in that high dose selectively upregulated the expression of pro-apoptotic genes such as NOXA, IL-11, and spermidine/spermine N1-acetyltransferase. Whereas low dose (10 J/$m^2$) UV exposure prompted a selectively transient upregulation of growth, cell-cycle, metabolism, and transcription factor genes such as serum-inducible kinase, SOX4, and IFI30. This provides further notion that highly distinct sets of genetic responses are invoked by low and high doses of DNA damage, specifically, UV-C. Interestingly, the gene transcripts that were upregulated during high UV exposure were downregulated during low UV exposure, and vice versa (65). An additional study exclusively illustrated that using a host cell reactivation assay, found that NER and NER-associated gene(s) (p21waf1/cip1) was increased immediately after low doses but not following high dose UV radiation. Further, induction of apoptosis and apoptotic-associated genes (Bax, bcl-2) was only visible proceeding high dose, but not low dose UV-B exposure (66). The discernable response to low and high dose UV irradiation, combined with UV quality (UV-C versus UV-B), suggests that cells retort vital cellular “SOS” signals that are entirely dependent upon UV dose and UV quality.

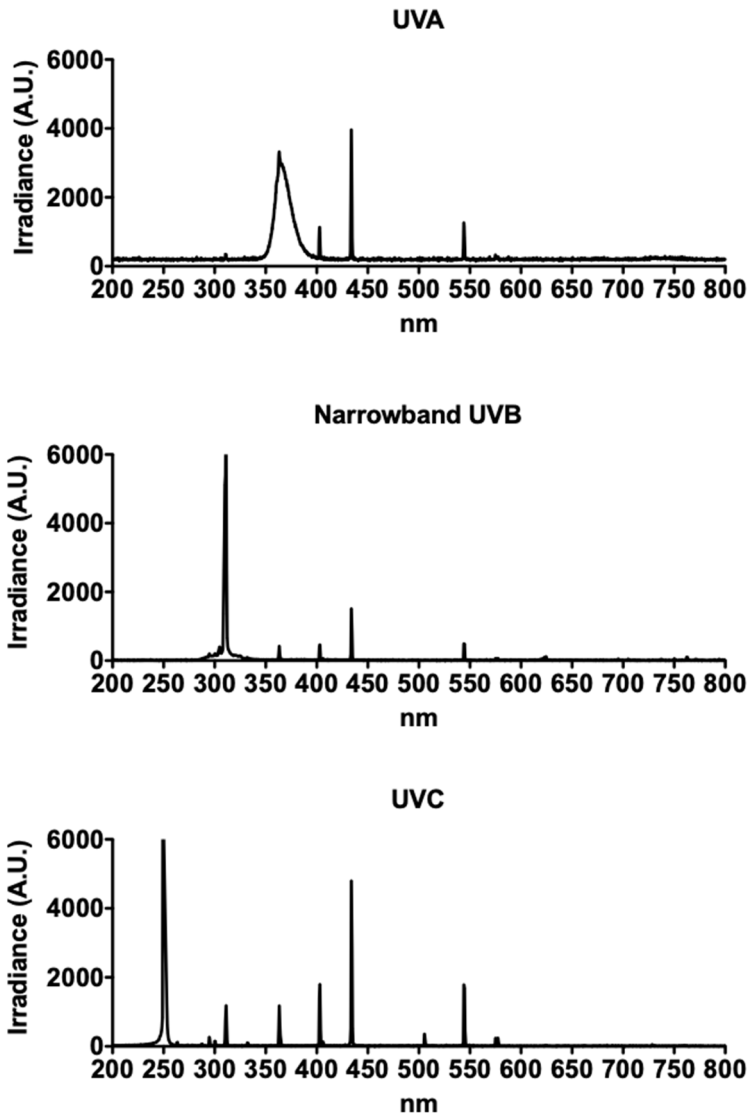


**Figure 12. Wavelength spectrum of UV-A, Narrowband UV-B, and UV-C**

### *1.3.12. Fluorescence light*

Often, the lighting predominately used in businesses, schools, factories and other establishments is composed of fluorescent or incandescent lamp fixtures. Since fluorescent lighting is considerably more energy efficient than its counterpart, incandescent fixtures, it is the favored lighting source in nearly all institutions. The components of a representative fluorescent lamp retain a long narrow glass tube caped with metal caps. Encapsulated within the tube is a

noble gas (argon, neon, or krypton) (67). Lastly, a few drops of mercury are placed within the gas. Mercury is preferred because of its inherent efficiency to emit fluorescence once struck by energetic electrons. In a more physical sense, in order to convert electrical energy into radiant fluorescent light inelastic scattering of electrons must occur. Moreover, this occurs when an electron collides with a mercury atom in the lamps gas. After such collision, the majority of mercury atoms will emit an ultraviolet photon, associated with the 185-254 nm UV wavelength (67). However, the photons released have wavelengths that are not visible to the human eye. Thankfully, the ultraviolet photons emitted are entirely absorbed by a fluorescent phosphor coating, exciting the electrons of the phosphor; thus, production and emission of "safe" fluorescent lighting. In addition, for scientific purposes, visualization of fluorescently labeled proteins and other DNA entities requires the use of a fluorescent microscope. Meaning, one must be careful in regard to the exposure of fluorescently labeled molecular entities to fluorescent light of the microscope. If a fluorophore is subject to high intensity fluorescent radiation, for an extended period of time, photobleaching irreversibly destroys fluorophores stimulated by radiation within this excitation spectrum, thus eliminating potentially useful information (68). For sake of this report, a standard 5-1/4" Coolwhite bulb (15 Watt, Model F15T8 4100K T-8 G-13 base (flux rate=$3.8 \times 10^{-5}$ J/sec/cm$^2$)) was used to carry out all fluorescent light irradiations, wavelength spectrum fig. 13.

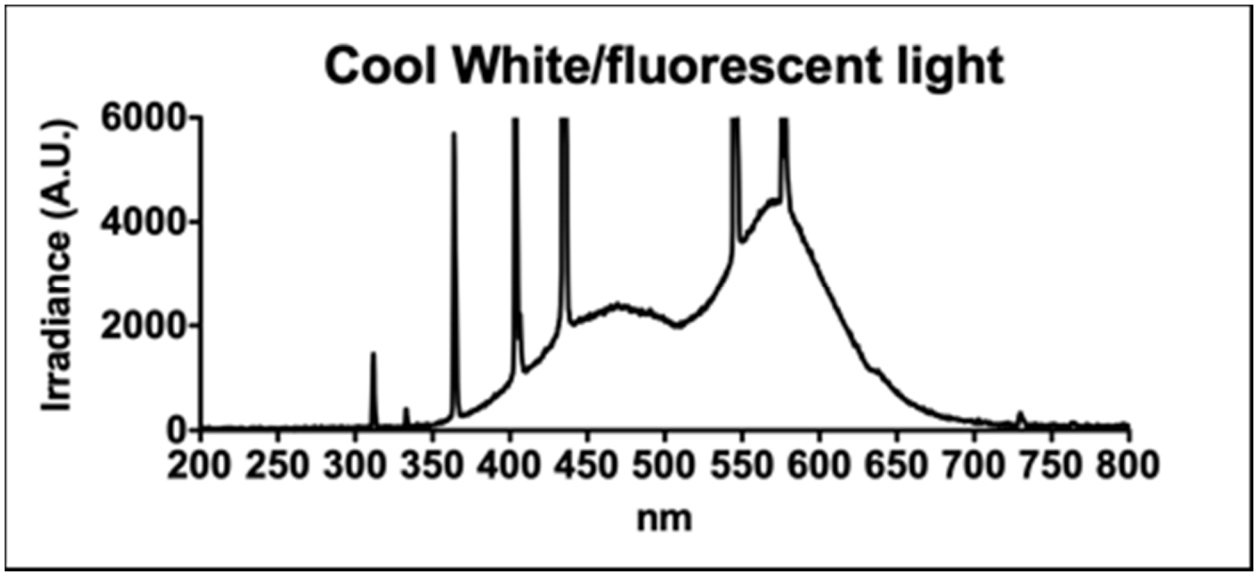


**Figure 13. CoolWhite/fluorescent light wavelength spectrum**

## 1.4 Fundamental biology, damage, and reparation of Deoxyribonucleic Acid

### *1.4.1. Deoxyribonucleic Acid*

In 1869, Friedrich Miescher discovered a mixture of compounds in the cell nucleus, of which he coined "nuclein" (69). Excitingly, he proposed that an integral portion of the nuclein was Deoxyribonucleic Acid (DNA). The crude structure of DNA, and related ribonucleic acid (RNA), was introduced in the late nineteenth century. Later, in the mid-1940s, the fundamental structures of DNA and RNA were elucidated (8). Intrinsically, DNA is composed of nucleotides, which are broken down into four nitrogenous bases; adenine (A), cytosine (C), guanine (G), and thymine (T) (Note: thymine is substituted for uracil (U) in RNA) (8). Each of which contain at least one phosphate group and a five-carbon sugar (deoxyribose). When a chain of nucleotides is covalently linked together via a phosphodiester bond, forming a sugar-phosphate backbone, a nucleic acid is formed. Essentially, nucleic acids are biopolymers that are composed of monomers of nucleotides. Nucleic acid polymers base pair with a complementary nucleic acid, ensuring that adenine and thymine pair via two hydrogen bonds, and guanine and cytosine pair via two hydrogen bonds. Note that the nucleic acid strands run in opposite directions in

directional relation to each other, therefore, they termed anti-parallel (8). The joining of two nucleic acid strands, thus, creating one molecule of DNA. However, the process is not yet completed. The final DNA product comprises of two strands of DNA, creating the iconic DNA double helix.

### *1.4.2. DNA double strand break*

Largely, DNA double strand breaks can arise from an array of deleterious sources. DSB's, where both strands in the double helix are severed, are the most dangerous type of DNA lesion (70). The human genome comprises 30,000+ intricately intertwined genes. However, these genes materialize from a simpler entity: DNA. Moreover, our DNA is subject to constant exogenous and endogenous cytotoxic agents (71). For example, in order to hike a mountain a human relies on their cell's ability to metabolize foodstuff to provide the necessary energy required to conquer a mountain. Interestingly, cellular metabolism creates harmful endogenous by-products known as reactive oxygen species (free radicals). Further, free radical reactive oxygen species are generated by our body by various endogenous systems (72). Free radicals are one of the primary sources by which DNA lesions are created. These lesions can result in a double strand break, where both strands of DNA have been "cut" through. Or, such lesions can act as a barrier to DNA replicative machinery, not permitting DNA replicative machinery to proceed through, resulting in a DSB (73). An alternate method by which DSB's are induced is by external or internal radiation exposure (71). For example, an individual presents a tumor and is prescribed photon irradiation therapy. The individual receives ample doses of therapeutic x-rays to the tumor region in hopes of eradicating the tumor. The x-rays "strike" and deliver high quantities of energy to the tumor cell DNA; inducing DSBs, single-strand DNA breaks, and various DNA lesions. Significantly, DSBs are accordingly deleterious in nature, by appropriately

optimizing therapeutic dose, inhibition of tumor DNA repair machinery can increase or halt altogether, thus, cell death throughout regions of the tumor. It is now evident that DSB's present a cellular double-edged sword; in regard to a therapeutic standpoint or innate harmful endogenous agents.

### *1.4.3. Reparation of DNA double strand breaks and other forms DNA damage*

Through biological marvel, species have acquired unique mechanisms to resolve and repair DNA breaks, lesions, deletions, and many other damages to DNA. One of which is Non-homologous end joining (NHEJ). NHEJ is a repair pathway that has been evolutionary conserved throughout all known forms of life. However, the NHEJ proteins and complexes used by prokaryotes and eukaryotes differ, but function almost virtually the same. DNA double-strand breaks (DSBs) are one of the most harmful, if not most serious, forms of DNA damage. Further, NHEJ is the preferred route of repair when DNA damages are induced in $G_1$ phase of the cell cycle. This is due to the absence of a sister chromatid homolog to be utilized as a template for repair. While NHEJ is an important pathway for DSB repair, it is intrinsically error-prone (70). There are three hypotheses that support the latter: 1) highly error-prone alternative end-joining process 2) the inability of NHEJ to perfectly join complementary ends 3) the requirement to first process chemically incompatible DNA ends that cannot be ligated directly (74). On the other hand, Homologous Recombination (HR) is nearly absent in $G_1$ but is predominant and most active in S phase, and declines slightly moving into $G_2$/M phase (70). It should be noted that HR is critical both for repairing DNA lesions in mitosis and for chromosomal pairing and exchange during meiosis (75). HR is considered "error-free" and favored during S phase due to the presence of a sister chromatid homolog (76). Moreover, if these double strand breaks are not repaired correctly and timely, this can lead to cell death, DNA mutations, or ultimately disease

and cancer. Studies of rodent and human lineages have clearly demonstrated that the choice between NHEJ or HR pathways depend on the cell cycle stage (70). Therefore, one should take into careful consideration which portion of the cell cycle exogenous/endogenous agents are elicited. Without respective repair mechanisms chromosomal abnormalities are transparently inevitable. Appreciably, if lesions are left unrepaired, with inarguable knowledge that DSBs are one of the most detrimental DNA lesions, DSBs carry the propensity to propagate aneuploidy, increased frequency of malignancy, and chromosomal translocations (77). In large, the recognition, and sub-sequential processes following, of DNA damage follows an overall theme (fig. 14).

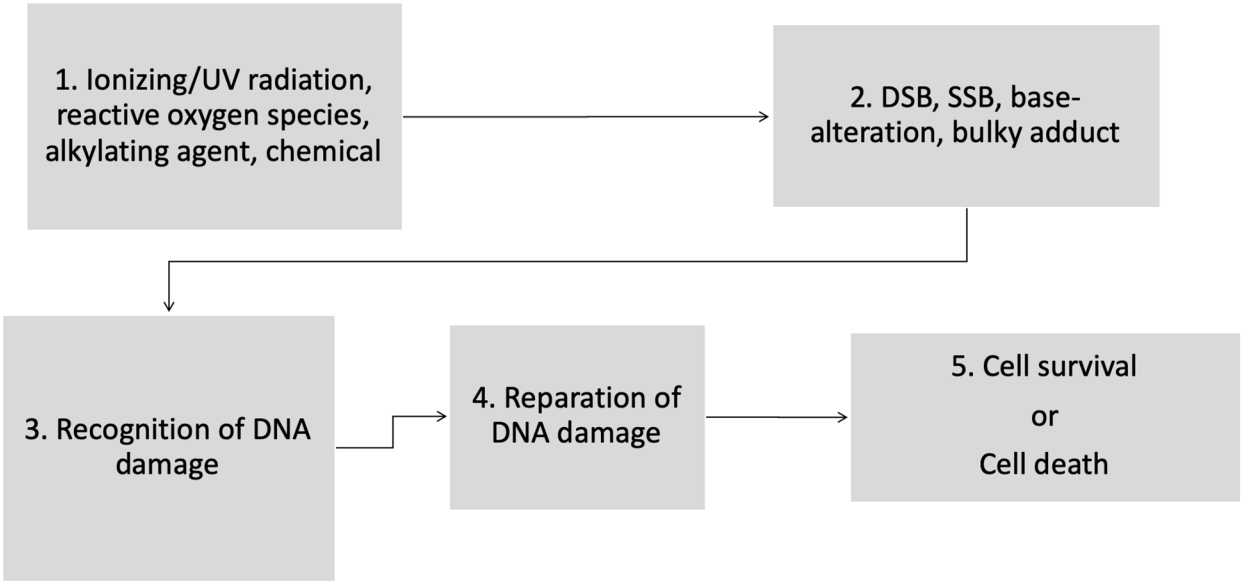


**Figure 14. Thematic representation of processing DNA damage, and sub-sequential steps**

### *1.4.4. Chromosomal abnormalities*

Fascinatingly, although such an incomprehensibly small object, an individual cell beholds an intricate molecular highway. Thousands of proteins, complexes, and processes must intercommunicate so that faithful and symmetric cellular division is accomplished. However, this

is not always the case. A plethora of deleterious structural and functional chromosomal mutations can occur while a cell proceeds through cellular division. Many diseases, if not all, arise from such mutations, and can drastically effect a diseased individual. Structural mutations may include, deletions, duplications, translocations, inversions, insertions, rings, dicentrics, breaks, and fusions. However, for purposes of this current report chromosome breaks, sister chromatid exchanges, and fusions (dicentrics) will be discussed exclusively (fig. 15). Chromosome breaks may arise through an array of impaired processes within the cell: (i) inability to restart stalled replication forks, (ii) inability to pair nucleotide bases correctly, (iii) deletion of a portion (or whole chromatid deletion) of a chromosome entity, (iv) inability to properly divide chromosomes during mitosis, (v) or improper DNA synthesis during S-phase of cellular division (78-80). Similarly, chromosome fusions give rise to several different forms of chromosomal abnormalities. An example of such is a chromosome ring, which arises when chromosome arms inappropriately fuse together, literally forming a ring. A centromere is an integral structural part of the chromosome where sister chromatids link together, and where the eventually split of the chromosome into two identical daughter chromosomes. Moreover, the centromere is a location where, commonly, unregulated, unfavorable cellular division can occur. A result of this may be the formation of a dicentric chromosome, where the chromosome has two centromeres (chromosome containing one centromere is normal). This is formed by the fusion of two chromosome segments, each of which containing one centromere. Of the previously listed chromosomal abnormalities, each arise through the inception of improper DNA synthesis or faulty DNA repair mechanisms.

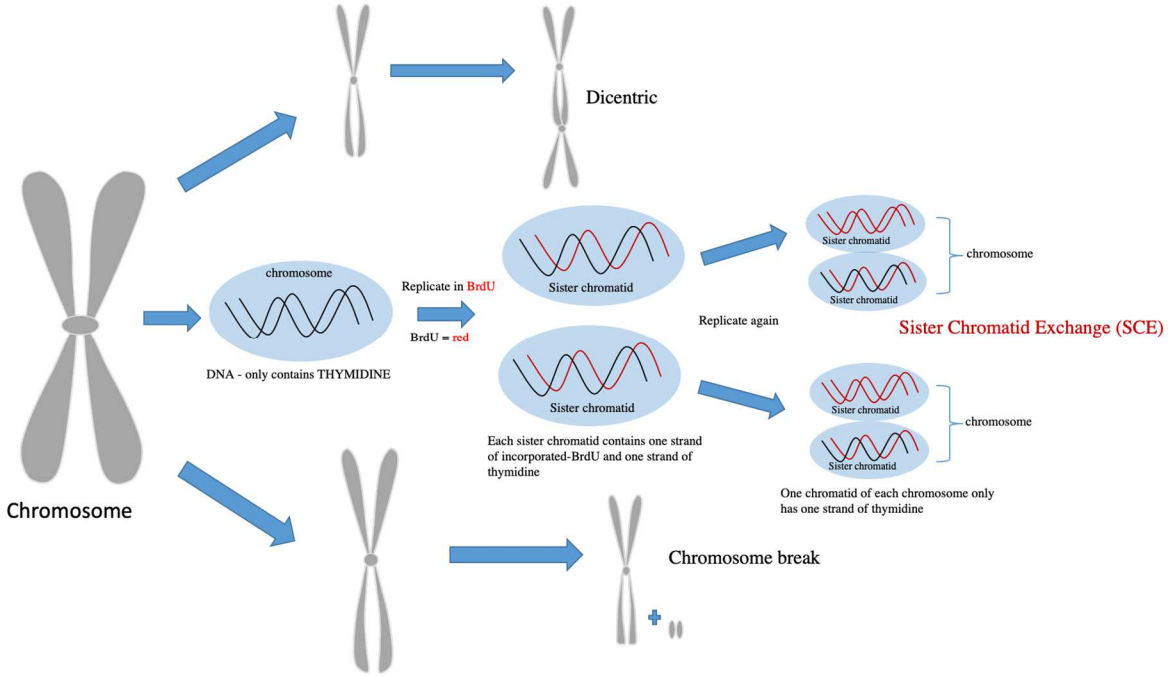


**Figure 15. Diagram of formation of dicentrics, sister chromatid exchanges, and chromosome breaks**

### *1.4.5. Conceptualizing non-homologous end joining repair pathway*

NHEJ repair pathway is one the primary route by which cells progress through in order to repair DSB's. Therefore, cells that are exposed to harmful endogenous/exogenous agents such as ionizing radiation, free radicals, and metabolic byproducts, during $G_1$ ($G_2$/M) phase preferentially route their repair pathway in the direction of NHEJ. As previously stated, this is due to the fact that there is an absence of a sister chromatid homolog during $G_1$ phase. Though, eukaryotes (us) and prokaryotes resemble two entirely distinct kingdoms, we both have commonalities through which we are alike. Except for limited protein homology between prokaryotes and eukaryotes, the nuclease, polymerase, and ligase components of NHEJ have arisen independently, however, they converge on the same underlying mechanistic themes in order to overcome and repair the burden of induced nucleotide base damages (81). The damages that NHEJ primarily repairs are DSBs. The recognition of a DSB initiates (fig. 16) the recruitment of specific repair proteins that

