INHIBITORY EFFECT OF BROMODEOXYURIDINE ON LONG-TERM CELL PROLIFERATION

By

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To my mom for her unconditional love, support, and friendship!

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The thymidine analog bromodeoxyuridine (BrdU) can be incorporated into newly-synthesized DNA and is commonly used to "birthdate" proliferative cells. It has been suggested that BrdU alters the stability of DNA thereby increasing the risk of sister-chromatid exchanges, mutations, and double-strand breaks. However, most of these effects are found only when BrdU incorporation is combined with secondary stressors. Early toxicity studies showed that BrdU can induce chromosomal breakage and increase the sensitivity of treated cells to ionizing radiation, and this radiosensitizing effect has continued to be pursued as an adjunctive therapy in the treatment of a variety of cancers. BrdU readily crosses the blood-brain barrier, and has been combined with conventional chemotherapy and radiation treatment in several clinical trials. While the clinical benefits of including BrdU as a radiosensitizer have been disappointing – showing, at best, modest improvements for some outcome measurements- it is possible that other therapeutic effects of BrdU were not appreciated, either because of interference by the other treatment modalities in these studies, or because finer analytical resolution is required to discern them.

Despite its extensive history there is no accepted consensus mechanism of action for BrdU. Surprisingly little attention has been focused on examining the influence that BrdU alone may

exert on cellular function. We report a novel anti-proliferative effect of BrdU on populations of stem and cancer cells that is independent of its role in radiosensitization. A single, brief in vitro exposure to BrdU induces a profound and sustained reduction in the proliferation rate of all cells examined. Cells do not die but variably upregulate some senescence-associated proteins as they accumulate in the G1 phase of the cell cycle. BrdU also impairs the proliferative capacity of primary tumor-initiating human glioma cells and may, therefore, represent a means of targeting cancer stem cells. Finally, conservative in vivo BrdU regimens (in the absence of any other treatment) significantly suppress tumor progression in a highly aggressive, syngeneic rat glioma model. Results suggest that BrdU may have an important role as an adjunctive therapeutic for a wide variety of cancers based upon new insights into its effect as a negative regulator of cell cycle progression.

CHAPTER 1
INTRODUCTION

Background

Bromodeoxyuridine (BrdU) is a thymidine analog that was introduced in the 1950s as a mutagen to target rapidly-dividing cancer cells (Hakala, 1959; Hakala, 1962; Djordjevic and Szybalski, 1960), but is now used ubiquitously to birthdate dividing cells. While there are myriad reports dealing with the consequences of BrdU incorporation into DNA chains (Littlefield and Gould, 1959; Stellwagon and Tomkins, 1971; Dunn and Smith, 1957; Terzaghi et al., 1962), the variations in dosage and exposure time in these studies make it difficult to compare individual results. Perhaps because of this, and because incorporating cells appear to maintain relatively normal function—at least in the short-term (Cameron and McKay, 2001)—BrdU is generally thought to substitute relatively benignly for thymidine. Recent work, though, suggests that BrdU may play a role in premature senescence induction in a wide variety of cells (Michishita et al., 1999; Suzuki et al., 2001).

Despite its extensive history there is no accepted consensus mechanism of action for BrdU. It has been suggested that BrdU alters the stability of DNA thereby increasing the risk of sister-chromatid exchanges, mutations, and double-strand breaks (reviewed in Taupin, 2006). However, most of these effects are found only when BrdU incorporation is combined with secondary stressors. Early toxicity studies showed that BrdU can induce chromosomal breakage and increase the sensitivity of treated cells to ionizing radiation (Djordjevic and Szybalski, 1960; Hsu and Somers, 1961; Erickson and Szybalski, 1963), and this radiosensitizing effect has continued to be pursued as an adjunctive therapy in the treatment of a variety of cancers. BrdU readily crosses the blood-brain barrier, and has been combined with conventional chemotherapy and radiation treatment in several clinical trials (Kinsella et al., 1984; Phillips et al., 1991;

Robertson et al., 1997a; Robertson et al., 1997b; Groves et al., 1999; Prados et al., 2004). While the clinical benefits of including BrdU as a radiosensitizer have been disappointing –showing, at best, modest improvements for some outcome measurements- it is possible that other therapeutic effects of BrdU were not appreciated, either because of interference by the other treatment modalities in these studies, or because finer analytical resolution is required to discern them.

Understanding the mechanism by which BrdU affects cells would allow scientists to maximize the efficacy of using BrdU in combination therapy. However, surprisingly little attention has been focused on examining the influence that BrdU alone may exert on cellular function. BrdU has been studied for over five decades but the results are dispersed over various subfields of research. Compiling all of the BrdU-related research is further complicated by the lack of a standard reference for this compound (Bromodeoxyuridine, 5-Bromodeoxyuridine, 5-Bromo-2'-deoxyuridine, BrdU, BrdUrd, BUdR, etc.). Revisiting the extensive literature may provide key clues for elucidating the effector mechanism of BrdU.

Mutagenecity and Toxicity

While most of the recent scientific literature pertaining to BrdU focuses on its use as a proliferative label, one should not ignore the fact that this synthetic thymidine analog was originally designed in the 1950s to perturb DNA synthesis in cancer cells. Within that decade scientists began characterizing how cells were affected by BrdU exposure. Hakala (1959) reported that once BrdU was incorporated in place of thymine in the DNA it creates an irreversible abnormality which prevents further synthesis of DNA. Shortly thereafter, Littlefield and Gould (1960) showed that when cells were incubated for 5 to 7 days with increasing concentrations of BrdU, an increasing amount of the analog was incorporated into DNA, and growth (as measured by cell number, total cell protein, and single cell plating efficiency) was progressively inhibited. Interestingly, they believed that the toxic effect of BrdU was related to

an interference with the *function* of DNA (i.e., transfer of information required for protein synthesis).