act as a docking site for further NHEJ proteins such as DNA-PKcs and the heterodimer, Ku70/80, which permits further recruitment of nucleases, polymerases, and ligases. Ku70/80 is thought to change conformationally when binding to broken DNA ends. This may be due in part to the need of Ku70/80 heterodimer to be mechanistically flexible when encountering a diverse array of DSBs (ionizing radiation, free radicals, bleomycin), and to protect broken DNA ends from further degradation. DNA-PKcs is a catalytic subunit that assists in aligning synapses between adjacent DNA region. Insofar that adjacent DNA ends are aligned appropriately, DNA-PKcs autophosphorylates itself and the Ku70/80 heterodimer, thus, promoting partial dissociation of Ku70/80 heterodimer. The phosphorylation of the Ku70/80 complex partially dissociates it from the DNA, so that it can carry out its function as a DNA helicase (unwinding of DNA, allowing further access of additional repair proteins). Further, after recognition and recruitment of repair proteins such as, artemis, end processing initiates (2). Artemis is an exonuclease that removes or trims away damaged DNA ends (5-20 nucleotides), preparing it for ligation. During end processing, two vital repair proteins, ataxia telangiectasia-mutated ATM kinase (ATM) and 53BP1, are recruited to the damaged site. ATM is exceptionally important, in that it recruits several proteins needed to initiate cell-cycle arrest in the event of a DSB. Proceeding end processing, DNA synthesis, resolution, and strand invasion begins (3). All while preparing for DNA synthesis and strand invasion, specific protein complexes and DNA polymerases are further recruited to the site of repair. These protein complexes are known as XRCC4 (x-ray repair cross-complementing protein 4) and DNA polymerase lambda and mu. The DNA polymerases, as it may seem, synthesize new DNA that will eventually be incorporated, replacing the resected/damaged DNA. XRCC4 serves as a docking site for DNA ligase IV. The last step (4) in NHEJ is ligation, where DNA ligase IV complex joins repaired DNA ends,

thereby constituting a newly identical (nearly or more often than not) repaired portion of DNA. In the prior sentence, we state in parentheses “nearly or more often than not” because NHEJ is error-prone, though this is still tabled for debate. This is due to the fact that NHEJ is not template-dependent, meaning this repair pathway does not utilize a sister DNA homolog as a template. Therefore, this pathway is basically synthesizing a “new” strand of DNA blindly, sort of speak. In addition, NHEJ, more often than not, results in “indels”, or insertions or deletions. Supporting the hypothesis, that NHEJ is error-prone, is that V(D)J recombination also employs nearly identical proteins as NHEJ. V(D)J recombination is a genetic mechanism that produces an extremely vast variety of lymphocytes from somatic cells. These lymphocytes are a subset of white blood cells. Moreover, lymphocytes comprise three key immune response cells: natural killer cells (cell-mediated, cytotoxic innate immunity), B-cells (humoral, antibody-driven adaptive immunity), and T-cells (cell-mediated, cytotoxic adaptive immunity). During the production of the latter cells, V(D)J recombination assembles, almost haphazardly, distinct and unique immunoglobulins and receptors necessary for the detection of potentially harmful exogenous and endogenous agents. Because our body’s immune system is constantly contending foreign agents such as bacteria and virus, our immunity is required to be adaptive and amenable. Though, the production of novel immune cells or responses does come at a cost. A cost which can weigh heavy on genomic integrity; resulting in mutations, deletions, and insertions. Therefore, it is reasonable to suggest that current and previous hypotheses regarding the integrity and fidelity of NHEJ are in fact true.

### *1.4.6. Conceptualization of HR repair (HRR) pathway*

Although homologous recombination repair (HRR) pathway is commonly thought of as a single, cut-and-dry pathway, it is interestingly divided into four discrete sub-pathways. Depending on the cell type, cell cycle, and severity of induced-damage double-strand break repair (double Holliday junction model), synthesis-dependent strand annealing, single-strand annealing, or break-induced replication pathway (82-84) are utilized. The aforementioned pathways do share the Rad51 protein, except break-induced replication. However, for purpose of simplicity, basic HRR will only be discussed here. It must be noted that homologous recombination is not necessarily isolated to repairing DSBs; its presence should be realized in DNA replication-fork rescue, meiotic chromosome segregation, and telomere maintenance (85).

In contrast to NHEJ, where damage mitigation during $G_1$ prefers the latter, HRR is most active during S phase and declines in $G_2$/M (70). HRR is preferred during S-phase-induced damage due to the inherent availability and proximity of sister chromatid homologs. Thereby, reducing cells to only one option of repair during S-phase, which is completely template-dependent. The most prominent damage HRR is responsible for is reparation of DSBs and interstrand crosslinks (ICLs). If these damages are left unnoticed by cellular machinery, they can promote large-scale rearrangement of chromosomes in somatic cells, which, in turn, can lead to cancer etiology (82). Also, HRR retains yet an additional task, telomere maintenance.

Once again, damage may be prompted by harmful chemicals, radiation, free radicals, radiation-induced free radicals, or other exogenous sources. The microscopic process of HRR has been evaluated and determined experimentally, however much is still up for debate and currently assessed. However, the basic conceptual scheme can be illustrated by the following (fig. 16). The induction of a DSB initiates recruitment of poly (ADP-ribose) polymerase 1

(PARP1) to the DSB site within a matter of 1 second (86). The localization of PARP1 to DSB sites initiates the recruitment of MRN complex (Mre11, Rad50, and Nbs1) to either side of the break within a mere 13 seconds (86). MRN holds several vital functions in regard to maintaining genomic integrity and overall structured repair, such as initial detection of the DNA damage, arresting the cell-cycle, determining the correct repair pathway, telomeric maintenance, and necessitate in the recruitment of supplementary protein complexes (77). In addition, a kinase termed ATM (ataxia-telangiectasia mutated) is recruited to site of break. After recruitment, end resection of the broken DNA begins via helicase and nucleases. Consequently, unwinding the DNA, rendering it accessible. Proceeding, RPA protein recruits to one of the exposed DNA strands to inhibit the strand from winding back on itself (87). RPA has high affinity for ssDNA (created by unwinding of dsDNA). Trailing RPA binding, with the help of associated proteins, Rad51 protein is recruited. As RPA is replaced by subsequent Rad51 recruitment, strand invasion by way of a spatially proximal sister homolog occurs. Rad51 is essentially a "bounty hunter" in search of a sister chromatid DNA template. Once a viable template is located, DNA polymerases can begin synthesizing a new undamaged DNA strand. A final quality control step is carried out, and a special DNA ligase, DNA ligase I, reanneals the newly formed DNA into the preexisting DNA. Therefore, a (fully) repaired DNA strand emerges.

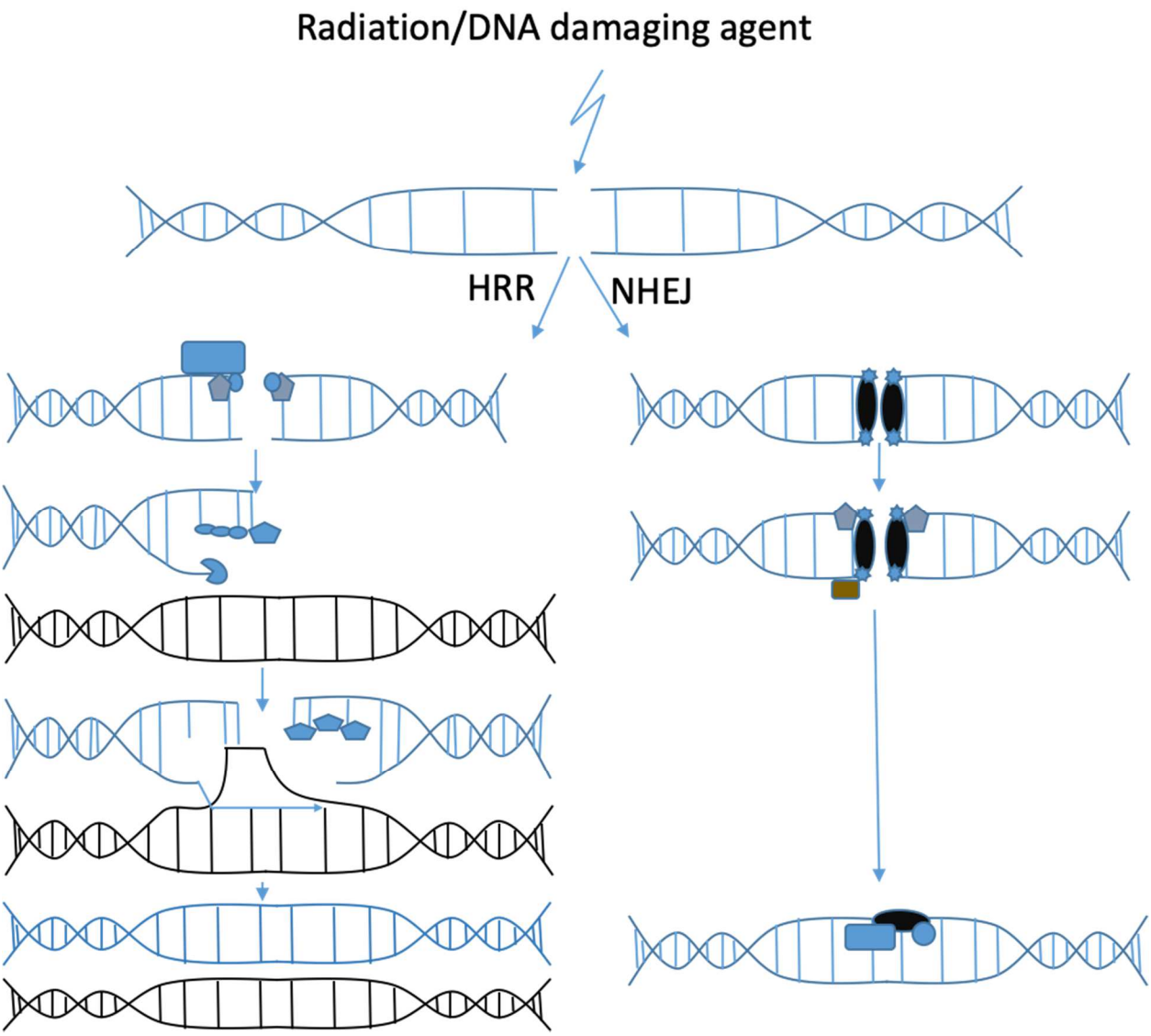


**Figure 16. Conceptualization of homologous recombination repair (HRR) and Non-homologous end joining (NHEJ) repair pathway**

### *1.4.7. UV mutagenesis and pyrimidine dimers*

As we go about our daily matters, we are exposed to sunlight, which contains UV light. This UV light attacks (88) our DNA inducing photochemical changes in our genetic information. Primarily, when cells are exposed to UVB and UVC radiation, pyrimidine dimers (PD) (or dipyrimidine) will arise. Data suggests that nearly 35% of mutations in the P53 gene arise from PD's (89). PDs are induced by absorption of UV light in the double bond in pyrimidine bases such as thymine. This results in the opening of the double bond allowing the bond to react with

an adjacent thymine, or pyrimidine. It is this adjacent pyrimidine that has potential to form a direct covalent bond with the neighboring pyrimidine; thus, a covalent linkage, or PD, is formed. Moreover, these PDs create lesions and bulky adducts within the DNA that induce torsional and steric strain, and difficulty for DNA repair mechanisms such as, NER. However, evolution (91) has developed a means to fix these PD-induced lesions; Nucleotide Excision Repair (discussed in next section). However, Xeroderma Pigmentosum, a rare autosomal recessive disease, lacks Nucleotide Excision Repair to fix UV-induced PDs. Moreover, it is critical that these affected individuals avoid extreme light/UV exposure.

### *1.4.8. What pathway(s) are activated proceeding UV exposure*

As one goes on a leisurely run on a sunny day, one most likely fails to recognize the harmful carcinogens they are exposed to. May those carcinogens be air pollution, chemicals from a residing factory, or, specifically, sunlight-containing UV light. It is UV light that induces PDs, interstrand crosslinks, or bulky adducts, in our foundational DNA. Moreover, mammals are fortunate to have a repair mechanism to fix damages induced by UV light: Nucleotide Excision Repair (NER). Mammalian NER is more complex and intricate compared to prokaryotic NER. NER is an innate DNA repair mechanism; human diseases arise from deficient or null NER capacity. NER is a very important DNA repair mechanism that fixes and removes PDs induced by UV exposure. UV exposure induces bulky DNA adducts that must be repaired promptly and efficiently in order for precise replication to occur. There are 9 major proteins that are involved in mammalian NER, however, those are beyond the scope of this discussion. Still, there are two major sub pathways of human NER: global genomic NER (GG-NER) and transcription coupled NER (TC-NER). It can involve all lesions throughout the genome (GG-NER), or it can be confined to the transcribed strands in genetically active regions of the genome (TC-NER).

Importantly, the mechanisms of these two forms of NER share several aspects in common, however, the method by which recognition of damaged DNA bases differs considerably (8). The recognition of damaged DNA bases, through GG-NER, is done by XPC protein. Whereas, TC-NER, recognition of damaged DNA bases is carried out by RNA polymerase. Therefore, the initial recognition of damaged DNA bases, during GG-NER, is carried out by XPC-hHR23B complex (or TC-NER coupled RNA polymerase). The cascade of NER process require common core proteins. For sake of simplicity of this report, the NER proteins will not be discussed in depth. In either kind of NER, DNA polymerase ε or δ fills in the gap left by the removal of the damaged DNA fragment, and DNA ligase seals the DNA (8) (fig. 17).

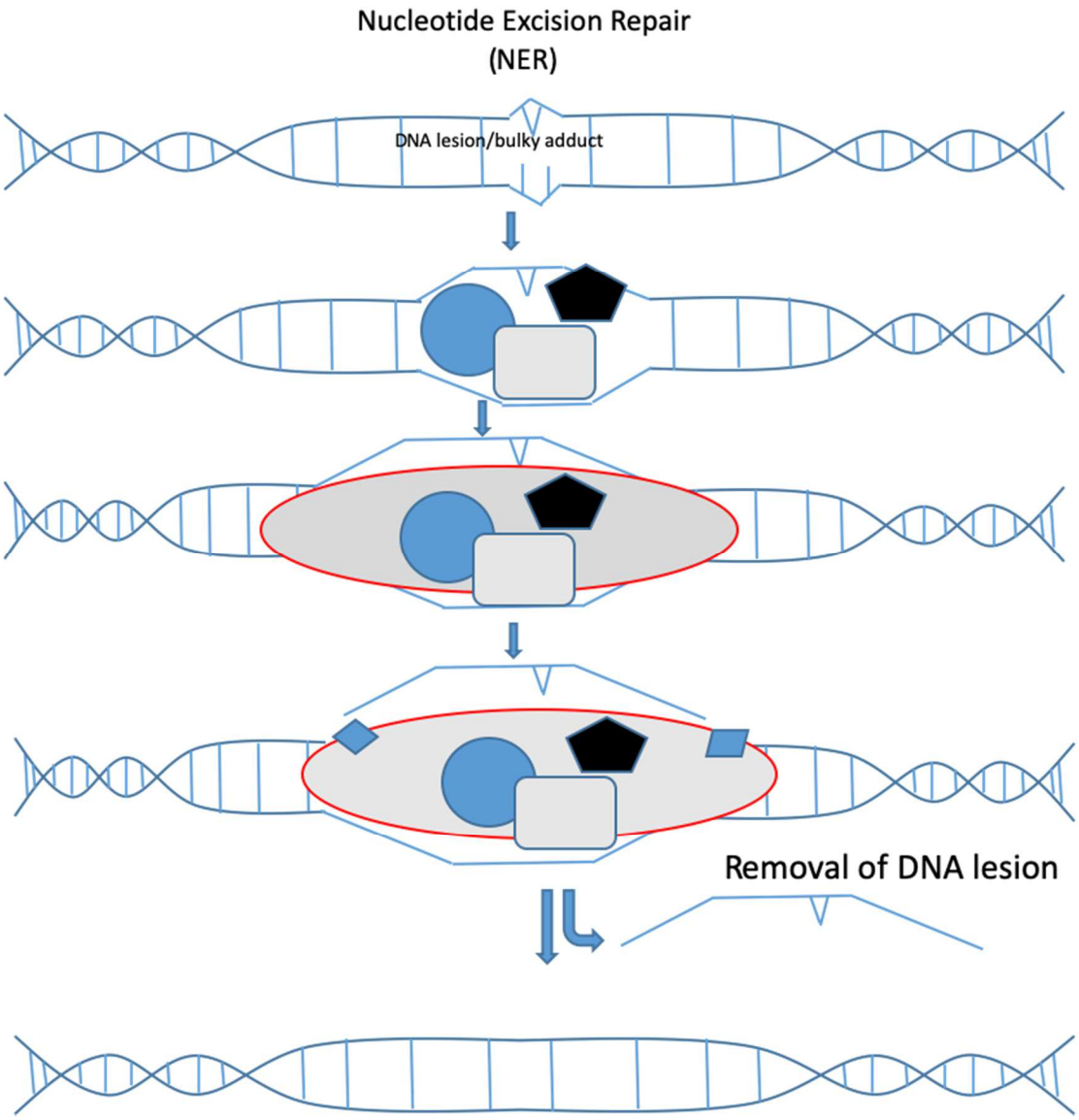


**Figure 17. Conceptualization of Nucleotide Excision Repair (NER) pathway**

### *1.4.9. Reparation of damage incurred by ultraviolet radiation*

Much of our information about UV radiation repair in humans has come from the study of congenital defects in DNA repair such as, Xeroderma Pigmentosum (XP). When an organism is exposed to UV radiation, cross-links are created between adjacent pyrimidines. Thus, the advent of pyrimidine dimers. It is these PDs that are harmful to a subjected organism, cell, or patient. However, evolutionary, earthly organisms have developed an ingenious repair mechanism by which PDs are nearly 100% repaired: NER. Bulky base damages, PDs, can be

easily removed and repaired via NER. The first step in NER is recognition of a distorted DNA double helix. Here, a complex involving XPC-hHR23B, RPA, and XPA recognizes the PD and initiates "melting" of the DNA. Following this, TFIIH (DNA helicase) binds damaged region and promotes increased DNA "melting". Moreover, RPA aids in positioning two endonucleases (ERCC1-XPF) and XPG on either side of the damage, these endonucleases clip (remove) away at the ends of the damaged DNA site. Lastly, once the damaged region is removed, DNA polymerase fills the gap with "good" DNA, while DNA ligase seals the final nick.

## 1.5 Hypersensitive radiation-provoked diseases

### *1.5.1. Xeroderma Pigmentosum and PD's*

Xeroderma Pigmentosum (XP), one of several genodermatoses (92), is a rare autosomal recessive disease lacking NER pathway. NER persists as the primary route by which bulky DNA damage (PD's) is repaired (93). Moreover, several epidemiological studies show that NER (and related proteins)-lacking individuals develop various melanomas 10,000-times higher the frequency than individuals who retain functional NER, therefore, suggesting that PD's are intimately connected with XP and human skin cancer development (93-95). While normal individuals who retain functional NER are affected by UV light to a lesser degree, XP individuals lack the NER machinery to repair damage induced by ultraviolet light. Symptoms of this disease may include keratitis, progressive cognitive impairment and neurological degeneration, and corneal ulcerations (94, 96). Moreover, frequent diseases acquired (1: 250,000-USA and 1: 20,000-Japan) by XP patients include basal cell carcinomas, metastatic malignant melanoma, and squamous cell carcinoma (96, 97). Altogether, deficiencies in NER are associated with the extremely skin cancer-prone inherited disease, XP (98).

### *1.5.2. Ataxia-telangiectasia*

Ataxia-telangiectasia (AT) Is a severe neurodegenerative disease that is noticeably apparent in early childhood (99). Individuals effected by the disease are predisposed to a gamete of diseases such as, chronic lung disease, leukemia, lymphoma, breast cancer, and telangiectasia. Of relevance to this report, AT patients are hypersensitive to ionizing radiation; in particular, X- and γ-irradiation. The latter is the result of a defunct Ataxia-telangiectasia mutated (ATM) gene. ATM is responsible for mediating/modulating cell-cycle progression in the presence of DSB's. If there is sufficient DNA damage (DSB's), ATM will activate (or indirectly inactivate) subsequent downstream substrates (P53, P21, MDM2, E2F, Cdk's, BRCA1, and so on) so that the cell will discontinue division, allowing for proper DNA repair to ensue. Exposure to ionization radiation is followed by subsequent activation of ATM (100). However, if an individual or cell is ATM deficient, the entity cannot inhibit cell-cycle progression appropriately after radiation-induced DSB's. Therefore, permitting propagation of un/misrepaired DNA, and accumulation of mutations resulting in a plethora of serious diseases.

### *1.5.3. Nijmegen Breakage Syndrome*

While AT and Nijmegen Breakage Syndrome (NBS) share many common chromosomal characteristics, NBS is a disease of its own (99, 101). NBS is a rare autosomal recessive syndrome of chromosomal instability. At the genomic level, and underlying *founder* mutation in the NBN gene is held responsible (102). The propagation of genetic loss or gain (deleterious or not) in a selective small, new population of individuals from a large population, is known as the Founder effect. NBS physiological and anatomical characteristics include, small size, microcephaly, mental retardation, skin lesions, serious immunodeficiencies, and hyperradiosensitivity. NBS patients are particularly sensitive to X-irradiation. In addition, the inhibition of DNA synthesis is less pronounced in NBS-derived cells compared to normal human

lymphocytes after X-irradiation. Investigative findings report and describe phenotypic/genotypic NBS as: spontaneous chromosomal breakage peripheral T-lymphocytes, sensitivity to ionizing radiation, biallelic (both copies of gene are mutated) hypomorphic mutations in NBN gene, and severely reduced NHEJ repair function (102).

### *1.5.4. Cockayne Syndrome*

Cockayne Syndrome (CS) is an unfavorable disorder characterized by a surfeit of clinical features including cachectic dwarfism, severe neurological manifestations including microcephaly and cognitive deficits, retinopathy, cataracts, sensory deficiencies, deafness, and ambulatory and feeding difficulties, often leading to an unfortunate adolescent death (~12 years old) (103). CS is a rare autosomal recessive disease, with a prevalence of approximately 2.5 million worldwide. Outstandingly, a clinical feature of CS is accelerated aging, progeria. Interestingly, yet not surprising, CS patients have overlapping clinical features, as well. At the most basic molecular platform, CS patients lack transcription-coupled NER. There are two common types of CS (CSA and CSB), however, extrapolation of the two subcategories are beyond the scope of this report, since recent reviews discuss this in extent. Though, it is important to note the defective proteins responsible for CSA and CSB: ERCC6 and ERCC8, respectively. CS cells may present damage in proxy of ionizing radiation, intercalating agents, profiled chemotherapeutics, but most prevalent, UV radiation (104). Evidence demonstrates that the source of error lies within defunct transcriptional machinery. DNA polymerases encountering a DNA lesion such as, a bulky adduct or (6-4) photoproduct, lack the ability to correctly mitigate further DNA repair. Collectively, ERCC6 and ERCC8 have selective N-and C-terminus that preferentially binds RNA polymerase II and RNA polymerase II-associated proteins (104). Disturbance of terminus results in inhibition of translocation of these proteins to the cell's

nucleus. Thus, inhibiting propagation of RNA polymerase activity and consequently, insufficient NER-DNA repair at site of UV-induced damage. The ineptitude of CS cells has certainly been demarked, but the overlapping gene and protein deficiencies between XP and CS patients remains an anomaly. The cross interplay between shared genes involve a deeper mechanism of understanding.

## 1.6 Cytogenetic and mutagenetic assays

### *1.6.1. Hypoxanthine-guanine phosphoribosyltransferase assay*

Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is a transferase, and is a necessary enzyme in humans which catalyzes salvage of purine bases into their respective nucleosides' (105); which are essential precursors for DNA and RNA. A major advantage of this assay is to selectively isolate mutant cells and determine the molecular basis of their deficiencies (106). The HPRT assay is used to test mutagenicity and carcinogenic potential of an array of compounds, drugs, chemotherapeutics, and various queried chemicals. This assay is an *in vitro* cell gene mutation test. Hypoxanthine-aminopterin-thymidine (HAT) is administered to cell cultures, which blocks the nucleotide salvage pathway, ultimately rendering cells reliant on the HPRT pathway. HPRT gene is located on a sex chromosome. For clarification of this report, cells (Chinese hamster ovary) that were elicited have one functional copy of the gene that encodes for the HPRT enzyme; during DNA synthesis, HPRT enzyme is necessarily important. Finally, drug or agent of interest is administered to cells. As a consequence of induced-mutations, mutant cells are elected by introduction of the selective toxic agent, 6-thioguanine (6-TG). Cells expressing a non-mutated HPRT gene incorporate 6-TG into their DNA, thus, leading to cell death. However, cells that are $HPRT^-$, or mutated, exclude 6-TG from their DNA and survive (107). Accordingly, compounds that produce or exacerbate a mutation in the HPRT gene, exhibit a cellular viability advantage and are deemed mutated, in the presence of this assay.

### *1.6.2. Doubling-time assay*

Doubling-time (DT) assay is one of the most fundamental and widely used assay for testing toxicity of a chemical or substance. It is crucial that researchers know the potentiated affects that a compound has on cells. More specifically, how a compound affects the cells ability to divide, or replicate. Examining the values of received DT data can supply a better understanding of the effects of a certain agent, modality, or compound on cellular kinetics. A reduced, increased, or marginal DT does not implicitly indicate that an experiment or protocol went awry, depending on the researchers aims and goals are. The name of the assay essentially explains itself in that, one selects a compound of interest and administers it to cell/tissue cultures. Moreover, the researcher will choose time points of interest (1 hr, 6 hr, 12 hr, 24 hr, etc.) after the chemical is administered and extract samples from the cell culture and quantitatively determine the cell count via Coulter counter or hemocytometer. One will then calculate how long it took the cells to divide once. Also, one must take into consideration the innate and unique DT of the cell line. For example, gastrointestinal cell DT differs appreciably than that of red blood cell, and so forth. Importantly, individual cell lineages have unique metabolic, repair, transcription, and kinetic rates. At any rate, it is critical that one defines a specific DT in order to correctly "seed" initial concentration of cells. If inappropriate concentrations of cells are initially seeded, complete overgrowth is foreseeable. Meaning, the desired effect of a chemotherapeutic, drug, or chemical may not be accomplished. Insofar that no cell death is appreciated. Moreover, in order to obtain correct DT values, the following simple equation can be utilized:

$$N(t) = 2C^{t/d}$$

- N(t)=number of cells present at time t
- C=initial number of cells

- d=cell lineage doubling period
- t=time

or

$$DT = \frac{\ln(2)}{growth\ rate}$$

- Growth rate= $N(t)$

### *1.6.3. Clonogenic survival assay*

Yet, again, the clonogenic survival assay is the most straightforward assay used for testing toxicity of a chemical, compound, therapy, or substance. This assay tests the ability of a single cell to propagate and divide into a colony (>50 cells/colony), or to determine cell viable after exposure to a compound (108). One begins by exposing cells to an agent of interest. Depending on the individual's cell plating protocol, the cells are initially manually counted for the zero-time point (zero hour). Subsequently, cells are placed into an incubator and allowed to incubate for an arbitrary amount of time (1-3 weeks). After an allotted incubation period, cell cultures are removed. Cultures are then fixed (washed with 0.9% NaCl & 100% ethanol) and stained (crystal violet 0.5% w/v), permitting visual interpretation and counting of colonies under light microscope (fig. 18). Following counting, one can mathematically assess plating efficiency (PE):

$$\text{PE}= \frac{\#\ colonies\ counted}{(\#\ of\ cells\ seeded)} x\ 100$$

(11) and survival fraction (SF): $SF = \frac{\#\ colonies\ counted}{\#\ cells\ seeded * PE}$

PE is postulated as the ratio of the number of untreated colonies counted to the number of cells initially seeded (pre-irradiation). In addition, SF is simply defined as the number of cells that have grown into viable colonies (post-irradiation) divided by the number of cells initially seeded multiplied by the PE. From values obtained, one can determine how effective a compound was on the cells ability to grow into colonies, and construct a corresponding survival curve. Again, a decrease or increase in PE or SF is not necessarily an indication of faulty experimental protocol or research error; it is entirely reliant on researchers aims and goals, and overall sterility applied when culturing cells. An entire experiment may be deemed nonsense or unusable by simply mistakenly utilizing unsterile culture dishes, pipettes, or gloves.

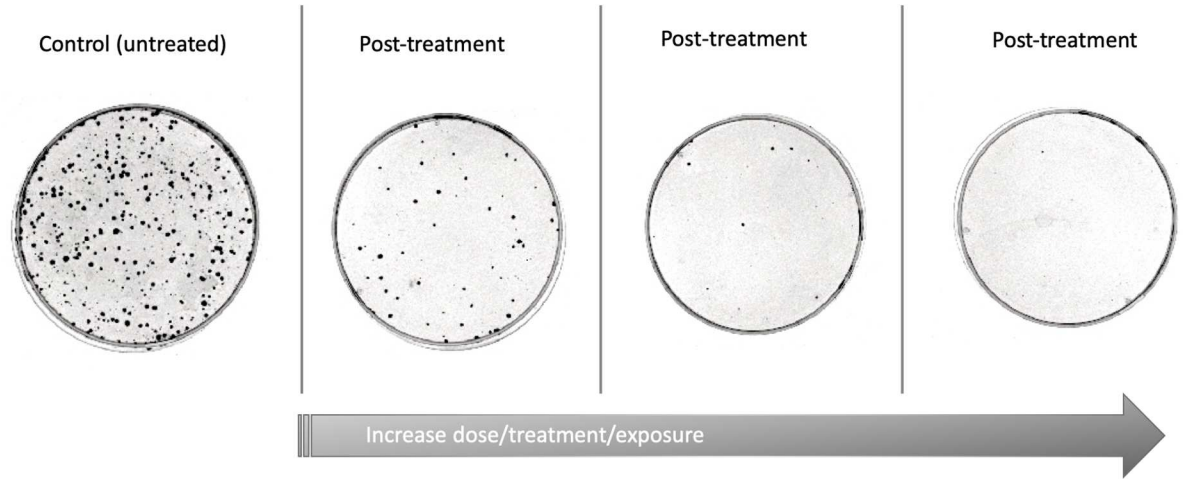


**Figure 18. Example of cell survival assay with UV-exposed CHO cell culture petri dishes**

### *1.6.4. Sister chromatid exchange assay*

Sister chromatid exchange (SCE) is a quintessential assay in that, it detects genotoxicity and chromosome alterations induced by endogenous and exogenous agents, compounds, or chemicals. Essentially, an SCE is created during DNA replication when two sister chromatids break at random DNA locations, rejoin, and exchange equal genetic information (109). However, since SCE's can occur randomly, it can be inferred that potential mutagens/genotoxins may indeed provoke the onset of abnormal SCE frequencies. Moreover, SCE assay is an important

assay for quantitatively and qualitatively assessing the efficiency, or deficiency, of HRR (76). After exposure to compound of interest one can score, visually, the frequency of SCE's produced by said compound and assess the rate at which SCE's were produced. Ultimately, evaluating the potential mutagenic/genotoxic effects a compound has on cell's DNA machinery. The protocol for obtaining SCE is relatively cumbersome, however, it is finely like that of the protocol established in this reports materials and methods (section 2.2.5.). This method utilizes a UV-sensitive dye.

## 1.7 Cell lines utilized-CHO, CHO DNA repair deficient cells, and A549

### *1.7.1. Chinese Hamster Ovary*

Chinese Hamster Ovary (CHO) cell line has been the primary cell lineage used throughout mammalian cell studies since its advent in 1957. CHO cell line is very diverse, mutable, and conveniently and easily maintained. The following CHO mutants only display a small percentage of the available deviant CHO available. In fact, 100s of CHO mutant cell derivatives are emerging, while some may never due to their proprietary nature. CHO cells observe a low chromosome number of 2n = 21. CHO wildtype cells have a doubling time approximately equal to ~12-15 hours. Thus, implicating them as a means to rapid and efficient experimental turnover. CHO cells' phenotype and genotype permits them to be relatively hardy, reliable, and the ability to retain a high plating efficiency (70-100%), which permits them as a suitable in vitro system (110, 111). In addition, the parameters for which CHO cells can be manipulated and mutated is boundless. Meaning, 100s, if not 1000s of isolated CHO mutant cell lines have been derived from the parental origin cell line. However, are the CHO cells that we, researchers, use today identical to the original parental cell line? We may not know for certain, due to the fact that over many passages, cells tend to acquire genetic variations and mutations. Meaning, that if one chooses to propagate a CHO cell line over the course of, for instance, one,

three, or many months, it wouldn't be frivolous to suggest that the endpoint-cells are entirely unique of the original plated cells. A great example of this: HeLa cells; derived from Henrietta Lack's blood sample in 1951. There are over 101,000 published scientific articles that retain the usage of "HeLa" cells in the title, dating as far back as 1953. It is only reasonable to suggest that, over the past 8 decades, countless passages, and genetic alternations, HeLa cells used in current studies are not identical to the original cell sample drawn in 1951. CHO and CHO DNA repair mutants utilized in this report are summarized in table 1.

**Table 1. The characteristics of Chinese hamster ovary (CHO), PADR9, and A549 cells**

| Cell line | Mutated gene | Mutated protein | Affected pathway |
|---|---|---|---|
| CHO10B2 | Wild type | Wild type | Wild type |
| V3 | Prkdc/XRCC-7 | DNA-PKcs | NHEJ |
| XRS-5 | XRCC5 | Ku80 | NHEJ |
| IRS-1sf | xrcc3 | XRCC3 | HRR |
| 51D1 | rad51d | RAD51D | HRR |
| KO40 | fancg | FANCG | Fanconi Anemia |
| PADR9 | parp mRNA | PARP (17% WT) | NHEJ/HRR |
| A549 | ABCG2 | Lung resistant protein (increase) | Multiple pathways |

*(CHO)V3*

V3 cell line is characterized by being spontaneously immortalized. In addition, V3 is markedly sensitive to x-ray ionizing radiation. But, are also markedly sensitive to bleomycin and $H_2O_2$. Studies illustrate that this mutation is acquired through null or nonsense mutations. Moreover, this cell line displays a tremendous decrease in NHEJ repair pathway activity; pronounced decrease ability to repair DSBs. This is due to a defect of the DNA-PKcs (XRCC7) catalytic subunit (112). DNA-PKcs is a critical, necessary protein responsible for high fidelity

DNA replication. In addition, V3 cells are deficient in V(D)J recombination, showing markedly decrease in T-and B-lymphocytic development in rodents (113).

### *(CHO)IRS-1SF*

IRS-1SF, a cell line that displays hypersensitivity to x-ray irradiation, has a defunct HR repair pathway. IRS-1SF is defective in the DSB repair protein XRCC3 (114). Moreover, XRCC3 is a paralog of RAD51 and aids in chromosome stability and DNA repair damage. This cell line is hypersensitive to X-rays and has a reduced ability to rejoin single-strand breaks; therefore, indicating it is additionally defective in NER. IRS-1SF has an increased rate of spontaneous and x-ray-induced chromatid abnormalities (115). IRS-1SF cells appears to exhibit only 50% x-ray-induced DNA damage repair of the parental line, indicating a lack of excision repair. Lastly, IRS-1SF was isolated via mutagenized colony screening system.

### *(CHO)51D1*

51D1 cell line is highly deficient in HR repair pathway. This cell is defective in the repair mediated protein, rad51. 51D1 is, in part, characterized by being considerably sensitive to gamma-rays, UV-C radiation, and methyl methanesulfonate (MMS) (116); with the inability to suitably repair DSBs produced by the latter. Importantly, 51D1 cells report a 12-fold increase in HGPRT gene, and a 4-to 10-fold increased rate of gene amplification at the dhfr and CAD loci, respectively (116).

### *(CHO)XRS-5*

XRS-5 cell line is markedly sensitive to x-ray, heavy charged particle (117), and UV-irradiation, ethyl methanesulphonate (EMS), methyl methanesulphonate (MMS), and bleomycin. This CHO DNA repair deficient mutant cell line was isolated by way of sterile ‘tooth-picking’ method. XRS-5 cell line is defective in DSB repair and V(D)J recombination (118). More

specifically, XRS-5 is affected in NHEJ pathway. The deficiency is due to a mutation in the XRCC5 gene which, ultimately affects Ku80 protein (119). Ku80 relays critical role in regard to DNA-dependent protein kinase catalyst subunit (DNA-PKcs) activity. XRCC5 is important in repair, in that it interacts with DNA ligase IV; thus, promoting efficient and proper repair/ligation of DSBs. Importantly, although the interplay between Ku80 and DNA-dependent protein kinase catalyst subunit DNA-PKcs remains to be delineated, it is hypothesized that, Ku directly targets the DNA-PKcs complex to the ends of damaged DNA (120). Thus, permitting activation of the DNA-PKcs complex, and further propagation of proper DNA repair.

### *(CHO)KO-40*

KO-40 cell line is a FANCG knockout cell line (121). FANCG is one of 17 Fanconi Anemia (FA)-like genes. Importantly, FA-like genes overlap with those of homologous recombination through FANCD1/BRAC2. However, previous literature may not agree on the exact role of FANCG in respect to cellular integrity. Tebbs et al. suggests that FANCG is not necessary for DNA replication that deals specifically with processing DNA crosslinks or promoting efficient homologous recombination; but, maintaining chromosomal continuity by stabilizing replication forks that encounter DNA lesions (122). Garcia et al. report that FANCG, and homologous recombination repair deficient cells, are increasingly sensitive to alkylating agents such as acetaldehyde (123). Moreover, they believe that FANCG is necessary for alkylating-induced lesions.

### *(CHO)PADR9*

PADR9 cell line displays approximately 17% of the wild-type level of poly-(ADP-ribose)-polymerase (PARP) activity (124). PADR9 was isolated a replica-plating procedure and a $^{32}$P-NAD+ permeable screening assay. Although PADR9 exhibits γ-ray sensitivity similar to

parental CHO cell line, it displays an increased sensitivity to killing by DNA-alkylating agents such as EMS, MMS, and DMS. PARP enzyme is required for proper DNA repair and regulation (124). Past research suggests that PARP facilitates chromatin unwinding, thus, allowing nuclear enzymes and regulatory proteins access to the DNA. In addition, the poly ADP-ribosylation of proteins catalyzed by PARP is involved in a number of cellular metabolic activities (125).

### *A549*

A549, adenocarcinomic human alveolar basal epithelial cells, is a cell line that was first developed through the removal and culturing of cancerous lung tissue from a 58-year-old Caucasian male (126). A549 is a subclass of stem cell-like cancer cells. A549 cells are particularly resistant to doxorubicin (DOX) and methotrexate (MTX) treatment (127); conferring multidrug resistance. A549 cells display an upregulation of ABC transporter genes, specifically, ABCG2. A549 cell line is to be considered immortal.

CHAPTER TWO

# EVALUATING THE GENOTOXIC AND CYTOTOXIC EFFECTS OF SYNTHETIC NUCLEOSIDES IN VITRO

## 2.1 Introduction

Instinctively, humans are inquisitive beings. The 'unknown' and untouched encourages human innovation, creativity, and proclamation of novel ideas. For example, cancer cell immortality has dumbfounded scientists for an innumerable number of decades. On-the-other-hand, humans were enticed by how we are able to maintain our bodily and physiological functions. It is widely accepted that "hijacking" normal cellular DNA replication and division are the pillars and mainstay by which cancer cells are equipped with the ability to thrive and gain immortal replicability. Although, humans are privy to being mortal beings, it is conclusively a mystery as to why. For as long as research and human inquiry dates back, we have come to understand that cancer cells require indefinite division. The elementary, yet novel method to investigate the latter, was not established until mid-1950s (reproducibly and accurately). To date, investigation of the elementary principles of cell biology remain high notoriety. Every day, the cells that implicate our body are subject to 100,000s of iterative divisions and replications. Ranging from red blood cells, white blood cells, lymphocytes, brain cells, muscle cells, and so forth; all participate in daily physiological maintenance. Cellular division, to the lay-eye, seems rather trivial and, more-or-less obsolete. On the contrary, cellular mechanisms that ensure and develop proper progression of humans, from birth to inevitable death, remain vaguely misunderstood. The advent of labeling actively dividing cells and DNA, amidst the biological "boom", occurred during the mid-1950s at Brookhaven National Laboratory. Hence, the introduction of tritiated thymidine into the biological realm.

Within the cell is a structure known as the "nucleus". The nucleus approximates ~30% of total cell composition. A further dissection of the nucleus, one will find the elaborate fabric of our very existence, DNA. Which is subject to division, replication, and maintenance daily. As more and more effort was put forth to examine such, novel methods were developed. The groundbreaking arrival of tritiated thymidine ($^3$H -TdR) introduced a level of microscopic identity that was unheard of, and was iconic within the biological society. By way of this method, researchers acquired the ability to autoradiographically visualize dividing cells in S-phase. As previously stated, cancer cells primarily impart their damage upon organisms' due to their innate ability to divide infinitely by invading and seizing a host cell's DNA replication machinery; ultimately, utilizing this to their advantage and gaining immortality. Therefore, scientists and researchers alike, sought to take advantage of this novel method in order to investigate and synthesize anticancer compounds that solicited, damaged, and killed cancer cells in S-phase. Attractively, S-phase is inherently the most sensitive portion of the cell cycle. Because, of the innate vulnerability of DNA. However, labelling cells with $^3$H -TdR was extremely cumbersome, time-consuming, and exposed scientists to unnecessary radiation.

In order to bypass the myriad of impasse pitfalls of labeling cells with $^3$H -TdR, in 1975, researchers from the University of Miami and CalTech developed an antibody specific for 5-bromo-2-deoxyuridine (1). BrdU, like $^3$H -TdR, is incorporated into DNA during S-phase, however, its lack of radioactivity was alluring. Nonetheless, nothing is perfect. Although BrdU possessed greater and safer-conducive biological qualities than its counterpart, $^3$H -TdR, it's methodology and detection followed suit that of tritiated thymidine: cumbersome and carcinogenic. In addition, BrdU detection necessitates partial denaturation of the DNA so that the antibody is permitted physical and sterical access to the DNA-BrdU complex, and complete

binding of conjugated BrdU-antibody is accomplished. The latter represents an imminent probability of improper base-pairing and annealing after reannealing. Moreover, BrdU is exceedingly cytotoxic, carcinogenic, and can permeate through human skin. Thus, physical-skin contact should be avoided at all costs; intercalation of BrdU in DNA may occur otherwise. Clearly, revelations provoked the need of safer biological inquiry.