It has been over five decades since scientists began reporting the effects of BrdU and yet the mechanism of action remains unknown. The integration of a bromine atom into the DNA alters its stability, increasing the risk of sister-chromatid exchanges, mutations, DNA double-strand breaks, and lengthens the cell cycle of incorporating cells (reviewed in Taupin, 2006). One of the earliest explanations (based on experiments with prokaryotic systems) is that BrdU-induced mutations occur as the result of occasional mispairing of the analog during DNA replication. BrdU has ambiguous pairing properties due to the bromine atom, and it can shift from a *keto* form (which pairs with adenine) to an *enol* form (which pairs guanine) thereby causing a mispair (Peng et al., 2001). Hopkins & Goodman (1980) argue that the effects of BrdU mutagenesis are actually two-fold, involving the perturbation of normal deoxyribonucleoside triphosphate pool sizes (Meuth and Green, 1974) and base-pairing ambiguity. BrdUTP is an inhibitor of ribonucleotide diphosphate reductase. This inhibition mainly affects the reduction of cytidine disphosphate (CDP) to deoxycytidine diphosphate (dCDP), and high levels of dTTP, dGTP, and dATP are generally synergistic with BrdUTP in this inhibition (Meuth and Green, 1974; Moore and Hurlbert, 1966). Therefore, high BrdUTP concentrations will prevent the formation of the cytosine substrate for DNA synthesis, dCTP. With a decrease in dCTP pools, BrdUTP becomes increasingly competitive for sites opposite template guanines during DNA replication.

Davidson et al. (1988) elaborated on this idea by suggesting that the BrdU-related mutagenesis results from a sequence of events: (1) there is an induction of a high BrdUTP/dCTP molar ratio (due to the inhibition by BrdUTP of the ribonucleotide reductase catalyzed reduction

of CDP to dCDP); (2) then BrdU mispairs or misincorporates opposite a guanine residue (due to the excess of BrdU and the lack of dCTP); and (3) the resulting BrdU-induced G-C → A-T transitions are driven by nucleotide pool perturbation.

Many early studies tried to combat the theoretical nucleoside pool imbalance by adding exogenous deoxycytidine to BrdU-treated cultures. The belief was that the mutagenicity, toxicity, and even the effects on differentiation associated with BrdU treatment might be thwarted if the presence of dCTP causes BrdUTP to compete less effectively for sites opposite guanine on DNA (Davidson and Kaufman, 1977; Davidson and Kaufman, 1978; Hopkins and Goodman, 1980; Kaufman and Davidson, 1979; Meuth and Green, 1974). Unfortunately, the results from such experiments are conflicting; while some results suggest a "rescue" effect, others report no difference.

Proliferation Label

The ability to label dividing cells and quantify DNA synthesis is fundamental to the study of cell biology, specifically cancer biology. Understanding the exact kinetics of the S-phase could maximize the efficacy of cell cycle-specific chemotherapeutic drugs that selectively kill tumor cells. Initially, scientists used techniques such as tritiated thymidine with autoradiography to measure the percentage of S-phase cells and/or label mitotic cells. The analysis of cell cycle was greatly improved by the introduction of flow cytometry because this technique allowed for the precise measurement of DNA content of a statistically significant population of cells. However, flow cytometers are not well suited for conventional autoradiographic measurements (e.g., tritiated thymidine) thus necessitating a new method (Leif et al., 2004).

Over four decades ago, the collaboration between Robert Leif, Howard Gratzner, and Albert Castro resulted in the production of a good affinity-purified polyclonal antibody to BrdU. The creation of this antibody allowed scientists to label cells with BrdU and subsequently

determine S-phase populations using flow cytometry. To improve specificity and affinity, a mouse monoclonal antibody to BrdU was produced. The use of the BrdU antibody has facilitated cell cycle studies, and the knowledge of S-phase content and timing continues to be useful for the creation of better cancer therapies (Leif et al., 2004).

While cancer was the original focus for utilizing BrdU as a proliferative label, it is now commonly associated with stem cell research. BrdU labeling represents a relatively easy method for tracking stem and progenitor cell proliferation in various regenerative tissues. In fact, BrdU labeling is the most commonly used technique for studying adult neurogenesis in situ (Taupin, 2006).

Neurogenesis

The adult brain was once thought to be relatively static, lacking the plasticity and genesis of the developing brain. This dogma persisted for nearly a century despite numerous reports suggesting the birth of new neurons in the adult brain. The first suggestion of adult neurogenesis came from early studies with [^{3}H]-thymidine autoradiography that showed neurogenesis in the adult rodent brain. These results were further supported by studies conducted with electron microscopy that reported autoradiographic and ultrastructural evidence of new neurons in adult rats as well as high levels of neurogenesis in the avian brain (as reviewed in Gross, 2000). Despite continuing evidence for neurogenesis, the dogma persisted. It was not until the 1990s, when advances in techniques to study neurogenesis were discovered that the longstanding notion that no new neurons were born in the adult brain was finally put to rest. Interestingly, the introduction of the synthetic thymidine analog BrdU was a major milestone in neurogenesis research because it allowed for the stereological estimation of the total number of cells as well as demonstration that the new cells express markers of specific cell types (Gross, 2000).

It is now well established that neurogenesis occurs throughout adulthood in the mammalian brain (Gage, 2000). There are two main regions in the adult mammalian brain that undergo neurogenesis, the dentate gyrus of the hippocampus and the subventricular zone (Taupin and Gage, 2002). Being able to label proliferating cells is requisite for studying neurogenesis. BrdU immunohistochemistry has been instrumental for studying the development of the nervous system and to confirm that neurogenesis does occur in the adult brain. The benefits of BrdU immunohistochemistry include faster processing, utilization of a monoclonal antibody rather than a radiolabeled substance, and its usability with relatively thick tissues (Gratzner, 1982; West et al., 1991). However, knowing that BrdU affects incorporating cells, it is important to distinguish cell proliferation and neurogenesis from other events (e.g., DNA synthesis, DNA repair).