With molecular biology transcending many other scientific fields at the time, it was necessary to establish a more accurate, safe, and reliable biomarker: EdU. Halogenated pyrimidines such as BrdU, IdU, and CldU have been utilized in other scientific sectors. However, EdU was originally employed as a means to treat viruses, tumors, and to identify bacteriophage (14,15). Remarkably and significantly, EdU was readily available as a biomarker for actively dividing cells, in conjunction with click chemistry. EdU provides accurate, quick, safe, accessible, and reliable labeling.

When contrasting and comparing BrdU and EdU there are similarities and differences. However, it is necessary to address the potential, if any, adverse effects that halogenated pyrimidines have on cellular integrity, function, and human wellbeing. Although, this report clearly indicates that BrdU and EdU, in the presence of ionizing or UV irradiation, have potential harmful effects, the implications of such in absence of external radiation modalities have remained rather misunderstood. Cells treated with concentrations >50 μM of BrdU for 24 hours displayed some inhibitory effect. Even more so, after 100 μM BrdU administration, cellular kinetics were greatly inhibited (128). Treatment of >50 μM BrdU for 1 to 2 hours did not display any noticeable inhibitory effect during first cell-cycle. Effectively, CHO cells exposed to 50, 100, and 500 μM BrdU demonstrated a prominent delay in progression to the next cell-cycle, with nearly all cells arrested in at the 3$^{rd}$ cell-cycle (<100 μM BrdU). And, an agreed conclusion

that propensity of SCE and centromeric exchange increased linearly with increased BrdU concentration (129), with 500 μM BrdU accumulating highest SCE ratio. The "click chemistry" approach utilizing EdU as a DNA precursor was introduced as a means to assess DNA replication. However, it was observed that once EdU was incorporated into DNA, induction DNA damaging signaling (DDS) was seen through phosphorylation of ATM, histone H2AX, P53, and Chk2 (130). A recent study reports that, pulse-labeling cells, throughout S-phase, at EdU concentrations recommended by suppliers did not affect S-phase structure or the rate of fork progression. However, continuous-labeling cells with EdU, resulted in clear disruption of the cell-cycle, with cells accumulating in $G_2$/M-phase. Correlating cell-cycle progression and cell number, a dramatic decrease in cell population in respect to cell-cycle retardation, with inhibition of proliferative capacity resulting in apoptosis (131). These data will surely aid in the advancement of cellular research

In this report, we aim to identify and fill in the voids pertaining to EdU and its effects on cell cytotoxicity and genotoxicity. More importantly, the involvement of EdU in regard to induction of cytotoxicity, SCE formation, HPRT mutation, PARP inhibition, and overall cellular clonogenicity, in CHO wildtype and CHO DNA repair deficient mutants. By way of incorporating various cellular mutation and intrinsic cell dynamic-morphological techniques, we were better able to establish and delineate a more profound synergistic effect of EdU on cell-cycle integrity and maintenance. The ramifications of EdU on cell-cycle dynamics is clearly illustrated throughout this present study.

## 2.2 Materials and Methods

### *2.2.1. Cell Cultures*

**Chinese hamster ovary (CHO-10B2) (wild-type)** and Five DNA repair deficient CHO mutant, 51D1 (Rad51D gene deficient), **PADR9** (17% wild-type PARP activity), **KO40** (FANCG knockout), **IRS-1SF** (XRCC3 gene deficient), **XRS-5** (XRCC5 gene deficient), and **V3** (DNA-PKcs gene deficient) cells, were kindly supplied by Dr. Joel Bedford (Colorado State University, Fort Collins, CO) and Dr. Larry Thompson at the Lawrence Livermore National Laboratory (Livermore, CA, USA). Cells were cultured in MEM-alpha (Gibco, Indianapolis, IN) supplemented with 10% heat inactivated fetal bovine serum (FBS, Sigma, St. Louis, MO) and 1% antibiotics and antimyotics (Gibco, Indianapolis, IN), and they were maintained in a 37°C humidified incubator with a supplemented ambient 5% $CO_2$ administration. Sub-cultured cells were seeded in a T25 flask at 2.0-5.0 x $10^5$ cells/flask, and incubated for approximately 1-2 days prior to start of experiment to ensure cell confluency and attachment.

### *2.2.2. Applied Chemicals*

5-bromo-2'-deoxyuridine (BrdU) was purchased from Sigma (St. Lous, MO) and stock solution was prepared in 100% DMSO as 1 mM. 5-ethynyl-2'-deoxyuridine (EdU) was purchased from Invitrogen (Waltham, MA) and stock solution was prepared in 100% DMSO as 10 mM.

### *2.2.3. Clonogenicity assay of CHO-nucleoside/nucleotide deficient medium*

This assay was implemented to discern the effect of nucleotide/nutrient deficient medium vs. nucleotide rich medium in the presence of thymidine analogues. Cell survival assay was carried out using standard cell culture dishes, and abides by previously described protocol. Briefly, exponentially growing cells were plated on P60 cell culture dishes at a density designed to yield approximately 100 viable colony-forming cells. BrdU and EdU were delivered in

increasing concentrations (0 to 1000 μM) accordingly, to an α-MEM-nutrient rich medium or MEM-nutrient depleted medium. Cells were then placed in 37˚C humidified incubator with supplemental ambient 5% $CO_2$ for approximately 7-8 days. Surviving colonies were washed with 0.9% NaCl, fixed with 100% ethanol and stained by 0.1% crystal violet dye. Each colony consisting of 50 or more cells was scored a viable colony. Cell survival curves were obtained by GraphPad Prism 5 software (GraphPad, La Jolla, CA) with linear quadratic regression for UV and ionizing radiation exposed cells. The $D_{10}$ values, which represent a dose at which is necessary to reduce the population by 90%, or achieve 10% survival, were obtained by GraphPad Prism 5 software.

### *2.2.4. Induced HPRT mutations in presence of thymidine analogues*

Prior to use in the HPRT mutation assay, CHO wild type cells were grown in HAT medium (supplemented medium with $2x10^{-4}$ M hypoxanthine, $2x10^{-7}$ M aminopterin, and $1.75x10^{-5}$ M thymidine) for three days in order to reduce the level of spontaneous HPRT mutations. The cells were then incubated in 37˚C humidified incubator with supplemental ambient 5% $CO_2$ for approximately 2-3 days. After incubating cells, each drug/control (EdU or BrdU and control) was administered to its corresponding cell culture dish (1μM EdU and 1 μM BrdU). After drug exposure, cells were diluted and grown in a nonselective medium for a period of time sufficient to allow phenotypic expression prior to plating for determination of mutant fraction index. Phenotypic expression time is approximately 7-10 days for clear HPRT mutation. Following, cells were then plated were plated in P60 cell culture dishes in the addition of a selective agent, 6-thioguanine (6-TG, 5μg/ml 6-TG). Cells from corresponding dishes were then plated at a low density (300 cells/ml) in the absence of the selective agent to determine plating efficiency. All cell cultures were incubated for 10-12 days, permitting sufficient growth period,

prior to scoring colonies. Each HPRT mutation experiment was carried out a minimum of 3 times, to ensure reproducibility. Colonies were then counted, collected, and data/graph was calculated and obtained from GraphPad Prism 5 software.

### *2.2.5. SCE induction by substituted thymidine analogues*

CHO wild type and CHO DNA repair deficient cells were synchronized into the $G_1$ phase using a mitotic shake-off procedure (132). Synchronized mitotic cells were subcultured in T25 flasks and incubated for two hours in 37˚C humidified incubator with supplemental ambient 5% $CO_2$. Cells were then treated with 10, 100, or 300 μM BrdU; or 1, 10, 30, 50, or 100 μM EdU for two cell cycles. 0.2 mg/ml of colcemid (Gibco, Indianapolis, IN) was added to cells to permit M-phase accumulation, and allowed to incubate for an additional 6 hours. Cells were harvested during metaphase, trypsinized, and then re-suspended in 4 ml of 75 mM KCl solution warmed to 37˚C and placed in a 37˚C water bath for 20 minutes. A fixative solution of 3:1 methanol to acetic acid was added to the samples according to the standard protocol (133). Fixed cells were dropped onto slides and allowed to dry at room temperature. Differential staining of metaphase chromosomes was completed using the fluorescence plus Giemsa technique for BrdU or EdU-"click" reaction with fluorescent microscope (134). Differentially stained metaphase chromosome images were scored under a Zeiss Axioskop microscope equipped with SPOT CCD camera RT 2.3.1 (Diagnostic Instrument, Inc.) and SPOT basic software or Zeiss Axiophot fluorescent microscope with Q-imaging cooled CCD camera. A minimum of 50 metaphase cells were scored for each chemical treatment concentration, and each experiment was conducted at least 3 consecutive times. Data presented are the mean of SCE frequency per chromosome.

### *2.2.6. PARP inhibitory assay*

Poly (ADP-ribose) polymerase (PARP) colorimetric assay kits (Trevigen, Gaithersburg, MD) were used to measure and quantify PARP activity as depicted by manufacturer's instructions. PARP was incubated in a 96-well microplate with a reaction mixture containing 50 μM β-NAD+ (10% biotinylated β-NAD+), 90% unlabeled β-NAD+, 1 mM 1,4-dithiothreitol, and 1.25 mg/ml nicked DNA. The formation of poly (ADP-ribose) polymers was detected with a peroxidase-labelled streptavidin and 3,3',5,5'-tetramethylbenzidine (Invitrogen, Carlsbad, CA). PARP inhibition was assessed by the addition of thymidine and its analogues at various dosages (.01-10 mM) in the reaction mixture. PARP activity was directly proportional to absorbance at 450 nm and measured by a NanoDrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA). Lastly, IC50 values of PARP inhibitory activity were derived by fitting a dose-response curve using a sigmoidal dose response equation. PARP inhibitory assay, in presence of thymidine and its analogues, was replicated minimum three times.

### *2.2.7. Doubling time assay*

Exponentially growing CHO and CHO DNA repair deficient cells (~10,000 cells/ml) were plated in DMSO (control), and presence of BrdU and EdU; concentrations ranging from 0 to 150 μM. Cells were place in a 37°C humidified incubator with supplemental ambient 5% $CO_2$ and cell number was counted by a particle analyzer, Coulter Counter Z1 (Beckman Coulter, Indianapolis, IN) every 12 hours. Cell doubling time was calculated by using GraphPad Prism 5 (GraphPad, La Jolla, CA). Doubling time assay, in presence of thymidine and its analogues, was replicated minimum three times.

### *2.2.8. Interpretation of data and data analysis*

All data obtained from experimental procedures were analyzed and processed through GraphPad Prism 5$^{TM}$ (Graphpad, La Jolla, CA). Statistical comparison of mean values was performed using a t-test or one-way analysis of variance (ANOVA) when comparing groups. Differences with a p-value <0.05 were considered to indicate a statistical significance. Error bars indicate standard error of means (SEM) or standard deviation (SD). One-way ANOVA, Dunnett's Multiple Comparison Test was performed to provide P-values ($P<0.05$) statistically significant differences from control

## 2.3 Results

### *2.3.1. HPRT induced mutations through synthetic nucleotide substitutions*

Nucleotide substitution effects on induced HPRT mutation frequency was demonstrated by way of *in vitro* CHO cell HPRT assay. In absence of external radiation modalities, an increased HPRT mutation frequency was clearly demonstrated by DNA-incorporation of EdU. On-the-other-hand, BrdU substitution demonstrated an enhanced production and increase in HPRT compared to CHO wildtype. While CHO wildtype-unlabeled cells exhibited nearly zero (~1) HPRT mutations, EdU-induced HPRT mutation was approximately 65-fold increase that of wildtype frequency. In addition, EdU provoked HPRT mutation frequency depicted 3-fold increase that of BrdU-induced HPRT mutations (fig. 19). Therefore, it is reasonable to suggest that in the process of rectifying stalled replication forks and translocations, in regard to homologous recombination repair, these morphological changes can be visualized as chromosomal aberrations or HPRT mutations. Even at concentrations as low 1-10 μM EdU, there was a recognizable increase of HPRT mutation. In the same respect, concentrations ranging 1- 10 μM BrdU, still, a noticeable induction of HPRT mutations. Contrasting CHO control, no

HPRT mutations were apparent, even at exceedingly high concentrations. However, HPRT mutation analysis was not carried out in other CHO DNA repair deficient mutant cell lines.

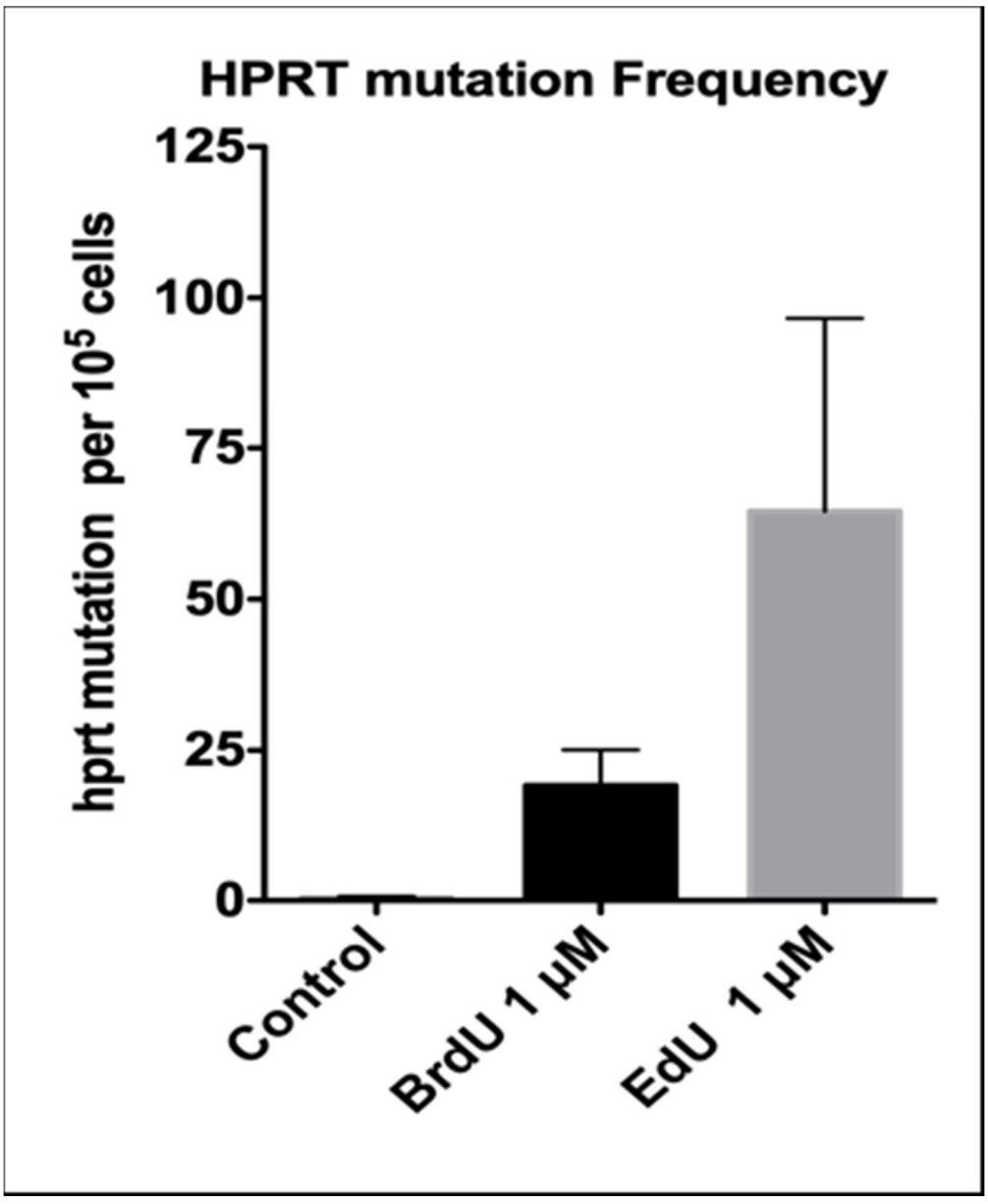


**Figure 19. EdU and BrdU increase HPRT mutation induction. Control cells did not appear to be affected. However, BrdU-HPRT mutation induction was on the order of ~25 HPRT mutations/$10^5$ cells. EdU-HPRT mutation induction was on the order of ~60 HPRT mutations/$10^5$ cells. Approximately, EdU had 3-fold increase HPRT mutation induction compared to BrdU. Error bars represent the mean of three independent experiments. One-way ANOVA, Dunnett's Multiple Comparison Test was performed to provide P-values ($P<0.05$) Statistically significant differences from control**

### *2.3.2. Elevated concentrations of EdU inherently incite increased doubling time*

In the presence of substituted nucleoside pyrimidines, EdU or BrdU, CHO wildtype and CHO DNA repair deficient mutant cells displayed a varying cellular effect. Insofar that respective doubling times varied significantly; of which, are depicted in figure 20 & 21. Both EdU and BrdU slowed down cellular kinetics by decreasing cellular division and increasing doubling time. However, BrdU to a lesser degree. At concentrations ranging from 0 to 100 μM ($P<.0001$) EdU or BrdU, CHO wildtype doubling time did not represent any significant differences. Oppositely, in HRR deficient cells, IRS-1SF and 51D1, a dramatic increase in doubling time at EdU concentrations ranging 10 to 50 μM ($P<.0001$). Although, transparent induction of decreased cellular division kinetics in HRR deficient cells, there remains a caveat. Nearing 50 μM EdU, data suggests a saturation effect (fig. 20), where there was no visible increased doubling time in HRR deficient cells. Comparatively, no saturation effect was observed through BrdU administration. Table 2 displays a coherent representation of effected CHO wildtype and CHO DNA repair deficient mutant cell lines doubling times.