BrdU can be administered by various methods including intracerebroventricular (i.c.v.), intraperitoneal (i.p.) and intravenous (i.v.) injection, or orally (in drinking water), for studying neurogenesis. While the mechanism of BrdU transport into the brain remains unknown, *in vitro* experiments suggest that BrdU uses the same nucleoside transporter as thymidine (Lynch et al. 1977). Thymidine, and likely BrdU as well, crosses the blood-brain barrier through a facilitative low affinity, high-capacity carrier-mediated nucleoside transport system (Taupin, 2006; Thomas nee William and Segal, 1996; Thomas et al. 1997; Spector and Berlinger, 1982). Hayes and Nowakowski (2000) suggest that BrdU is only available for labeling cells in the adult brain for approximately two hours following systemic injection.

However, with all of the benefits of BrdU labeling, one must not forget that BrdU can be toxic. It is known that high doses and/or multiple exposures of BrdU can trigger cell death in embryonic and neonatal development and, when given to pregnant rats, affect the litter size,

body weight and mortality of the offspring. These BrdU regimens also reduce the size of the cerebellum and produce defects in proliferation, migration, and patterning of the cerebellum in adult progenies (Sekerkova et al., 2004). This is in contrast to the fact that single-injection low-dose BrdU does not appear toxic to the development of certain brain regions (Miller and Nowakowski, 1988; Takahashi et al., 1995). While there is no report of toxicity using BrdU to study adult neurogenesis, it is certainly plausible that even low doses of BrdU are likely to have toxic effects on newly generated neuronal cells in the adult brain – particularly when multiple doses are administered (Sekerkova et al., 2004). Even with its extensive history of mutagenicity and toxicity, BrdU remains the most commonly used method for studying neurogenesis.

Cancer

The focus of BrdU in cancer biology is generally related to its role as a proliferative label and radiosensitizer. However, there have been reports of BrdU inducing differentiation in a variety of cancers including neuroblastomas and small cell lung carcinomas. Chen et al. (2007) show that BrdU treatment causes morphological changes in non-adherent cancer cells such that a new adherent subpopulation forms. Additionally, this adherent subpopulation shows reduced colony-forming capacity (both in size and number). Finally, when the BrdU-treated adherent subpopulation was tested for its ability to form tumors *in vivo*, the authors found a reduction in tumor development. Interestingly, it appears that the differentiation events can take place in the absence of DNA synthesis, suggesting that BrdU need not be incorporated into DNA to alter the phenotype of the cell (Schubert and Jacob, 1970).

Early experiments using BrdU suggest that BrdU weakens the DNA thereby increasing the risk of sister-chromatid exchanges, mutations, and DNA double-strand breaks. Understanding the potential of those events in possibly weakening and/or killing cancer cells, scientists combined BrdU treatment with radiation to determine if their combination had a synergistic

effect. The results showed that BrdU "radiosensitizes" cells, making them more susceptible to the damage induced by radiation. This discovery held great promise in improving the efficacy of radiation as a cancer therapy and continues to be studied in various clinical trials.

Radiosensitization

Combining radiation therapy with radiosensitizing drugs has been a focus for oncologists for many decades. The idea is that the drugs increase the sensitivity of treated cells to radiation thereby improving treatment. However, there has been limited success with the countless clinical trials looking at the combination of chemical/biological agents and radiation. Steele and Peckham (1979; 1988) categorized these types of clinical trials according to the nature of how the radiation and drug(s) interact: spatial cooperation, toxicity independence, protection of normal tissue, and enhancement of tumor response (Coleman and Mitchell, 1999). One of the biggest hurdles in achieving success is the lack of preclinical studies that would elucidate the optimal timing and dosing regimen for the various agents. Additionally, proper analysis of biological tissue during the treatments would greatly enhance the quality of these studies. Eisbruch et al. (1999) utilized data from preclinical studies to explore a unique schedule of BrdU delivery to cervical cancer patients receiving radiation treatment. By collecting multiple biopsies of tumor and normal tissue the investigators were able to obtain substantial results regarding the incorporation of BrdU into normal and tumor tissues that supported the rationale of their study (Coleman and Mitchell, 1999).

Cells exposed to halogenated thymidine analogs are more sensitive to ultraviolet light and radiation than untreated cells. The incorporation of the halogenated deoxyuridine, BrdU, is known to increase the radiosensitivity of a variety of organisms that include certain bacteria, bacteriophages, and mammalian cells (Greer and Zamenhof, 1957; Kozinski and Szybalski, 1959; Djordjevic and Szybalski, 1960). Compared to other thymidine analogs, the molecular

structure of BrdU most closely resembles that of thymidine and was therefore targeted for clinical use. Over the last three decades there have been myriad clinical trials designed around the use of BrdU as a radiosensitizer. These studies were designed to find the most effective mode of administration and dosing regimen for maximizing the radiosensitization effect of BrdU without producing toxic side effects.

Scientists and clinicians delivered BrdU via intravenous infusion or intra-arterial infusion at doses ranging from 25 mg/kg/day to 1000 mg/m^2/day for various time periods. Some of the reported side effects included varying levels of anorexia, fatigue, ipsilateral forehead dermatitis, blepharitis, iritis, and nail ridging. However, the most common toxicities occurred in the bone marrow and skin (e.g., myelosuppression and maculopapular skin rash) (Kinsella et al., 1984; Greenberg et al., 1988). Many of the trials were able to minimize these toxicities to acceptable levels upon finding ideal dosing regimens, and concluded that BrdU was able to significantly enhance radiotherapy in malignant brain cancer, pancreatic cancer, hepatobiliary tumors, and colorectal liver metastases (Kinsella et al., 1984; Greenberg et al., 1988; Matsutani et al., 1988; Hegarty et al., 1990; Robertson et al., 1997a; Robertson et al., 1997b).