**Table 2. Summarization of EdU and BrdU effects on CHO and CHO DNA repair mutants doubling time**

| Cell line | 5'-Edu DT affect | 5'-BrdU DT affect | Saturation affect |
|---|---|---|---|
| CHO | n/a | n/a | n/a |
| PADR9 | 100 μM - 2x | n/a | n/a |
| XRS-5 | 100 μM - 3x | n/a | n/a |
| V3 | 50 μM- 2x | n/a | Slight (EdU) |
| IRS-1SF | 12 μM- 2x<br>30 μM- 3x<br>48 μM- 4x | Slightly linear | Yes (EdU) |
| 51D1 | 10 μM- 2x<br>30 μM- 7x<br>50 μM- 8x | 100 μM- 2x | Yes (EdU) |
| KO40 | 100 μM- 2x | n/a | n/a |

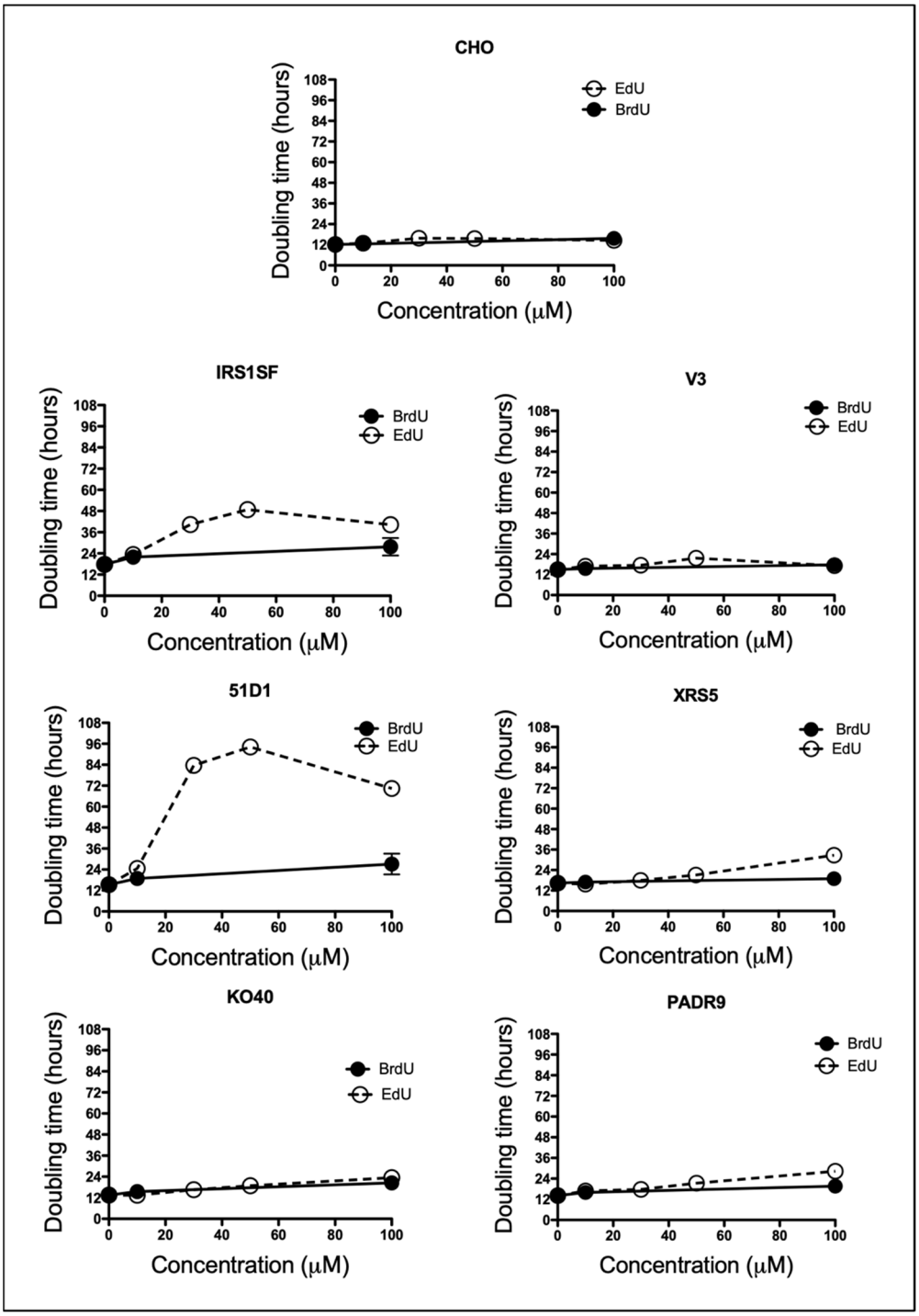


**Figure 20. EdU increases doubling time of CHO DNA repair deficient mutants: HRR deficient cells, IRS1SF and 51D1, demonstrated the highest increase of doubling time, with a noticeable saturation effect at 50 μM EdU. Though, slight increase in doubling time in PADR9 and XRS5 cells. BrdU did not result in significant alterations in cell doubling time. Error bars represent the mean of three independent experiments. One-way ANOVA, Dunnett's Multiple Comparison Test was performed to provide P-values. P<0.050. Statistically significant differences from control**

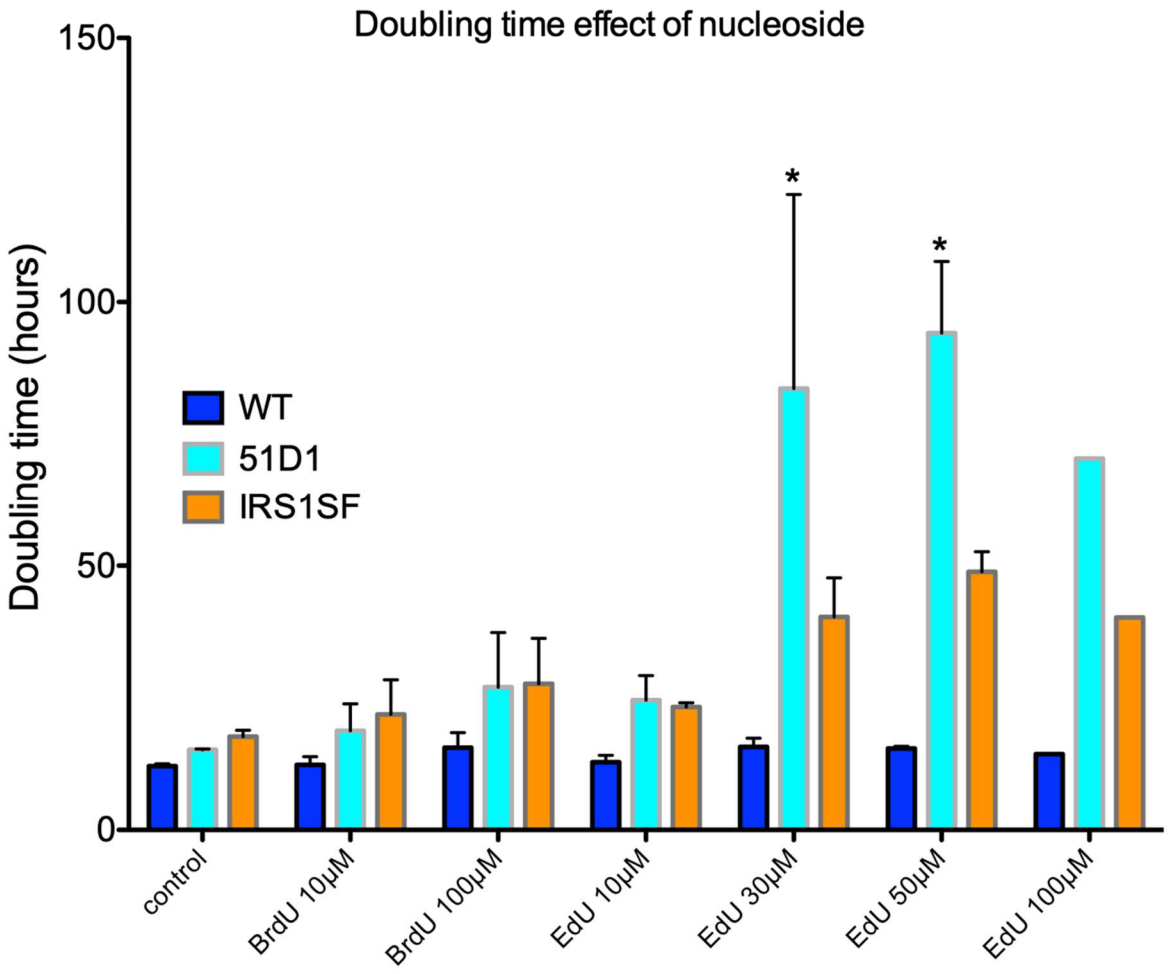


**Figure 21. Increased doubling time in HRR deficient IRS1SF and 51D1 CHO cell line. CHO HRR mutants, IRS1SF and 51D1 (orange and cyanic blue, respectively), display a dramatic increase in cellular doubling time. With a marked increase shown for 51D1 cells. BrdU did not result in an appreciable doubling time increase, even at 100 μM. Error bars indicate standard error of the mean of three independent experiments. One-way ANOVA, Dunnett's Multiple Comparison Test was performed to provide P-values. (*) denotes significance. P<0.05. (*) Statistically significant differences from control.**

### *2.3.3. Continuous exposure of EdU kills homologous recombination deficient cells*

Colony size, expressed as diameter, was used to evaluate the estimated EdU-induced cell growth suppression and killing. Continuous cell exposure to EdU clearly demonstrated that HRR deficient cells, 51D1 and IRS-1SF, were inhibited greatly (fig. 22). Specifically, at 10 to 30 μM EdU exposure, 51D1 cells were unable to maintain viability, thus, resulting in no colony

formation. Moreover, IRS-1SF cells exposed to 10 to 30 μM EdU displayed an appreciable decrease in colony formation/colony size, with no colony formation at 30 μM EdU. While CHO wildtype did not present any negative results from EdU or BrdU exposure, control CHO DNA repair deficient mutants did. In contrast, Fanconi deficient cells, KO40, and Poly (ADP-ribose) polymerase cells deficient cells, PADR9, did display slight attenuation of colony formation at 30 μM EdU. With all considered, data suggests that homologous recombination repair is held partially responsible for EdU hypersensitivity. And, Fanconi Anemia pathway and PARP signaling plays vital interplay in EdU hypersensitivity. With respect to continuously exposing cells to EdU, 3-day exposure expresses less of an obvious clonogenic-inhibitory effect.

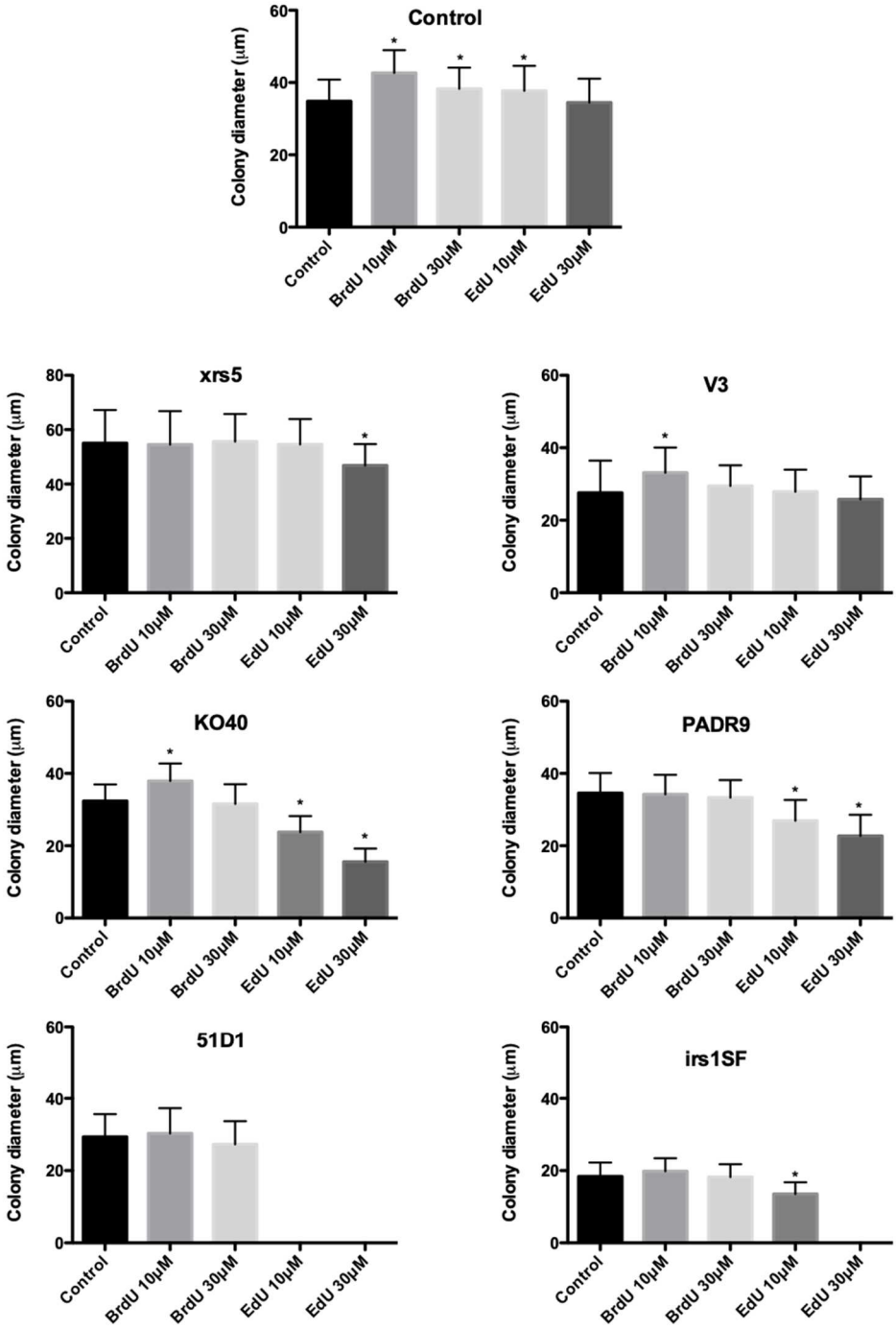


**Figure 22. Increased EdU incorporation affects clonogenicity and colony diameter size: Clonogenic propensity and their respective diameter size is significantly inhibited by administration of 10 μM EdU; complete absence of HRR cells at 30 μM EdU. One-way ANOVA, Dunnett's Multiple Comparison Test was performed to provide P-values. (*) denotes significance against control within each cell group.**

### *2.3.4. PARP formation inhibited by BrdU and EdU*

Thymidine, EdU, and BrdU were compared for their effect on PARP inhibition. EdU exposure did not present superior PARP inhibitory effect compared to BrdU or thymidine (fig. 23). While BrdU and thymidine displayed similar dose-dependent PARP inhibitory effect, ranging from 1 mM to 5 mM, EdU remained constant. A decrease in PARP production of 1.22 to 0.44 PARP units, was observed at 1mM and 5 mM BrdU, respectively. Intriguingly, elevated levels of thymidine administration did present a linear decrease in PARP production formation. Thymidine-PARP production (or inhibition) is dose-dependent, as well as BrdU. Our results suggest that high SCE frequency, chromosomal aberrations, and HPRT mutation frequency are not a result of PARP inhibition. Although, there is qualitative and quantitative differences in PARP activity, however, PARP activity assay was only carried out once. Therefore, statistical differences were not achievable. Error bars represent standard deviation (SD) of one assay.

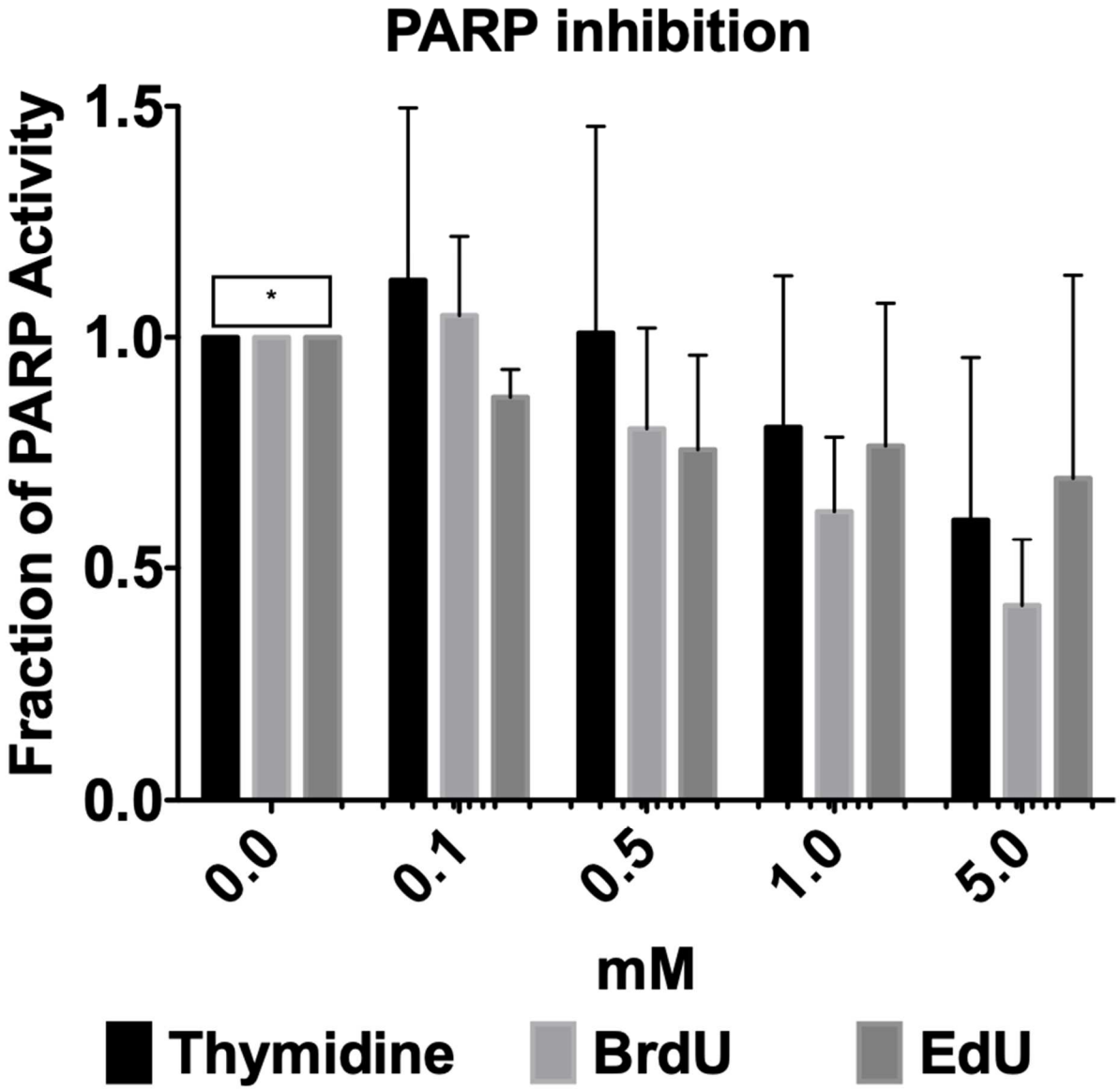


**Figure 23. Effects of nucleoside substitution on PARP inhibition: At each incremental increase of thymidine, BrdU, or EdU concentration, PARP activity was reported. CHO cells exhibited a linear decrease in PARP activity with dose-dependent thymidine and BrdU concentrations. (*) denotes baseline (0 mM). There were no statistical differences between baseline and 0.1, 0.5, 1.0, 5.0 mM concentrations of thymidine, BrdU, or EdU. Error bars represent SD of one experiment.**

### *2.3.5. Effect of nucleoside or nucleotide supplemented medium*

AlphaMEM medium, supplemented with nucleoside and nucleotide, contains 10.0 mg/L thymidine (0.041 mM) and thymine. In order to understand the cytotoxic effects of EdU, MEM

medium (nucleotide/nucleoside deficient) lacking thymidine and thymine was utilized during colony formation. BrdU cytotoxicity was enhanced in the absence of thymidine and thymine. BrdU $IC_{50}$ values changed from 150 μM in alphaMEM to 40 μM in MEM (fig. 24). Dramatically, on-the-other-hand, $IC_{50}$ values for EdU drastically changed from alphaMEM to MEM medium; 75 μM to 0.05 μM, respectively. This dramatic alteration in $IC_{50}$ values suggests that cells can tolerate BrdU incorporation into DNA, and that DNA can be replicated when BrdU is conformed in the template strand. On the contrary, EdU is inherently cytotoxic, and cells cannot use EdU-containing strand as a template during DNA replication. Likely, EdU-containing strand leads to replication fork stalling or collapse during DNA replication. Also, our data suggests that, when cells are subjected to choose between BrdU or thymidine as template, there is little or no competition.

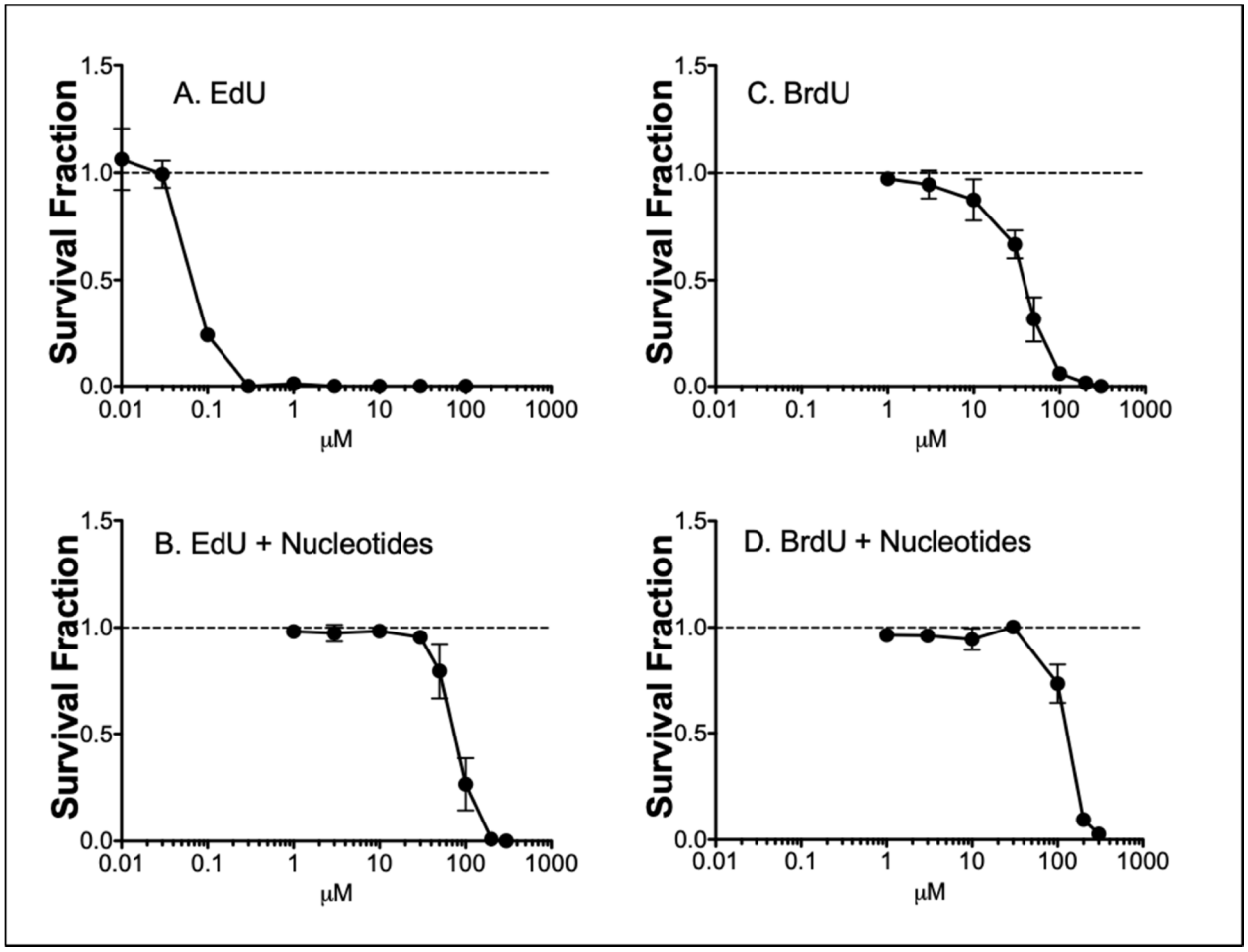


**Figure 24. AlphaMEM (nucleotide) or MEM medium (-nucleotide). CHO wildtype cells displayed a shift in cell survival when cultured in AlphaMEM versus MEM medium. Effectively, $IC_{50}$ values for increasing EdU-supplemented AlphaMEM and MEM medium decreased from 75 μM to 0.05 μM, respectively. $IC_{50}$ values for increasing BrdU-supplemented AlphaMEM and MEM medium from 150 μM to 40 μM, respectively.**

### *2.3.6. Increase SCE production in presence of EdU*

EdU induced replication fork stall was confirmed by analysis of sister chromatid exchange assay (fig. 25). CHO wildtype did display a dose-dependent increase of SCE frequency. Where, average SCE induction per cell was over 100 for CHO wildtype administered 100 μM EdU, there was absolutely no induction of SCE in CHO DNA repair mutant cells at the same conditions. Suggesting that all cells were unable to retain viability, resulting in cell death,

thus, no SCE appearance. Even 100 μM BrdU-induced SCE production was apparent in all CHO wildtype and CHO DNA repair deficient cell lines, 300 μM BrdU induced a noticeable increase in SCE per cell. Although, there was an increase in BrdU-induced SCE per cell, it was considerably lower than that of EdU. IRS-1SF SCE per cell data was not shown for BrdU because all cells died or failed to provide a suitable SCE spread. There was approximately 6 SCE per cell in the presence of 100 μM BrdU. Even more so, 10 to 28 SCE per cell were counted in the presence of 300 μM BrdU. Error bars represent the SEM for three independent experiments. There was no statistically significant induction of SCE when compared to control for both EdU and BrdU.