BrdU was also combined with radiation and chemotherapy in a series of Phase II and III clinical trials that failed to show any positive effect of adding BrdU to the therapy. While the clinical benefits of including BrdU as a radiosensitizer have been mixed –showing, at best, modest improvements for some outcome measurements- it is possible that other therapeutic effects of BrdU were not appreciated, either because of interference by the other treatment modalities in these studies, or because finer analytical resolution is required to discern them.

Senescence

Senescence was originally described by Hayflick and colleagues (1961) in studies showing that normal human fibroblasts have a finite ability to proliferate. Cellular senescence is confined

to mitotic cells which can stall for long intervals in a reversibly arrested state. These cells remain viable awaiting the appropriate signals to resume proliferation. Interestingly, senescent cells undergo alterations in gene expression and often become resistant to apoptosis. A hallmark of senescence is the inability to progress through the cell cycle. To achieve this, senescent cells maintain a delicate balance of expressing cell cycle inhibitors (e.g., p21 and p16) and repressing genes that encode proteins that stimulate cell cycle progression (e.g., cyclins, c-Fos, PCNA).

Most of the observations regarding senescence have been made using cultured cells; however, the importance of senescence *in vivo* has gained support over the last decade. A major limitation in studying senescence is the fact that the markers used to identify senescent cells are not exclusive to this state. Despite this limitation in identifying senescent cells, the significance of this state is highlighted in two main areas of research, cancer and aging. Populations of senescent cells have been found in renewable tissues in various species as well as at sites of chronic age-related pathology and benign dysplastic or preneoplastic lesions (reviewed in Campisi and d'Adda di Fagagna, 2007).

Senescent cells may contribute to the aging process by altering the expression of tumor-suppressive (i.e., pro-aging) proteins that suppress stem cell proliferation and tissue regeneration, and by upregulating genes that encode extracellular-matrix-degrading enzymes, inflammatory cytokines and growth factors that influence the local environment. These secreted factors can actually stimulate the growth and angiogenic activity of nearby premalignant cells (Campisi, 2005; Campisi and d'Adda di Fagagna, 2007). This relationship between mitotically inactive senescent cells and highly replicative cancer cells seems paradoxical. However, this may explain the rise in cancer development in aging organisms.

Cellular senescence probably suppresses tumorigenesis by limiting cell proliferation. This is supported by the presence of senescent cells in preneoplastic lesions where they are presumed to be halting the progression to malignancy. However, senescence may not be permanent and the reversal of this state may allow damaged, stressed, or oncogene-expressing cells to proliferate. Many of the stimuli that induce senescence are potentially oncogenic and cancer cells must acquire mutations that allow them to avoid senescence. Interestingly, this does not necessarily result in malignant transformation. The loss of the senescence response appears to be critical, albeit insufficient, in the development of cancer (reviewed in Campisi and d'Adda di Fagagna, 2007).

There is no defining marker for senescent cells. However, senescence is typically characterized by a flat, enlarged cell shape, the induction of senescence markers such as senescence-associated beta-galactosidase (SAβGal), and resistance to apoptosis (Cristofalo and Pignolo, 1993; Dimri et al., 1995; Wang, 1997). While the initiating events leading to senescence and the resulting molecular signature are not well understood, the following potential causes have been evaluated: degradation and/or dysfunctional telomeres, DNA damage, chromatin perturbation, activation of oncogenes, and stress (reviewed in Campisi and d'Adda di Fagagna, 2007).

The Ayusawa group has shown that BrdU induces an immediate senescence-like phenotype in any type of mammalian cell. In fact, they suggest that this effect may occur in all eukaryotic cells as the effect seems to carry over into thymidine-auxotrophic yeast as well (2002). Their results show that BrdU induces flat and enlarged cell shape, characteristic of senescent cells, and senescence-associated beta-galactosidase in mammalian cells regardless of cell type or species. Interestingly, human cell lines lacking functional p21, p16, or p53 behaved

similarly. This is particularly surprising considering the well-documented relationship between alterations in the expression of these proteins and the induction of senescence. Additionally, while telomerase activity was suppressed in positive cell lines, accelerated telomere shortening was not observed in tumor cell lines. These results suggest the BrdU activates a common senescence pathway present in both mortal and immortal mammalian cells. However, it is unclear whether this BrdU-induced senescence is permanent and the authors fail to define mechanism for this change.

Masterson and O'Dea (2007) report that BrdU induces a DNA damage response that involves the activation of Chk1/2 and p53, subsequent cell cycle inhibition, and phenotypic changes consistent with senescence. Additionally, BrdU causes alterations in the expression profiles of many of the quintessential senescence markers (e.g., p21, p27, SAβGal staining). Interestingly, despite inducing a DNA damage response, the authors believe that the downstream effects of BrdU influence cell cycle dynamics, differentiation, and senescence, but do not initiate the apoptotic cascade. Similarly, Peng et al. (2001) showed that continuous exposure to 20μM BrdU for 48 hours or more markedly inhibited the growth of cancer cells irrespective of p53 status, and that the growth suppression is mainly due to cell cycle arrest rather than cell death.

Cancer Stem Cells

The theory that cancer might arise from a rare population of cells that display stem cell characteristics was postulated over 150 years ago and various studies have been conducted to isolate this proposed subpopulation. However, the majority of these studies have been limited to *in vitro* assays that measure proliferation rather than true self-renewal. Advances in stem cell biology and animal models now allow scientists to more directly measure self-renewal, a central tenet in defining stem cell populations (reviewed in Wicha et al., 2006).