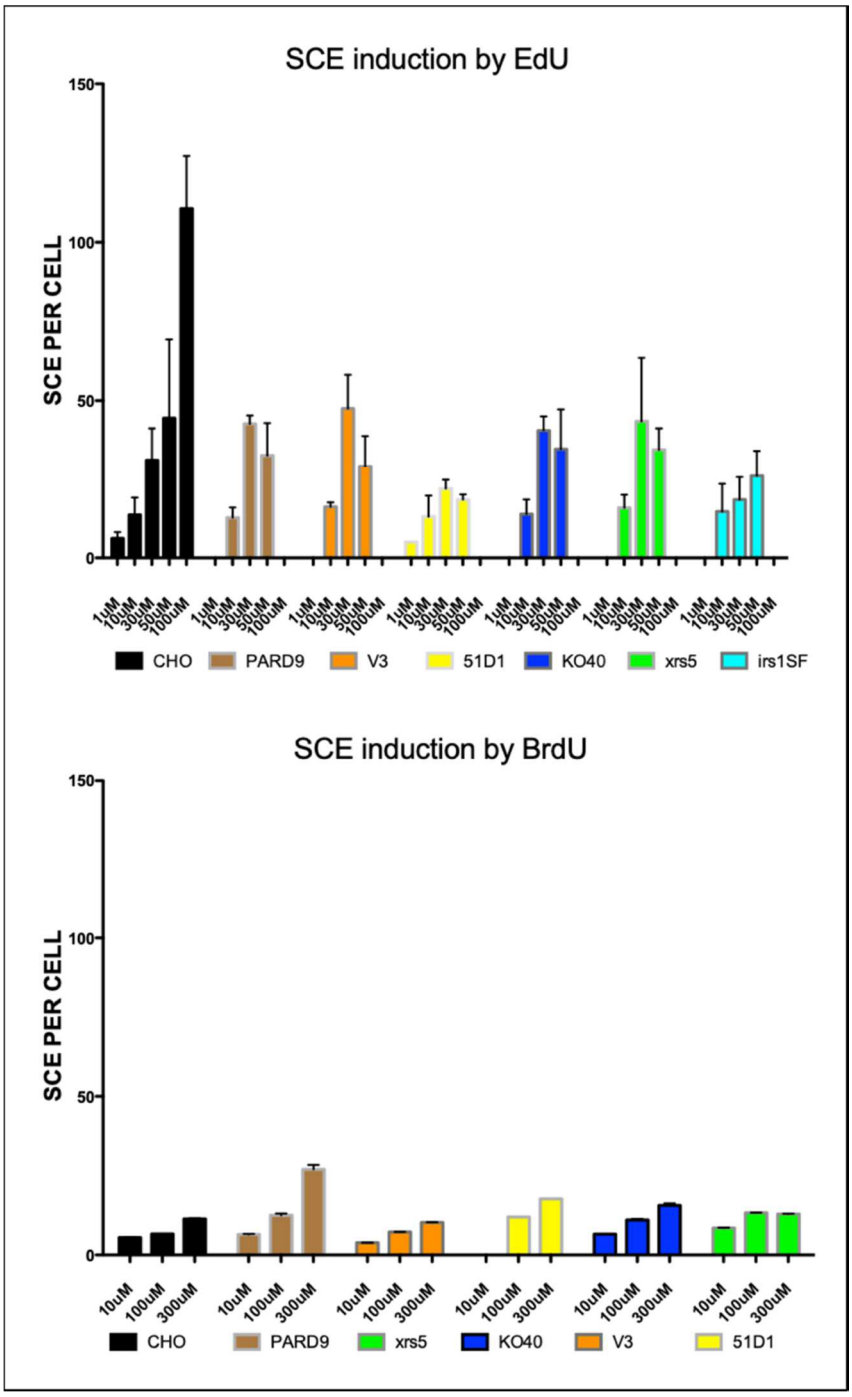


**Figure 25. (Top graph) EdU increases SCE induction at lower concentrations. 10-50 µM EdU increased SCE induction in all CHO/CHO mutants. No SCE presence at 100 µM EdU. (Lower graph) BrdU, in respect to CHO wildtype, did not induce an increase in SCE.**

## 2.4 Discussion

The primary objective of this study was to discern the effects that synthetic halogenated pyrimidine analogues have on cellular cytotoxicity and genotoxicity. Specifically, 5-ethynyl-2'-deoxyuridine and 5-bromo-2'-deoxyuridine. Moreover, the first portion of this report implements CHO wildtype and various CHO DNA repair mutant cell lines to demarcate the significance of DNA repair pathways in regard to nucleotide substitution. It is commonly agreed upon in the scientific realm that BrdU induces cellular genotoxicity, and sensitizes cells to ionizing radiation, UV-radiation, and fluorescent light (14, 20, 135). Though, other halogenated pyrimidines exhibit elevated levels of radiosensitization. While other studies have proven BrdU and EdU to be carcinogenic, cytotoxic, and genotoxic, this investigation reports similar results, but significantly, purposeful findings.

The proposed mechanism of EdU-induced disruption of cellular replication suggests that EdU directly consequences genomic instability. However, this may be a misleading virtue and particularly untrue. EdU alone, does not conclude genomic instability nor radiosensitization. A cascade effect, that when EdU is substituted as a thymidine analogue into DNA, this results in a replication fork stall or collapse. Collapse of a replication fork is known to be detrimental toward cellular replication and endpoint-viability. We report that when homologous recombination repair pathway is unable to resolve DNA DSB constituents, such as CHO DNA repair deficient 51D1 and IRS1SF (Rad51d paralog mutants) cells, incorrect DNA reparations can lead to aberrant chromosomal formations, sister chromatid exchanges (fig. 25), and contorted structures, aiming a cell toward cell death. On the contrary, cells with an active and properly functioning homologous recombination repair pathway are keen to sensing and mitigating the genotoxic/cytotoxic-inducing DNA DSBs. Thus, resolution of DNA DSBs. Though, methods to

distinguish morphological and kinetic rates of replication fork progression have formatted such as sister chromatid exchange (SCE) analysis. Specifically, a high increase in SCE frequency is correlated with replication fork collapse. EdU is an antimetabolite precursor in DNA nucleotide salvage pathway, that disrupts DNA integrity and function. Moreover, after continuous labeling of 1 μM, 10 μM, 20 μM EdU, necrotic cell death increased by 22.4, 46.3, and 84.7%, respectively. For comparison and relation to induction of necrotic cell death, after 1-10 μM pulse of EdU for 24 hours followed by 120-hour chase resulted in high SCE index (6). These concentrations are comparative to this report. In that, our data conclusively reports that 10-50 μM EdU promotes an increase of SCE frequency encompassing all CHO DNA repair deficient mutants, even CHO wildtype. In fact, CHO wildtype depicted a dose-dependent linear increase of SCE induction, 1-100 μM EdU. Thus, we suggest that EdU incorporated into DNA promotes error-prone template synthesis, conferring a quantitative increase in SCE frequency. More so, 100 μM EdU-SCE data insinuates EdU as a main contributor toward IRS-1SF, 51D1, PADR9, KO40, V3, and XRS5 cells ability to form viable colonies (fig. 20). Comparison of EdU-induced increase of SCE frequency to that of BrdU, displays a slight increase in favor of EdU. We suggest that EdU is integrated as DNA template, however, DNA replication machinery is incapable of correctly using EdU as a template, resulting in replication fork collapse. Although BrdU cannot be voided as a means to increased SCE frequency in of itself.

However, our data is similar to that of a previous report (136). To the extent that, if AlphaMEM medium is preferentially applied versus MEM medium (nucleotide/nucleoside deficient), EdU incorporation will result an increased cell viability ($IC_{50}$=75 μM EdU) or decreased cell viability ($IC_{50}$=0.05μM EdU), respectively (fig. 24). Meaning, cells will preferentially uptake EdU as DNA template, ultimately resulting in 1,500-fold decrease in $IC_{50}$

values and cell survival. By no mistake, there is strong correlation promoting the premise that, decrease in cell survival by EdU is buttressed on high SCE-induction frequency. In addition, 10 μM BrdU has markedly proven to be clastogenic, affecting subcellular regulation pathways such as DNA replication, metabolism, apoptosis, and cell death. Therefore, protocols that necessitate thymidine analogues near or at manufacturers suggested concentration, should be queried at the consumers discretion. In order to prevent undesirable biased results or "failure" of experiment.

Commonly, EdU has been conducted as a simple, reproducible, and effective means to analyze DNA division. Moreover, EdU can also improve detection of unscheduled DNA synthesis (UDS), which is an assay performed to measure a population of cells propensity in global-NER. Moreover, a human virus known as hepatitis B virus (HBV) utilizes an intrinsic hepatitis B viral protein (Hbx) to downregulate UDS by 25% (137). Thus, acquiring the ability to surpass vital cellular checkpoints and survive. Additionally, Human Herpesvirus-1 and Human Herpesvirus-2 (HSV-1&2) utilize viral thymidine kinase enzyme (V-TK) to propagate pathogenicity in humans. V-TK is non-preferential towards nucleoside incorporation, such as 5-substituted 2'-deoxyuridines, whereas human TK is highly specific. Data suggests that incorporation of 5-substituted 2'-deoxyuridines inhibits the proliferative capacity of HSV-1, by deregulating and/or inhibiting V-TK; administering 5-substituted 2'-deoxyuridines to HSV-1&2 cells exhibits $IC_{50}$ values ranging from 0.3-51 μM (138). Also, it was established that approximately 9.8 μM 5-substituted 2'-deoxyuridine was required for viral inactivation. Originally, EdU was used as an agent for viral inactivation. Significantly, 0.3-51 μM is extremely similar to concentrations recommended by manufacturer (10 μM EdU).

Not only is EdU significantly related to the induction of SCE, BrdU is as well. High concentrations of BrdU is known to induce global-genomic scale cytotoxicity. Furthermore, it

has been demonstrated that certain media supplements can induce SCE in cultured cells in vitro, and an abnormal SCE frequency with supplemented 0.24 μM BrdU (136). SCEs visualized by fluorescence plus Giemsa, differential staining method with BrdU increases SCE in an increasing dose-dependent manner. Before BrdU differential staining method was available, tritiated thymidine ($^3$H -TdR) was used as a means to visualize SCE through autoradiography. However, $^3$H -TdR administered during first cell-cycle, resulted in an increase in SCE frequency. And, even more so, when incorporated during the second cell-cycle. However, interestingly, BrdU adjuvant administration with $^3$H -TdR, resulting in a decrease in SCE induction. Suggesting that, $^3$H -TdR competes with an additional halogenated pyrimidine, when excess nuclear "pools" of $^3$H -TdR is present (3). It is pertinent, that when precisely a halogenated pyrimidine is implemented, in respect to cell-period, and concentrations of said halogenated pyrimidine, that correct measures are used.

We have demonstrated that EdU and BrdU conclusively induce an elevated frequency of HPRT-mutation (fig. 19) compared to CHO wildtype-unlabeled cells. Other studies have demonstrated likewise, however, it is critical upon distribution of cell-cycle and temporal distribution of said halogenated pyrimidines (4). Specifically, two hours after initiation of DNA synthesis (S-phase). And, that mutation frequency is protein-level-dependent and enzyme-dependent (4). Therefore, BrdU-induced mutations are associated with mutations in genes or proteins that are necessary for the production of HPRT gene and $Na^+$ $K^+$ ATPase enzyme (4). Also, decrease in plating efficiency is observed in cells that are administered halogenated pyrimidines during S-phase. Reports have suggested and displayed concrete evidence pertaining to BrdU-induced HPRT-mutations. While others are temporal and gene-specific, ours gyrated around cells synchronized in $G_1$-phase. Though discrepancies between our methods and others

have been acknowledged, it is transparent that BrdU induces significant HPRT-mutations. More importantly, our data, novel it may be, leads to a conclusion that EdU results in an accumulation of HPRT-mutations, even at concentrations (1 μM )10-fold less than manufacturer suggested levels.

Evidently, BrdU and EdU are considerable genotoxic and cytotoxic agents. In regard to PARP inhibition, our data suggests that EdU is not a cytotoxic factor (fig. 23). Though various analogues of deoxythymidine and deoxyuridine have been demonstrated as potent inhibitors (3-aminobenzamide) of PARP enzyme. Of which, are potent antiviral/cancer compounds (125). To our knowledge, EdU has not been entirely established as means to inhibit PARP-1 activity. Our data suggests, that EdU (unmodified) is not an intrinsic potentiated inhibitor of PARP production. Others have claimed that the 2'-, 3'-, and 5'-position of the substituted deoxyuridine moiety significantly effects the metabolic activity. Which is reasonable to suggest, given knowledge that steric location of a substituent greatly affects the compatibility of enzymes and co-factors with other cellular substituents. ADP-ribosylation of proteins has implicated its importance as role in cellular metabolism, such as DNA repair. Inhibition of PARP activity in diseases such as AIDS, hepatitis B, and HPV-1 may lead to potential 'cures'. However, the concentrations and $K_i$ constants of 5-substituted deoxyuridines are too high for potential use within the medical realm. Further experimentation and synthetization of thymidine derivatives, may, in fact, result in novel-therapeutic approaches, across different disciplines of medicine.

This portion of experiments imply importance and influence of halogenated pyrimidines on overall cell viability. Moreover, CHO DNA repair deficient cells are respectively sensitive to EdU and BrdU in regard to PARP production, SCE frequency, clonogenicity, HPRT-mutation induction, doubling time, and medium specificity.

CHAPTER THREE

# EVAULATING RADIOSENSITIZING EFFECTS OF EDU AND BRDUU ON A549 AND CHO WILDTYPE CELLS

## 3.1. Introduction

Molecular revelations transcending several aspects of cellular biology permitted the institution of labeling actively dividing cells by way of radioactive tritiated thymidine ($^{3}$H-TdR) incorporation. However, $^{3}$H-TdR labeling was known to be cumbersome, carcinogenic, and, unsurprisingly, radioactive. All of which, deemed the method unsafe, inefficient, and dispensable. While $^{3}$H-TdR labeling is archaic, though still engendering an effective labeling procedure, the technique is seldomly applied in current biological practices. Therein lies 5-bromo-2'-deoxyuridine (BrdU) as an effective and efficient method to reliably label actively dividing cells-though it seems.

Seemingly, resolute analysis of the facets of BrdU provokes many of its actions and consequences underlying cellular function, however, one in particular requests attention: radiosensitization. Importantly, radiosensitization has remained hallmark in cancer radiotherapy, yet the physics, biology, and chemistry delineating radiotherapy are convoluted, to say the least. The latter provides an understated, and all-too-often, misrepresented view of radiotherapy. The simplest and most trivial explanation of radiotherapy can be illustrated as the delivery of powerful and energetic radiation-external or internal-to defective or cancerous cells that reside in an organism, in hopes of disrupting cancer cell kinetics and division thus, diminishing tumorigenic areas. The most frequented type of radiation recalled during radiotherapy is known as ionizing radiation-explained in previous passages. Although radiotherapy is extremely effective in the treatment of tumors and diseases, implicit stalemates are encountered and must

be apportioned accordingly such as, hypoxic tumorigenic microenvironments (lack of oxygen) and tumor cell repopulation, redistribution, and repair. All of which, incur potential complications in regard to cancer cell susceptibility. Primary objectives of cancer therapy are to implicate the least invasive means of treatment. However, more-often-than-not, this is not the case; due to the inherent aggressive and paradoxical nature of cancer cells. Therefore, adjuvant or additional therapeutics necessitate the process of cancer radiotherapy. Oncologists, researchers, and physicians alike, aim to reduce cancer cells to vulnerability, ultimately enhancing the radiosensitivity of said cells. By way of sensitizing cells, simply provoking higher susceptibility toward cell death, cancer eradication may be achieved.

Achieving purposeful radiosensitivity may be accomplished through several methods; in particular, administration of conventional pyrimidine analogues. Such as, but not limited to, cisplatin, 5-fluorouracil, or Gemcitabine. Pyrimidine analogues retain distinguishing features such as structure and activity, all accompany dysregulation of cellular metabolism, communication, and functionality. Consequently, and hopefully, lowering the apoptotic threshold of devious cancer cells. Likewise, halogenated pyrimidines such as BrdU, IdU, and CldU, have been noteworthy in radiotherapy, more specifically, inducing radiosensitization. Specifically, BrdU was of the first established courses of labeling actively dividing cells. Not until further examination of the potential facets of the chemical composition of BrdU, did the beneficial chemotherapeutic effects become apparent. Administration of BrdU alone, has resulted in decrease cell survival on many occasions. Moreover, 5 μM BrdU (concentrations likened to this report) has resulted in approximately 50% reduction of cell survival compared to control (21). Additionally, CHO cells treated with concentrations >50 μM of BrdU for 24 hours displayed specific inhibitory effect. Even more so, after 100 μM BrdU administration, cellular

kinetics were greatly inhibited (128). Though, treatment of >50 μM BrdU for 1 to 2 hours did not display noticeable inhibitory effects during first cell-cycle. Effectively, CHO cells exposed to 50, 100, and 500 μM BrdU demonstrated a prominent delay in progression to the next cell-cycle, with nearly all cells arrested in at the 3$^{rd}$ cell-cycle (<50 μM BrdU). Investigation of the mechanisms involved in BrdU-induced cell survival reduction indicate that replacement of thymidine by BrdU is partially responsible for cellular alterations. As previously established, cells are most sensitive to modifying agents when exposed during S-and-$G_2$-phase; exactly when BrdU can be administered to cells. Genuinely, BrdU beholds an extensive history in remedying specific skin, dermal, and subdermal diseases. Such as, suppression of gliomas (19), psoriasis, melanomas, and various hyperproliferative skin disorders. Though the precise mechanism by which halogenated pyrimidines exert their cytotoxic effects remains undisclosed, and still to be queried.

Largely, it is evident that BrdU, in of itself, is sufficient in introducing cellular lethality. It is therefore imminent and probable of a synergistic cell-killing effect of BrdU and ionizing radiation. Classically, as mentioned, radiation had been delivered singly. Increasing evidence suggests that, radiotherapy alone, is not as efficacious as administering a concomitant chemotherapeutic, such as BrdU. Exponentially growing XRS-5 and CHO cells exposed to 10 μM BrdU and 2-Gy gamma-irradiation resulted in a significant decrease in cell viability (100-fold decrease) (21). Likewise, ultraviolet radiation (UVR) produces photoproducts, DNA-protein/protein-protein crosslinks, DSBs, and nucleobase lesions in DNA, rendering potential mutagenic areas. UVR fitted phototherapeutic properties in treating and targeting particular hyperproliferative skin disorders (2). Utilizing a TUNEL assay (detection of apoptotic propensity), 2.5 μg/ml BrdU and 10 J/m$^2$ UVR combination resulted in ~59% TUNEL-positive

cells. Whereas, control, UVR, and BrdU exposure resulted in 3.9, 9.1, and 29.4% TUNEL-positive cells (14). In addition, fluorescent light (FL)-irradiation provides an additional external modality in the treatment of skin diseases. Following exposure to FL-irradiation, V79-753B-3M fibroblast cells ensued a dramatic ~30% reduction in plating efficiency (5). Supporting, 10 μM BrdU followed by 21 minutes FL-irradiation, incurred a $D_{10}$ equating to 5.7 minutes, while 1.0 x $10^{-5}$ μM BrdU under the same exposure conditions resulted in a $D_{10}$ approximately equal to 3.4 minutes. Under the same conditions, 10 mM cysteamine (supposed radioprotectant) and 1.0x $10^{-5}$ μM BrdU were applied to cells, interestingly restoring the cell survival curve (5). The latter demonstrates that, in the presence of BrdU, it proves to radiosensitize cells, but, concomitant cysteamine application, cell survival is presently normal. However, due to the indiscriminate nature of radiation, cells juxtaposed to the area of interest are subject to supposed therapeutic radiative energy. Contrary, concomitant administration of chemotherapeutic agent such as BrdU, to effected area of interest, may reinstate a confined, focalized cell-killing.