Stem cells and cancer cells share several important properties that include (a) capacity for self-renewal, (b) ability to differentiate, (c) active telomerase expression, (d) activation of anti-apoptotic pathways, (e) increased membrane transporter activity, and (f) the ability to migrate and metastasize (Wicha et al., 2006). Stem cells can undergo either symmetric (production of two identical stem cell progeny) or asymmetric (production of one exact copy and one daughter cell that undergoes differentiation) division. The symmetric divisions produce the cells that are responsible for tumor infiltration and metastasis while asymmetric divisions produce the cells that comprise the tumor bulk. The regulation of self-renewal is critical for development and repair and is believed to be influenced by environmental signals present in the stem cell niche. If the proper inhibition cues are not available, stem cells will continue to proliferate. This deregulation may represent an early stage in carcinogenesis, and is supported by the fact that signaling pathways involved in stem cell self-renewal (e.g., Wnt, Notch, and Hedgehog) are implicated in various human cancers as well. Alterations in these pathways have been shown in colon, pancreatic, gastric, prostate, skin, cervical, blood, and breast carcinomas (reviewed in Wicha et al., 2006).

While the focus remains on the rare stem cell population, it is certainly plausible that the progenitor cells may also be responsible for tumorigenesis. Whether these cells transform and/or de-differentiate, it is likely that they acquire the capacity for self-renewal. Certain oncogenic mutations might deactivate the control sequences involved in differentiation, cell-cycle inhibition, or cell death. Wicha et al. (2006) propose that both types of cells are important because self-renewal drives tumorigenesis whereas differentiation (albeit aberrant in tumors) contributes to tumor phenotypic heterogeneity. The maintenance of these states are controlled by paracrine signaling pathways, negative feedback loops that limit the response to mitogenic

signals, and pathways that suppress activation of the differentiation program. This suppression may be a result of translational repression or epigenetic changes that lead to repression of differentiation-associated genes (Clarke and Fuller, 2006). Ultimately, the mechanism of transformation remains unknown and the identification of the intracellular and extracellular events that support the cancer stem cells is critical.

Normal stem cells are programmed to survive and are inherently resistant to apoptosis. The assumption that cancer stem cells share this characteristic has therapeutic relevance because this resistance to apoptosis may explain the inefficiency of cytotoxic agents and radiation therapy in cancer treatment. This apoptosis resistance may be the result of alterations in cell cycle kinetics, DNA replication and repair, asynchronous DNA synthesis, and/or the expression patterns of anti-apoptotic and transporter proteins. Further characterization of these changes will allow for new targeted therapies. However, the similarity between normal and cancer stem cells poses a challenge – can a therapy efficiently target cancer stem cells without affecting normal stem cells? Promising results from recent studies involving signaling pathway and enzyme inhibitors support the feasibility of selectively targeting the cancer stem cell population (Wicha et al., 2006).

It is estimated that only a very small fraction of cancer cells (< 1%) is clonogenic. Hamburger & Salmon (1977) found that only 1/1000 to 1/5000 cells from tumors of the lung and ovary formed colonies in agar. While scientists have found cancer cells that express markers commonly associated with stem cells (e.g., CD133, nestin, Oct3/4), there is still no unique marker for the cancer stem cell. In fact, it is likely that the putative cancer stem cells within each type of tumor will possess a unique combination of cell surface markers. Cells possessing specific cell surface marker phenotypes have been isolated from blood, breast, and brain tumors,

and have been shown to generate tumors in secondary hosts (e.g., NOD/SCID mice) that are nearly identical to the parent neoplasm (reviewed in Huang et al., 2007).

There is great heterogeneity within tumors and the ability to identify and isolate the putative cancer stem cell will be critical for targeting these cells. It is difficult to design a therapeutic that will effectively treat a heterogeneous population of cells. This may explain why most current therapies can induce tumor shrinkage yet fail to actually *cure* cancer and prevent recurrence. An agent designed specifically to kill the cancer stem cell could be more effective. However, it is likely that a treatment that is toxic to cancer stem cells will also affect the normal stem cell populations.

Maintenance of normal tissue requires a balance between self-renewal and differentiation within the stem cell pools. A small number of stem cells are responsible for producing a large population of differentiated progeny. However, the stem cells are not the only cells that can proliferate; the transit-amplifying progenitor cells may undergo a few mitotic divisions before entering a fully differentiated, post-mitotic state. Stem cell replication is regulated by various internal and external stimuli but the alterations that cause a "normal" stem cell to become a cancer stem cell remain unknown. There are two ways that cancer stem cells may arise from normal stem cells. First, mutations may disrupt the regulation of replication thereby transforming the cell. Alternatively, mutations may allow the transit-amplifying progenitor cells to continue proliferating without entering a post-mitotic differentiated state. In either case, a population of self-renewing cells originates that is resistant to differentiation and susceptible to additional mutations (Clarke and Fuller, 2006).

Connecting Neural Stem Cells and Gliomas

Alterations in cellular functions such as proliferation, apoptosis, and tissue invasion can lead to cancer. Neural stem and progenitor cells are regulated by the same pathways that

influence many brain tumors. Therefore, it is reasonable to assume that these neurogenic populations of cells are potentially susceptible to transformation. In support of this theory is the fact that, in animal models, highly-proliferative regions of the brain are more sensitive to chemical or viral oncogenesis than are areas with a low proportion of proliferating cells. Additionally, many gliomas are found near germinal regions, such as the subventricular zone in humans, and frequently express markers that are associated with progenitor cells. While the precise mechanism of transformation is unknown, recent experiments have shown that changing the expression profiles of certain signal transduction proteins cause the formation of brain tumors in rodents (reviewed in Sanai et al., 2005). Alternatively, these cells may arise from a de-differentiation event occurring within the organ, allowing this subpopulation to adopt a stem cell-like phenotype.

Concluding remarks

Conclusions drawn from the literature review suggest that the incorporation of BrdU probably carries consequences for cellular viability. While the mechanism(s) for the varied effects of BrdU remain unknown, it is possible that BrdU functions in a cell type-specific manner. Aside from the early BrdU studies, very little research has been conducted on the consequences of incorporating BrdU in the absence of secondary stressors. Considering BrdU is the most commonly employed method for labeling proliferative cells, it is important to determine whether the incorporation of this thymidine analog has consequences for these populations.

CHAPTER 2
MATERIALS AND METHODS

Neurosphere Culture

Primary neurospheres (NS) were derived from neonatal C57BL/6 mice (P4 to P8). *In vivo* studies were conducted using young C57BL/6 mice (~P21). Animals were housed at the University of Florida's Department of Animal Care Services, and all procedures were in compliance with the regulations of the Institutional Animal Care and Use Committee. NS were generated as described (Laywell et al., 2002). Briefly, brains were removed from euthanized animals, incubated in Trypsin, and dissociated into a single-cell slurry with a series of decreasing bore glass pipettes. The slurry was plated overnight in growth media (DMEM/F12, 5% FBS, 10 ng/µl bFGF and EGF). In order to isolate NFC, the slurry suspension was aspirated, pelleted by centrifugation and incubated in Trypsin for 2 minutes. Cells were gently triturated, washed and resuspended. NFC were then plated in non-adherent flasks at clonal density (10,000 cells/cm2) in growth medium. The typical dose ranges for BrdU administration (based on the literature) are 10-50µM *in vitro* and 50-300mg/kg *in vivo*.

Neurosphere Quantification and Measurement

NS were assessed for frequency and size as a measure of NFC number and proliferative capacity, respectively. NS frequency was determined by counting random triplicate 50 µl aliquots using a 10X objective. Total NS number was extrapolated to total culture volume for each field counted. NS size was determined by measuring the diameter of ten randomly chosen spheres in each well (triplicate conditions) using calibrated measuring probes associated with digital image capture software (Spot Advanced).

Immunolabeling

For cleaved caspase-3 and γH2A.X immunolabeling cells were grown to ~75% confluency on polyornithine-coated glass coverslips. The media was removed and the cells were fixed by incubation in 4% paraformaldehyde in PBS at room temperature for 15 minutes then washed with PBS for 5 minutes. Cells were prepared for immunocytochemistry by first blocking at room temperature for 1 hour in PBS plus 0.01% Triton X-100 (PBSt) containing 10% FBS. Primary antibodies were then applied to the cells for one hour in PBSt + 10% FBS with moderate agitation at 37^{o}C. The antibodies used were either Cleaved Caspase-3 (1:400; #9661S; Cell Signaling Technology) or γH2A.X phospho-S139 (1:200; ab2893; Abcam). Residual primary antibody was removed by three 5 minute washes with PBS and secondary antibodies were applied at room temperature for one hour in PBSt + 10% FBS. Residual secondary antibodies were removed by three 5 minute washes in PBS. The cover-slips were placed onto glass slides and Vectashield mounting medium plus DAPI (Burlingame, CA: H-1200) was applied immediately prior to cover-slipping.

Cells were processed for BrdU immunolabeling as previously described (Laywell et al., 2005). Briefly, cells were incubated for 2 hours in a 1:1 ratio of 2xSSC:formamide at 65^{o}C. After a wash in 2xSSC the cells were then incubated for 30 min. at 37^{o}C in 2N HCL. Finally, the cells were equilibrated at room temperature for 10 minutes in borate buffer, followed by standard indirect immunofluorescence detection of BrdU with a rat anti-BrdU antibody (#ab6326, Abcam, Cambridge, MA).

Senescence-Associated β-Galactosidase Labeling

SAβGal activity was detected as described (Dimri et al., 1995). Briefly, tissue samples were collected from the neurogenic brain regions of animals receiving either BrdU (100mg/kg) or saline (0.9%) injections (1-3x/24h). The samples were incubated overnight at 37°C in buffer

containing 1 mg/ml 5 bromo-4-chloro-3-indolyl B-Dgalactoside (X-Gal), 40 mM sodium citrate pH 6.0, 5% dimethylformamide, 5% potassium ferrocyanide, 5 mM ferricyanide, 150 mM sodium chloride and 2 mM magnesium chloride. Tissue samples were mounted on slides, viewed with a compound microscope, and scored for SAβGal label as indicated by blue/green reactivation product over the cell soma. SAβGal(+) cells were quantified for all brain regions (tissue samples were in triplicate).

Cell Culture

Cell lines were obtained from American Type Culture Collection (www.atcc.org): H9, human cutaneous T-cell lymphoma (#HTB-176); MG-63, human osteosarcoma (#CRL-1427); Saos-2, human osteosarcoma (#HTB-85); TT, human thyroid tumor (#CRL-1803); BJ, human fibroblasts (#CRL-2522); and RG2, rat glioma (#CRL-2433). Primary human glioma cells were generated from a surgical resection. Experiments were performed in triplicate cultures maintained in a 37^{o} C humidified chamber containing 5% CO_2.

In Vitro Drug Treatment and Quantification of Proliferative Activity

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted: BrdU (#B9285); BrdU (#B23151 from Molecular Probes, Eugene, OR); 5-chloro-2'-deoxyuridine (#C6891); 5-iodo-2'-deoxyuridine (#I7125); 5-aza-2'-deoxycytidine (#A3656); 5-fluorouracil (#F6627); thymidine (#T1895); cytidine (#C4654). Exposure times ranged from 1 min. to 24 hours, after which the medium was aspirated and replaced with fresh medium without analogs. Control and treated cultures received the same number of medium changes.