Clearly, BrdU possesses definitive molecular roles, and has the ability to implicate critical cellular amendments. Beneficial or deleterious, they may be. Remit it would be to discount that, BrdU detection requires the proposition of an antibody. The latter consequences partial denaturation of the DNA structure through harsh, volatile chemicals, and several hours of incubation. Until relatively recently, 5-ethynyl-2'-deoxyuridine (EdU), as an alternative, was largely introduced as a molecular workhorse. EdU detection is remarkably specific, categorized, accurate, safe, and reliable, when labeling actively dividing cells. Though EdU was originally employed as a means to treat viruses, tumors, and to identify bacteriophage (14,15).

Distinctions do present themselves when speculating BrdU and EdU. Necessary it is to address the potential, if any, adverse effects that halogenated pyrimidines have on cellular

integrity, function, and human wellbeing. The “click chemistry” approach utilizing EdU as a DNA precursor was introduced as a means to assess DNA replication. It was observed that, EdU incorporation into DNA, initiated a DNA damaging signaling (DDS) seen through phosphorylation of ATM, histone H2AX, P53, and Chk2 (130). Recently, pulse-labeling cells, throughout S-phase, with EdU concentrations recommended by suppliers did not affect S-phase structure or the rate of fork progression. However, continuous-labeling cells with EdU, resulted in clear disruption of the cell-cycle, with cells accumulating in $G_2$/M-phase. Correlating cell-cycle progression and cell number, a dramatic decrease in cell population in respect to cell-cycle retardation, with inhibition of proliferative capacity resulting in apoptosis (131).

Although, this report clearly purports that BrdU and EdU retain mutagenic and cytotoxic properties, the implications of such, in presence of external radiation modalities have remained rather misunderstood. In this section, we aim to identify and fill in the voids pertaining to EdU and its function as a radiosensitizer. More importantly, the involvement of EdU in regard to cell viability, in the presence of supplemental external radiation modalities. Knowingly, BrdU has been established elsewhere as a potent radiosensitizer. Here we extend credit, that BrdU is an honorable radiosensitizer, but illuminate EdU as a potential radiosensitizer in CHO wildtype and human small cell lung adenocarcinoma cells (A549). By way of external radiation modalities, such as UV, gamma-irradiation, and fluorescent radiation, we were better able to establish and delineate a more profound causative effect of EdU on cell-cycle integrity and maintenance. The ramifications of EdU on cell-cycle dynamics, cytotoxicity, and mutagenicity is clearly illustrated throughout this extended study. However, its implicated profound radiosensitization effects will be further discerned.

## 3.2 Materials and Methods

### *3.2.1. Radiation conditions*

**UVC-irradiations (UVC-spectrum-fig. 12)** were conducted at Colorado State University UV radiation facility (MRB, Fort Collins, CO) using a Phillips germicidal UVC lamp with a dose rate of 1.1 J/$m^2$/sec and a wavelength approximately 254 nm at approximately 0.2 m point-source distance in room temperature (20˚C), with a rotating pedestal. **Gamma-irradiation** was performed at Colorado State University with a J.L. Shepherd Model Mark I-68 nominal 6000 $Ci^{137}$ Cs irradiator (J.L. Shepherd and Associates, San Fernando, CA, USA) at room temperature (20˚C), with a dose rate of ~2.5 Gy/min. **Fluorescent-irradiations** were conducted at Colorado State University fluorescent radiation facility (Dr. Kato Laboratory, CSU, Fort Collins, CO) using an Airclean 600 PCR workstation (ensuring sterility) with an auxiliary 5-1/4" Coolwhite bulb (15 Watt, Model F15T8 4100K T-8 G-13 base (flux rate=3.8 x $10^{-5}$ J/sec/$cm^2$) , CW Eiko (Shawnee, KS, USA)) at approximately 6" point-source distance at room temperature (20˚C) (CoolWhite/fluorescent light wavelength spectrum fig. 13).

### *3.2.2. Cell Cultures*

**Chinese hamster ovary (CHO-10B2) (wild-type)** and **human lung small cell adenocarcinoma (A 549)** cells, were kindly supplied by Dr. Joel Bedford (Colorado State University, Fort Collins, CO) and Dr. Larry Thompson at the Lawrence Livermore National Laboratory (Livermore, CA, USA). Cells were cultured in MEM-alpha (Gibco, Indianapolis, IN) supplemented with 10% heat inactivated fetal bovine serum (FBS, Sigma, St. Louis, MO) and 1% antibiotics and antimyotics (Gibco, Indianapolis, IN), and they were maintained in a 37˚C humidified incubator with a supplemented ambient 5% $CO_2$ administration. Sub-cultured cells

were seeded in a T25 flask at 2.0-5.0 x $10^5$ cells/flask, and incubated for approximately 1-2 days prior to start of experiment to ensure cell confluency and attachment.

### *3.2.3. Applied Chemicals*

5-bromo-2'-deoxyuridine (BrdU) was purchased from Sigma (St. Lous, MO) and stock solution was prepared in 100% DMSO as 1 mM. 5-ethynyl-2'-deoxyuridine was purchased from Invitrogen (Waltham, MA) and stock solution was prepared in 100% DMSO as 10 mM.

### *3.2.4. UV-irradiation procedure for clonogenic cell survival assay In vitro*

Prior to trypsinization, 3-T25 culture flasks were constructed and labelled accordingly: 1) "BrdU" 2) "EdU" and 3) "control". Cells were seeded in accord to section 2.2.2. Once cells were ensured confluency, cultured cells in a T25 flask were detached via 0.5% trypsinizing solution containing 1mM EDTA and maintained in a 37˚C humidified incubator with a supplemented ambient 5% $CO_2$ administration for approximately 5 minutes ensuring cell detachment. Cells were then re-suspended in ~25 mL fresh growth medium containing α-MEM with 10% FBS and pipetted thoroughly to ensure homogeneous cell mixture. Once cells were re-suspended, P30 dishes were prepared, prepped with 2 mL fresh α-MEM, and labelled accordingly: 4 x EdU-0, 5, 10, 15 seconds, 4 x BrdU-0, 5, 10, 15 seconds, and 4 x control-0, 5, 10, 15 seconds. Cells from original appropriately designated T25 flasks were then aliquoted proportionally to each labelled P30 dishes. Then administration of parallel drugs of interest was carried out: 2 µL EdU-10mM (final [10 µM]), 20 µL BrdU-10mM (final [10 µM]), and control-no drug. Cells were then incubated in a 37˚C humidified incubator with supplemental ambient 5% $CO_2$ for approximately 24 hours to ensure complete incorporation of nucleoside(s) into cellular DNA. Then P30-containing cell dishes were aspirated and washed with phosphate-buffered solution (PBS), leaving 1 mL PBS in dish. Then cells were transported to the UV-irradiation facility to carry out

necessary UV-exposure. Cell dish lids were removed (in room) prior to UV-irradiation. Then, each exposure (0, 5, 10, 15 seconds) was carried out. Cell dishes were then transported back to cell culture hood for necessary extraction and seeding into P60 cell culture dishes. Prior to seeding, each individual P30 dish cell concentration was counted by a particle analyzer (Coulter Z1). Then, 5000, 5000, 500, and 300 cells were extracted and seeded for 5, 10, 15, and 0 seconds, respectively. Samples were then incubated in 37˚C humidified incubator with supplemental ambient 5% $CO_2$ for approximately 10 days, and then cells were rinsed with PBS, fixed with 10% formalin solution for 10 min, washed with tap water, stained with 1% methylene blue solution and air dried. Colonies comprising >50 cells were counted as viable cells. Each UV-irradiation experiment was carried out a minimum of 3 times, to ensure reproducibility. Colonies were then counted, collected, and a clonogenic survival curves were calculated and obtained from GraphPad Prism 5 software.

### *3.2.5. Gamma-irradiation procedure for clonogenic cell survival assay in vitro*

The gamma-irradiation procedure was nearly identical to section 2.2.3., with appropriate substitutions. As follows: 3 x EdU-0, 4, and 8 Gy, 3 x BrdU-0, 4, and 8 Gy, and 3 x control-0, 4, and 8 Gy. P60 cell culture dishes were prepared. Cells from original appropriately designated T25 flasks were then aliquoted proportionally to each labelled P60 dish. Then administration of parallel drugs of interest was carried out: 2 μL EdU-10mM (final [10 μM]), 20 μL BrdU-10mM (final [10 μM]), and control-no drug. Cells were then incubated in a 37˚C humidified incubator with supplemental ambient 5% $CO_2$ for approximately 24 hours to ensure complete incorporation of nucleoside(s) into cellular DNA. Then cells were transported to the gamma-irradiation facility (Colorado State University, Fort Collins, CO, Molecular & Radiobiological Building). Then, subsequent gamma-exposure (0, 4, and 8 Gy) was carried out. Cell dishes were then transported

back to cell culture hood for necessary extraction and seeding into new P60 cell culture dishes. Prior to seeding, each individual P60 dish cell concentration was counted by a particle analyzer (Coulter Z1). Then, 500, 500, and 5000 cells were extracted and seeded for 0, 4, and 8 Gy, respectively. Samples were then incubated in 37˚C humidified incubator with supplemental ambient 5% $CO_2$ for approximately 10 days, and then cells were rinsed with PBS, fixed with 10% formalin solution for 10 min, washed with tap water, stained with 1% methylene blue solution and air dried. Colonies comprising >50 cells were counted as viable cells. Each UV-irradiation experiment was carried out a minimum of 3 times, to ensure reproducibility. Colonies were then counted, collected, and a clonogenic survival curves were calculated and obtained from GraphPad Prism 5 software.

### *3.2.6. Fluorescent-irradiation procedure for clonogenic cell survival assay In vitro*

The fluorescent-irradiation procedure was nearly identical to section 2.2.3., with appropriate substitutions. As follows: 4 x EdU-0, 1, 2, and 3 hours, 4 x BrdU-0, 1, 2, and 3 hours, and 4 x control-0, 1, 2, and 3 hours. P60 cell culture dishes were prepared. Cells from original appropriately designated T25 flasks were then aliquoted proportionally to each labelled P60 dish. Then administration of parallel drugs of interest was carried out: 2 µL EdU-10mM (final [10 µM]), 20 µL BrdU-10mM (final [10 µM]), and control-no drug. Cells were then incubated in a 37˚C humidified incubator with supplemental ambient 5% $CO_2$ for approximately 24 hours to ensure complete incorporation of nucleoside(s) into cellular DNA. Then cells were transported to the fluorescent-irradiation facility. Then, subsequent fluorescent-exposure (0, 1, 2, and 3 hours) was carried out. Cell dishes were then transported back to cell culture hood for necessary extraction and seeding into new P60 cell culture dishes. Prior to seeding, each individual P60 dish cell concentration was counted by a particle analyzer (Coulter Z1). Then, 300 cells were

seeded for each respective exposure. Samples were then incubated in 37˚C humidified incubator with supplemental ambient 5% $CO_2$ for approximately 10 days, and then cells were rinsed with PBS, fixed with 10% formalin solution for 10 min, washed with tap water, stained with 1% methylene blue solution and air dried. Colonies comprising >50 cells were counted as viable cells. Each UV-irradiation experiment was carried out a minimum of 3 times, to ensure reproducibility. Colonies were then counted, collected, and a clonogenic survival curves were calculated and obtained from GraphPad Prism 5 software.

### *3.2.7. Interpretation of data and data analysis*

All data obtained from experimental procedures were analyzed and processed through GraphPad Prism 5™ (Graphpad, La Jolla, CA). Statistical comparison of mean values was performed using a t-test or a two-way or one-way analysis of variance (ANOVA) when comparing groups. Differences with a p-value <0.05 were considered to indicate a statistical significance. Error bars indicate standard error of means. Dunnett's Multiple Comparison Test was used to calculate statistical differences between control and EdU or BrdU administration.

## 3.3 Results

### *3.3.1. BrdU UV, ionizing, and fluorescent radiation sensitization effects*

CHO-10B2 wildtype cells were administered 10 μM BrdU or EdU for one cell-cycle, to ensure nucleoside incorporation. Further, cells were irradiated in $G_1$ phase. Colony formation assay was then carried out in accordance to previously established methods. Data from colony exposure reported that, while BrdU produced an increase in radiosensitivity to UV-, gamma-, and photon radiation, EdU did not (fig. 26). Enhanced fluorescent radiosensitization through EdU exposure was not observed. The $D_{10}$ value, the dose required to produce 10% survival, and the $D_{37}$ value, the dose required to produce 37% survival, were calculated to compare the degree

of radiosensitization by adjuvant EdU or BrdU exposure. $D_{10}$ and $D_{37}$ values were used as parameter for biological equivalent doses. Data reports that 10 μM BrdU sensitizes cells to UV-C with a $D_{10}$ value of 12.044 J/m$^2$ (control) to 4.016 J/m$^2$, 2.99 sensitization ($P<0.05$). While, 10 μM EdU-UV-exposure increased $D_{10}$ values from 12.044 J/m$^2$ (unlabeled) to 14.311 J/m$^2$, 0.842 sensitization ($P>0.05$). Thus, EdU-induced sensitization was not observed. And, 10 μM BrdU sensitizes cells to gamma-irradiation with a $D_{10}$ value of 6.920 Gy (unlabeled) to 5.337 Gy, 1.2977 sensitization ($P<0.05$). Finally, 10 μM EdU-gamma-irradiation increased $D_{10}$ from 6.920 Gy (unlabeled) to 7.971 Gy, 0.868 desensitization. The $D_{37}$ values followed the same trend as $D_{10}$ values for gamma-irradiation; 4.095, 2.714, and 5.013 Gy, unlabeled-control, BrdU, and EdU, respectively. Likewise, $D_{37}$ values followed the same trend as $D_{10}$ values for UV-irradiation; 5.200, 1.734, and 6.180 J/m$^2$, unlabeled, BrdU, and EdU, respectively. Differences in $D_{10}$ values were determined to be statistically significant ($P<0.05$). Also, differences in $D_{37}$ values were determined to be statistically significant ($P<0.05$). Therefore, our data suggests that EdU incorporation does not sensitize cells to UV or gamma-irradiation. Summarization of $D_{10}$ and $D_{37}$ values, in regard to their relationship of the effects of incorporated nucleoside when exposed to dissimilar radiation qualities are shown in table 3. Dunnett's Multiple Comparison Test was used to calculate statistical differences between control and EdU or BrdU administration.

**Table 3. Effects of dissimilar radiations and EdU and BrdU on CHO cell survival**

| Relationship between incorporated nucleosides and $D_{10}$ and $D_{37}$ values for dissimilar radiation types (CHO) | | | | |
|---|---|---|---|---|
| Radiation type | $D_{10,37}$ | BrdU | EdU | Control$_{(unlabeled)}$ |
| Photon$_{(fluorescent)}$ ($3.8 \times 10^{-5}$ J/sec/cm$^2$) | $D_{10}$ | 120.104 min | >300 min | >300 min |
| | $D_{37}$ | 61.564 min | 256.660 min | >300 min |
| Gamma (2.5 Gy/min) | $D_{10}$ | 5.337 Gy | 7.971 Gy | 6.920 Gy |
| | $D_{37}$ | 2.714 Gy | 5.013 Gy | 4.095 Gy |
| UV-C (1.1 J/m$^2$/sec) | $D_{10}$ | 4.016 J/m$^2$ | 14.311 J/m$^2$ | 12.044 J/m$^2$ |
| | $D_{37}$ | 1.734 J/m$^2$ | 6.1800 J/m$^2$ | 5.2004 J/m$^2$ |

$D_{10}$ and $D_{37}$ values were calculated from GraphPad Prism 5. Experiments were carried out at least three times to obtain data

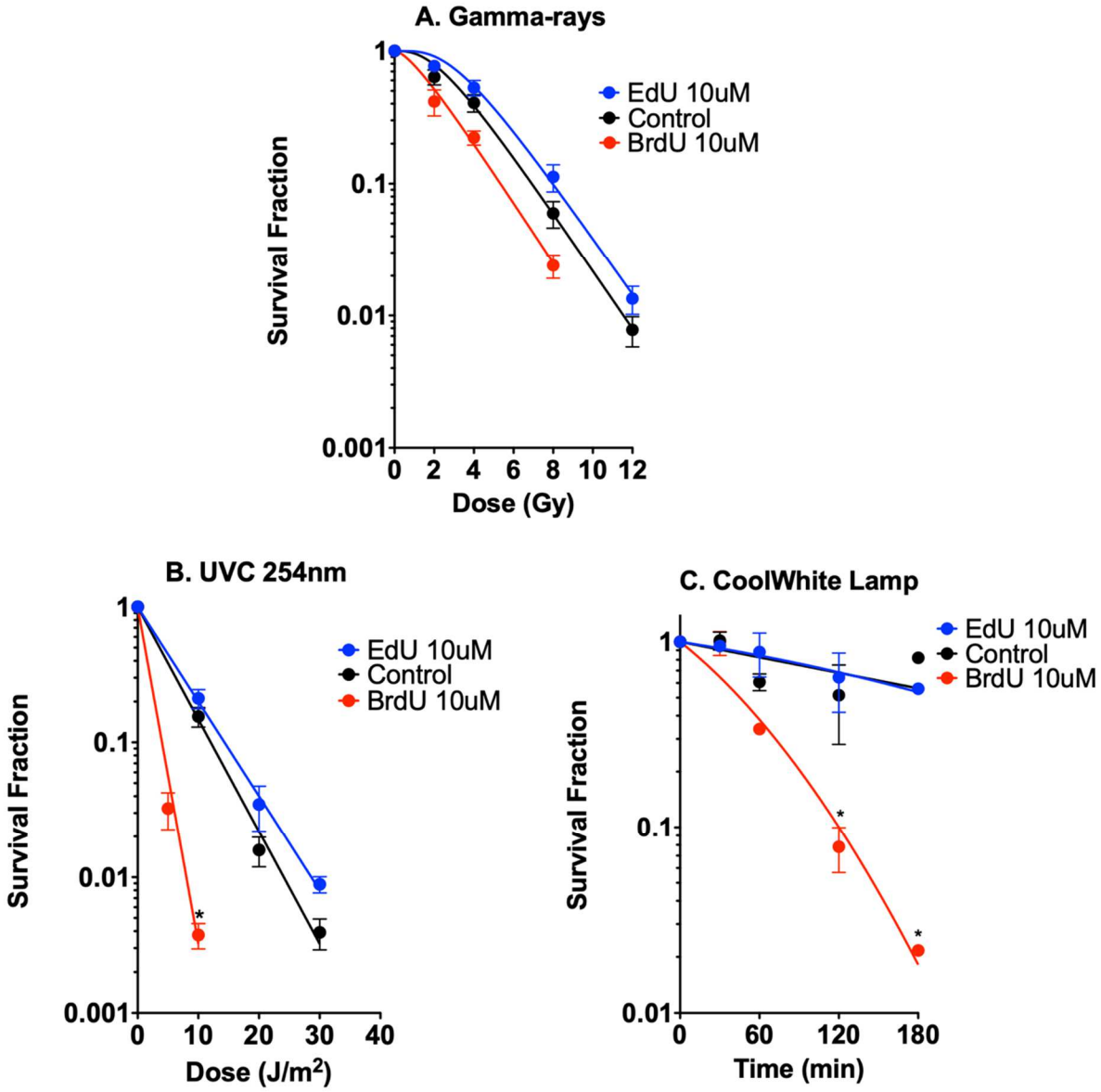


**Figure 26. BrdU radiosensitizes CHO wildtype cells to UV-, gamma-, and photon-irradiation. Cells exposed to (A) gamma-irradiation, (B) UV-C, and (C) fluorescent radiation were greatly radiosensitized in the presence of 10 µM BrdU. EdU administration did not result in radiosensitization. Error bars indicate standard error of the mean of three independent experiments. (*) Statistically significant differences from control, with a P value <0.05, are denoted.**

### *3.3.2 BrdU radiosensitizes A549 cells to gamma, UV, and photon irradiation*

A549 cells were administered 10 µM BrdU or EdU for one cell-cycle, to ensure full incorporation of nucleoside. Further, cells were synchronized in $G_1$-phase and irradiated. Cell

viability assay was then performed in accordance to previously established methods. Our data reports that BrdU did radiosensitize A549 cells to gamma-, UV-, and photon irradiation (fig. 27). However, EdU did present a slight degree of radiosensitization to 4 Gy gamma-irradiation, but no discernable radiosensitization at 10% survival compared to control. And, slight ($P>0.05$) radiosensitization to 4 and 10 J/m$^2$ UV-irradiation. Though, no apparent synergistic radiosensitization with photon-irradiation. Again, $D_{10}$ and $D_{37}$ values were calculated in order to compare magnitude of radiosensitization, and as a means to standardize a parameter for biological equivalent dose. We determined that BrdU decreased the $D_{10}$ value from 8.103 Gy (unlabeled) to 6.794 Gy (unifilar-BrdU) (1.1193 sensitization), during gamma-irradiation. Also, under the same conditions, EdU slightly enhanced $D_{10}$ values from 8.103 Gy (unlabeled) to 8.128 Gy (unifilar-EdU), 0.997 desensitization. A high degree of dichotomy was observed during BrdU-UV-exposure, where $D_{10}$ value started at 15.831 J/m$^2$ (unlabeled) and declined to $D_{10}$=8.156 J/m$^2$ (unifilar-BrdU), 1.941 sensitization. However, under the same conditions, $D_{10}$ values only slightly decreased, from $D_{10}$=15.831 J/m$^2$ (unlabeled) to $D_{10}$=14.076 J/m$^2$ (unifilar-EdU) (1.125 sensitization), for EdU administration. An extrapolation and interpolation of BrdU-photon exposure survival curve depicts, that a photon exposure of approximately 165 minutes is necessary to produce 10% survival for A549 cells. Effectively, prior EdU exposure followed by FL-irradiation, displayed no radiosensitization effect. A further verification of radiosensitization via BrdU administration was established by $D_{37}$ values, which paralleled $D_{10}$ values to some degree. BrdU-gamma, UV, and photon-irradiation $D_{37}$ values were 4.542 Gy, 3.522 J/m$^2$, and 96.057 minutes, respectively. Likewise, EdU-gamma and UV-irradiation $D_{37}$ values mirrored $D_{10}$ values accordingly; gamma- and UV-irradiation $D_{37}$ values were 4.887 Gy and 7.542 J/m$^2$, respectively. Therefore, our data suggests that EdU does not consequence any radiosensitization

effects when administered concomitantly with gamma, UV, or photon-irradiation. On the contrary, BrdU synergizes with gamma, UV, and photon-irradiation to invoke a high degree of radiosensitization. Summarization of $D_{10}$ and $D_{37}$ values, in regard to their relationship of the effects of incorporated nucleoside when exposed to dissimilar radiation qualities are shown in table 4. Dunnett's Multiple Comparison Test was used to calculate statistical differences between control and EdU or BrdU administration.