Cultures were initially plated at 2000 cells/cm^2, and were quantified with a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA) at various intervals after the removal of BrdU (range = 1 min. to several weeks). Neurosphere cultures of primary human glioma cells were established and maintained as described (Piccirillo et al., 2006).

Subcutaneous Tumors and In Vivo BrdU Administration

A bolus of either untreated or pre-treated RG2 glioma cells ($1x10^6$ cells in 250μL of PBS) was injected subcutaneously between the scapulae of anesthetized adult male Fisher 344 rats as previously described (Mariani et al., 2006). Pre-treated RG2 cells were treated with 50μM BrdU for 24 hours prior to implantation. Tumors were measured every other day in two dimensions with digital calipers, and tumor volume was calculated [$(\pi/6) \times W^2 \times L$ (W = shortest dimension and L = longest dimension)]. The experimental endpoint was defined as a tumor volume > $3000mm^3$. At endpoint euthanasia was performed by transcardial perfusion with 200 mL of 4% paraformaldehyde in PBS under deep sodium pentobarbital anesthesia (150 mg/kg, i.p.).

BrdU administration, i.p.

Untreated RG2 cells were implanted into 10 animals as described above. The BrdU regimen was initiated when palpable tumors had reached a volume of $200mm^3$. Half of the animals received 3 i.p. injections of BrdU (300mg/kg) per day for 2 days, while the other half served as controls and received an equal number and volume of sterile saline injections.

BrdU administration, oral

Again, untreated RG2 cells were implanted subcutaneously into 20 animals as described. Immediately after implantation, half of the animals were provided with drinking water containing BrdU (0.8mg/mL), and half received normal drinking water. All animals were provided with freshly prepared water (either with or without BrdU) each day for 7 days, *ad libitum*. On the eighth day after implantation, all animals were placed on normal drinking water for the duration of the experiment.

A dose of 300mg/kg corresponds to a clinical dose of $1800mg/m^2$. The rats received three of these doses per day for two days, thus receiving a total of 10,800 mg/m^2. The drinking water dose (based on the standard 20mL per day consumption by adult rats) is 640 mg/m^2/day for

seven days, or a total of 4,480 mg/m^2. By way of comparison, previous clinical trials (e.g. Kinsella et al., 1984) that included BrdU as a radiosensitizer as part of a multimodal therapy treated patients with 350mg/m^2 for continuous 12 hour infusions every day for 14 days, or 4900mg/m^2 total. Thus, the treatment range in our study is generally in accord with previous human clinical applications since, even though our injected BrdU was theoretically about twice what humans received, it is known that BrdU is active in plasma only for about 2 hours. Therefore, the continuous infusion employed in the human trials likely resulted in more widespread BrdU incorporation than our injection paradigm.

Flow Cytometry

Cell cycle analysis (Propidium Iodide)

Cells were fixed overnight in 70% ethanol and then incubated for 1 hour at 4°C in PBS containing 50μg/ml each of propidium iodide (#P-4170; Sigma-Aldrich) and RNase A (#R6513; Sigma-Aldrich). Samples were processed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed with FlowJo Flow Cytometry Analysis Software (Ashland, OR).

Annexin-V

Cells were harvested at various time points following BrdU administration (50μM) to assess Annexin-V staining using the Vybrant Apoptosis Assay Kit #9 (V35113, Molecular Probes). Briefly, cell pellets were obtained by centrifugation and resuspended at 1 x 10^6 cells/ml in 1X Annexin Binding Buffer (ABB). Annexin V (APC) and SYTOX green stain were added to the cell suspension and incubated at 37^{o}C, 5% CO_2 for 15 minutes. The cell suspension was diluted with 1X ABB, gently mixed, and analyzed by flow cytometry (530/660nm). Populations were separated based on high and low levels of red and green fluorescence.

JC-1

The JC-1 assay was performed to determine if BrdU exposure causes any changes in mitochondrial membrane potential. Control and BrdU-treated cells were suspended in warm medium at 1 x 10^6 cells/ml. The positive control sample was treated with CCCP and incubated at 37^{o}C for 5 minutes. All groups received 2μM JC-1 and were incubated at 37^{o}C, 5% CO_2 for 30 minutes. Cells were washed once and resuspended in 500μl of PBS. Samples were then analyzed on a flow cytometer with 488nm excitation using the appropriate emission filter for Alexa Fluor 488 dye and R-phycoerythrin. Cells were gated to exclude debris and standard compensation was performed using the CCCP-treated sample.

Western Blots and Densitometry

Proteins were isolated from adherent cell lines (RG2 and BJ) by incubation with RIPA extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% sodium dodecyl sulfate) containing protease inhibitor cocktail (Calbiochem, San Diego, CA) on flasks over an ice bath for 5 min followed by cell scraping. Extracted protein was quantified against a bovine serum albumin (BSA) standard using a DC protein assay reagent kit (Bio-Rad, Hercules, California). Equal protein was loaded onto Bis-Tris 4-12% density gradient gels and electrophoresis was performed using NuPAGE MES [12(N-morpholino) ethane sulfonic acid] reducing buffer system (Invitrogen) for 50 minutes at 200 V. Proteins were transferred onto nitrocellulose membranes for 1-1.5 hour(s) at 30 V. Non-specific binding was blocked with 5% bovine milk in Tris buffered saline plus 0.05% Tween 20. Membranes were incubated in blocking buffer at room temperature for 1 hour prior to being probed with the following primary antibodies: Phospho-specific (Ser249,Thr252) anti-retinoblastoma (1:500, PC640, Oncogene Research Products), p21 (1:500, ab7960, Abcam), anti-human retinoblastoma protein (1:200, #554136, BD Pharmingen) and anti-actin (1:2000, A-4700, Sigma). Membranes were incubated

in primary antibody at 4^{o}C overnight and then probed with an appropriate secondary antibody for one hour at room temperature. Finally, immunopositive proteins were detected by autoradiography using ECL reagents (GE Healthcare Life Sciences, UK) and densitometry was quantified with Image J software.