**Table 4. Effects of dissimilar radiations and EdU and BrdU on A549 cell survival**

| Relationship between incorporated nucleosides and $D_{10}$ and $D_{37}$ values for dissimilar radiation types (A549) | | | | |
|---|---|---|---|---|
| Radiation type | $D_{10,37}$ | BrdU | EdU | Control$_{(unlabeled)}$ |
| Photon$_{(fluorescent)}$ ($3.8\times10^{-5}$ J/sec/cm$^2$) | $D_{10}$ | 164.19 min | >200 min | >200 min |
| | $D_{37}$ | 96.06 min | >200 min | >200 min |
| Gamma (2.5 Gy/min) | $D_{10}$ | 6.974 Gy | 8.128 Gy | 8.103 Gy |
| | $D_{37}$ | 4.542 Gy | 4.887 Gy | 5.565 Gy |
| UV-C (1.1 J/m$^2$/sec) | $D_{10}$ | 8.156 J/m$^2$ | 14.076 J/m$^2$ | 15.831 J/m$^2$ |
| | $D_{37}$ | 3.522 J/m$^2$ | 7.452 J/m$^2$ | 10.403 J/m$^2$ |

$D_{10}$ and $D_{37}$ values were calculated from GraphPad Prism 5. Experiments were carried out at least three times to obtain data

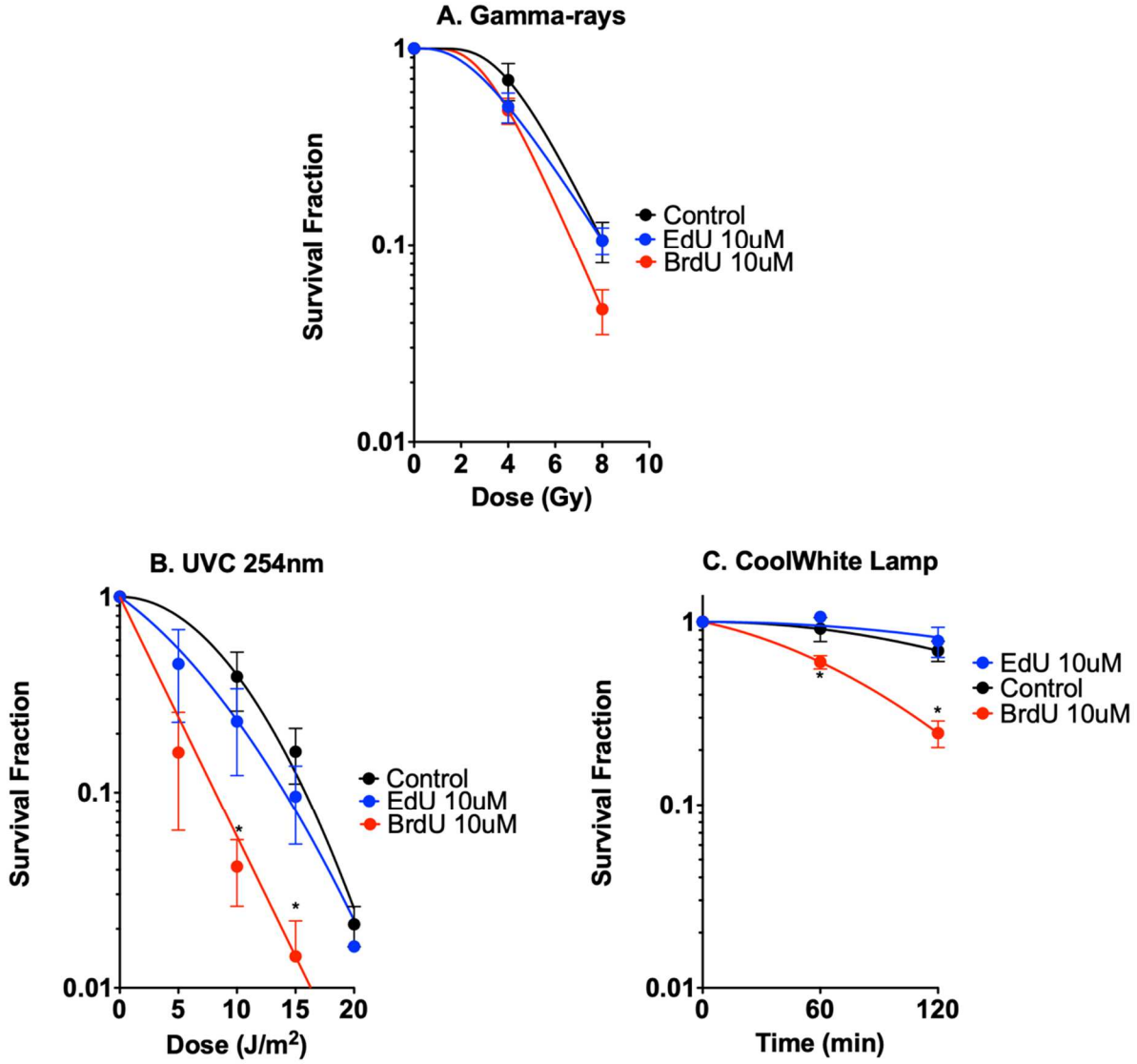


**Figure 27. BrdU radiosensitizes A549 cells to UV-, gamma-, and photon-irradiation. Cells exposed to (A) gamma-irradiation, (B) UV-C-irradiation, and (C) photon-irradiation, were significantly radiosensitized in the presence of 10 µM BrdU. 10 µM EdU did not present any significant radiosensitization effects. Error bars indicate standard error of the mean of three independent experiments. (*) Statistically significant differences from control, with a P value <0.05, are denoted.**

## 3.4 Discussion

This portion of our study aims to discern the effects that synthetic halogenated pyrimidine analogues have on cellular radiosensitization. Specifically, 5-ethynyl-2'-deoxyuridine and 5-bromo-2'-deoxyuridine. In addition, this study utilizes many conventional radiation

modalities such as gamma-, UV-, and photon-irradiation. Moreover, this section uniquely contrasts the first allotment of experiments (section 2), in that it implements CHO wildtype and human small cell lung adenocarcinoma (A549) cells and gamma-, UV-, and photon-irradiation, in order to distinguish nucleotide substitution and its direct effect on radiosensitization. Previous research indicates that BrdU induces cellular genotoxicity, and sensitizes cells to ionizing radiation, UV-radiation, and fluorescent light (14, 20, 135). Though, other halogenated pyrimidines such as, 5'-iododeoxyuridine and 5'-chlorodeoxyuridine, exhibit elevated levels of radiosensitization (139), in the presence of X-irradiation. Halogenated pyrimidines are a select class of radiosensitizers which, in order to introduce their radiosensitizing effects, must be incorporated into cellular DNA. It is hypothesized that radiosensitization may be completely reliant on a cells inherent repair proficiency of potentially lethal damage (PLD), DNA lesions, and DNA DSBs. Through distinctive methods, BrdU has proven to be an effective radiosensitizer (139). Continuing, cells exposed to 10 $J/m^2$ UV-irradiation and BrdU, displayed an enhanced expression of pro-apoptotic genes and cell death (14). In order to investigate the synergistic effects of combining select halogenated pyrimidines and external radiation modalities, we are better to delineate and fashion an array of radiosensitizing effects, if any.

In fact, $^3$H -TdR was of the first methods to monitor cellular division and kinetics. However, $^3$H -TdR is a low-energy β-emitting radioactive compound. Provided insightful knowledge on the effects of radiation *in vivo* and *in vitro*, one can assume that $^3$H -TdR has potential implications on cellular integrity and function. Despite $^3$H -TdRs innate radioactive properties and cumbersome labeling methodology, it has been widely applied throughout the cellular ecosystem. The analyses of modifying agents on structural rearrangements and clonogenicity has been up for debate for quite some time. Since $^3$H -TdR retains a short

traversing capability (<1-2 μM), deleterious effects are observed and confined mostly to nucleus where chromosomes are located. Incremental dosages of 0.125, 0.25, 0.50, and 0.75 μCi/ml of $^{3}$H -TdR was treated to cells. Longer durations and higher [$^{3}$H -TdR] resulted in a higher frequency of chromatid-type breaks and deletions per cell compared to low dosage (0.50, 0.75 μCi/ml; 10 and 38 chromatid breaks/per, respectively) (140). Also, if cells are affected by $^{3}$H -TdR uptake during S-phase, cells are insulted a higher frequency of intrachromosomal exchanges and breaks. Indeed, $^{3}$H -TdR has the propensity to induce structural distortions and rearrangements, partially due to its high specific activity. Specifically. $^{3}$H -TdR has been suggested to induce an abnormal frequency of SCE induction. To the extent that it increases sister chromatid exchanges to nearly 20-30 sites per nucleus (141). Additionally, cells applied a higher dose of 3H-TdR (0.5 μCi/ml), concluded a higher SCE frequency during second cell-cycle (142). Likewise, our results (fig. 24) depict EdU produces a dramatic increase in SCE frequency (10 to 120 SCE per cell). Though, $^{3}$H -TdR is radioactive, while EdU is not, both have the propensity to induce abnormal SCE frequency.

On-the-other-hand, EdU is advantageous compared to BrdU, in that it is considerably less cytotoxic in the presence of UV-irradiation. Phototherapeutically, BrdU is administered topically to treat particular skin disorders. Rightfully so, it is rational to suggest that BrdU, once incorporated into DNA, promotes pyrimidine dimer and cyclo-pyrimidine photoproduct formation. Both of which, are lethal DNA lesions. Insofar that it is mutagenic. In conjunction with UV-irradiation, BrdU enhanced radiosensitivity 2 to 3-fold ($D_{10}$=8.156 and 4.016 J/m$^{2}$, respectively) compared to EdU ($D_{10}$=14.056 and 14.311 J/m$^{2}$, respectively) in A549 and CHO wildtype cells, respectively. Although, CHO and A549 cells have different repair kinetics and viability, there is suggested susceptibility of BrdU to promote radiosensitization with UV-

exposure. Even in the absence of UV-irradiation, BrdU selectively decreased the expression of anti-apoptotic protein, bcl-2. Synergistic effects of BrdU and UV-irradiation, resulted in a respectable decrease in *bcl-2*, while notably increasing expression of pro-apoptotic protein, *Bax* (known to predict propensity to undergo cell death) (14). Not entirely established is EdUs facility to invoke UV-radiosensitization. Although there is finite evidence (nearly none) sponsoring EdU-radiosensitization, we conclude that EdU does not appear to produce any notable radiosensitization in cells in the event of UV-irradiation (fig. 26 & 27).

A direct narrative of BrdU and its facilitation of UV-radiosensitization has been endorsed. Albeit, the biological impact of gamma-irradiation modalities has been credited, the pharmacology of thymidine analogues has been established (143). Their uptake and metabolism is entirely dependent upon thymidine salvage pathway. From this salvage pathway, DNA analogues can be used as a template for further integration into DNA. Incorporation, seemingly is a prerequisite for radiosensitization by both UV and ionizing radiation in tumor cells as well as normal cells (14), and the extent of radiosensitization correlates directly with the %thymidine DNA replacement. Illustrated in previous work, as BrdU replacement of thymidine occurred, a 150-fold decrease in cell survival was observed compared to control. Additionally, subsequent 15 μM BrdU and 2 and 4 Gy gamma-irradiation, further necessitated a decrease in cell survival; $D_{10}$=0.55 and .33 Gy, respectively. Decrease in cell survival may be due to production of chromosomal breaks and malformations. These data reflect our data in a similar fashion, in that $D_{10}$ values decreased accordingly in the presence of 10 μM BrdU compared to control (unlabeled) (fig. 26 & 27). Nonetheless, our data portrays that BrdU-mediated radiosensitization is sufficient in of itself to decrease overall clonogenicity. Though, EdU exhibits little-to-no mediated radiosensitization in the presence of external gamma-irradiation.

Photons play integral in our lives; walking to the mailbox, turning on one's office light, or, in this report, exposure to cells. Photons may appear mundane, or ineffectual toward our well-being-wrong. However, combined with halogenated pyrimidines, may discretely insult a cells viability. What discerns X- and photon-irradiation, is the energies of said emitted photons. Methodologies akin to ours (approximately equivalent) report, that CHO wildtype cells exposed to *Coolwhite*-fluorescent light (flux rate=3.8x $10^{-5}$ J/sec/$cm^2$) and 10 μM BrdU display a tremendous 100-fold decrease in cell survival compared to unexposed-control cells. This study also incorporated an additional halogenated pyrimidine, 5-iodo-2'-deoxyuridine (IUdR), for comparison. Indeed, while IUdR represented a 5-fold decrease in cell survival compared to unexposed control, BrdU incorporation resulted in 20-fold decrease cell survival compared to IUdR (144). Although we did not report data pertaining to the comparison of SSBs induced by gamma-irradiation versus fluorescent light (FL) and subsequent BrdU incorporation, a study reports, that FL is 40-60 times more efficient in inducing SSBs and overall decreased cell survival compared to gamma-irradiation. And, that these SSBs persist longer through concomitant BrdU/FL-exposure (5). In our present work, we report that 10 μM BrdU with FL-exposure results in a $D_{10}$=165 and 120 min in A549 and CHO wildtype cells, respectively (fig. 26 & 27). Which, is equivalent to the above experiment. Also, since this study did not directly report the comparison of chromosomal breaks induced by gamma-irradiation versus FL-exposure, we were able to interpolate that, our findings were different from above. In that, combination of gamma-irradiation and BrdU exposure was considerably more lethal than FL-BrdU-exposure (fig. 26 & 27). Lastly, our evidence reports that CHO wildtype and A549 cells are not radiosensitized to FL-EdU-exposure. More detailed analysis may be required to define any discrepancies.

In conclusion, our findings suggest that, cells exposed to gamma-, UV-, and FL-irradiation and BrdU, are susceptible to radiosensitization. Although, concomitant EdU and gamma-, UV-, and FL-irradiation, did not depict any radiosensitization effects. Though, an agreeance, that halogenated pyrimidines create a hostile environment for cellular capacity, once incorporated, can be met. Nonetheless, it is important that, caution and consideration should be held to high regard when applying any form of halogenated pyrimidine, specifically, BrdU, to cell culture experiments. Even more so, when attempting to normalize data obtained from gamma-, UV-, and FL-irradiation and halogenated pyrimidine-labeling experiments. Without proper speculation and selection of concentrations, experiments may be insulted, and result in improper or misinterpreted data.

# CHAPTER FOUR

## CONCLUSIONS AND FURTHER DIRECTIONS

This study reports significant findings: CHO wildtype and CHO DNA repair deficient XRS-5 (XRCC 5 gene deficient), 51D1 (Rad51D gene deficient), IRS1SF (XRCC 3 gene deficient), KO40 (FANCG deficient), PADR9 (17% wildtype PARP activity), and V3 (DNA-PKcs gene deficient) cells display various levels of sensitivity in regard to nucleotide substitution, specifically, 5-bromo-2'-deoxyuridine and 5-ethynyl-2'-deoxyuridine. Specifically, these cells all report fluctuating vulnerability in respect to PARP activity, HPRT-mutation induction, SCE frequency, doubling time, and media-supplemented specificity.

Moreover, we report that, homologous recombination repair deficient cells, 51D1 and IRS1SF, are subject to a dramatic decrease in cell viability, and increased doubling time in the presence of BrdU or EdU incorporation. A saturation effect was observed at EdU concentrations suspended at 50 μM, suggesting that HRR is ensured for proper cell maintenance and propagation. Although CHO wildtype cells presented the highest dose-dependent increase in SCE frequency, all DNA repair mutants developed an abnormal SCE frequency induction, with EdU administration. BrdU did display an elevated SCE frequency, however 100 μM EdU did not produce any viable colonies in order for SCE detection. Thus, proposing that (mutant) cells replicative abilities are impressively inhibited or influenced by EdU incorporated as a DNA template; incompetent in completion of successive cellular divisions. At below recommended manufacturer concentrations (10 μM), EdU and BrdU (1 μM) produced an increase in HPRT-mutations, EdU having the highest frequency of HPRT-mutations. This study did not provide data on PADR9-PARP activity, as it retains an inherent decrease (17% wildtype) in PARP

activity. However, we found that PARP activity in CHO wildtype cell is not effected by EdU to any reportable degree. Contrary, BrdU reporting maximal decrease of PARP activity. Implying that, high SCE frequency, chromosomal aberrations, and HPRT mutation frequency are not a result of PARP inhibition.

CHO cells maintained and cultured for successive generations, in nucleotide-supplemented media (AlphaMEM), exhibited the highest retention of viability when administered 10 μM EdU or BrdU. Contrasting, MEM media (nucleotide deficient) with substituted EdU as DNA template, reported an exacerbated decrease in cell survival, as opposed to BrdU. Likely, EdU-containing strand leads to replication fork stalling or collapse during DNA replication. Therefore, researchers should query the extent and endpoint goals of their experiments when applying experimental halogenated pyrimidines.

Adjuvant application of external radiation modalities and BrdU materialized a decrease in cell survival in CHO wildtype and A549 cells. One of this study's primary objectives was to discern the effects that EdU implicated on cell radiosensitivity. In fact, our data revealed, that EdU does not promote radiosensitivity. However, our data does show radiosensitive dependency on BrdU incorporation. All radiation sources, gamma-, UV-, or FL-irradiation did, in fact, exhibit a radiosensitivity dependence when BrdU is incorporated into cellular DNA. Previous work from our laboratory reports similar conclusions.

There are numerous variables that affect cell susceptibility to incorporated halogenated pyrimidines. Such as, dehalogenation of halogenated pyrimidines, through nucleotide salvage pathway, registering free radicals, which directly consequence DNA . Also, biological or therapeutic endpoint conclusions and expectations are certainly dependent on cell-type, quality of radiation utilized, and concentration of drug (BrdU/EdU). For example, if one desires to

investigate the effects of compound "X" on breast cancer cell replicative ability (BrdU/EdU as labeling agent) in the presence of radiotherapy, they must be aware of the clonogenicity of breast cancer cells (inherently lower cell viability), or any cell line, for that matter. Moreover, our conclusions suggest that BrdU and EdU affect cell viability. Therefore, if one does not consider BrdU's radiosensitization effects, or EdU's capacity to result lower cell survival, their results may be incorrect or misinterpreted.

As previously stated, there are numerous variables influencing the verification of a study. Our future directions are focused on several items: free-radical production by EdU and BrdU, molecular receptors accounting for mediation of nucleosides, synthesis of more user-friendly halogenated pyrimidines, and application of various cell-lines. It is by these additional modifications, that we will better improve clinical and research-based applications of synthetic nucleosides. If there is to be relayed from this report finding, we strongly recommend that concentrations and desired biological-endpoints are established, while cautiously considering the cytotoxic and genotoxic effects of EdU and BrdU.

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