TUNEL Assay

Apoptotic cells from control and BrdU-treated cultures were visualized using a fluorimetric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (DeadEnd Fluoremetric TUNEL System G3250; Promega) according to the manufacturer's recommendations. This assay measures the fragmented DNA of apoptotic cells by incorporating fluorescein-labeled dUTP at the 3′ ends of DNA strands. Cells were counterstained with Vectashield + DAPI (H1200; Vector), and cell death was quantified by counting the number of TUNEL+ nuclei on each of three coverslips at five predetermined stage coordinates. The criterion for apoptotic cells was intentionally liberal to avoid undercounting (i.e., nuclei with even the faintest evidence of fluorescein label were considered positive).

Telomere Assays

Telomere Length

To determine if the effects of BrdU are related to changes in telomere length we performed a TeloTAGGG assay (#2209136; Roche). Briefly, genomic DNA was isolated (#11814770001; Roche) and digested with Hinf1 and Rsa1 enzymes. Following digestion, the DNA fragments are separated by gel electrophoresis and transferred to a nylon membrane by Southern blotting. The blotted DNA fragments were hybridized to a digoxigenin(DIG)-labeled probe specific for telomeric repeats and incubated with a DIG-specific antibody covalently coupled to alkaline phosphatase. Finally, the immobilized telomere probe was visualized by virtue of alkaline phosphatase metabolizing CDP-*Star*, a highly sensitive chemiluminescence substrate.

Telomerase activity

The TRAPeze Elisa kit (#S7750; Chemicon) assay was employed to determine levels of telomerase activity in our control and BrdU-treated cells. Briefly, the sample cells' telomerase adds a number of telomeric repeats (GGTTAG) onto the 3' end of the biotinylated Telomerase Substrate oligonucleotide (b-TS) and the extended products are then amplified by the polymerase chain reaction (PCR). The extension/amplification was performed with biotinylated primer and DNP-labeled dCTP. Thus, the TRAP products are tagged with biotin and DNP residues and the labeled products can be immobilized onto streptavidin-coated microtiter plates via biotin-streptavidin interaction, and then detected by anti-DNP antibody conjugated to horseradish peroxidase (HRP). The amount of TRAP products was determined by means of the HRP activity using substrate TMB and subsequent color development.

Statistics

All analyses were performed with Graphpad InStat & Prism 4 (San Diego, CA). Two-group comparisons of cell counts were performed with the Students T-test. Multiple group comparisons were performed with either a one-way or two-way analysis of variance (ANOVA). Tumor progression data is expressed as Kaplan-Meier survival curves.

For the compound statistical model (Figure 4-3) the ratio is estimated using mean (exp group)/mean (control group) for each cancer line/time point combination. The standard deviation was computed according to Cochran's equation 6.4 (Cochran, William G. 1977 Sampling Techniques 3rd Ed., Wiley, New York). Under the null hypothesis that the ratio is 1, the test statistic is a z-statistic, which has the standard normal distribution if the null is true. An asterisk (*) indicates significance using the Bonferroni correction for multiple tests.

CHAPTER 3
BRDU INDUCES A SENESCENT-LIKE STATE IN NEURAL STEM AND PROGENITOR CELLS

Stem cells are generally characterized by functional criteria that include the ability to proliferate, self-renew over an extended period of time, and generate progeny that can differentiate into the primary cell types of the tissue from which it is obtained (Reynolds & Rietze, 2005). To assess these characteristics scientists must design and employ proper assays. In the case of the neural stem cell, the neurosphere assay (NSA) has emerged as the assay of choice for the detection and expansion of neural stem and progenitor cells. Neural stem cells can be isolated from neurogenic regions of brain tissue (e.g., subventricular zone, hippocampus) that has been mechanically dissociated and plated on non-adherent culture dishes in appropriate media containing growth factors. In time, free-floating colonies of cells called neurospheres will appear. Neurospheres can then be dissociated into a single cell suspension and replated on culture dishes. Each series of growth and dissociation is referred to as a passage.

To assess the differentiation potential of the cells within the neurosphere, one must plate the cells in an adherent culture condition and withdraw serum and growth factors. Generally the cells at the periphery of the neurosphere will migrate and differentiate into three main cell types (astrocytes, oligodendrocytes, and neurons) while the cells at the center remain undifferentiated. Because the NSA allows for the expansion of both stem and non-stem cell populations, one must be cautious in the interpretation of the NSA because it does not provide a reliable or accurate readout of stem cell frequency (Reynolds & Rietze, 2005). However, this assay continues to be the standard for studying the biology of this particular population of stem cells. The NSA is critical in determining how various exogenous factors influence the proliferation kinetics and differentiation potential of neural stem and progenitor cells.

The halogenated thymidine analog 5-bromo-2-deoxyuridine (BrdU) can incorporate into DNA during S-phase of the cell cycle and can therefore be used to detect events including cell division, DNA repair and cell cycle re-entry. BrdU is commonly used to quantitate proliferative index or to birthdate, identify and track cycling endogenous and transplanted neural stem/progenitor cells. However, quantitative comparison of independent studies of neurogenesis is difficult because BrdU does not exclusively label dividing cells and there is no standardized dosing schedule (reviewed in Taupin, 2006). In addition to these technical issues, some data suggest detrimental downstream functional effects of BrdU incorporation. Exposure to BrdU has been shown to be toxic to precursor and mature neuronal cells and has also been shown to modulate the growth and differentiation of cultured neuroblastoma cells (Bannigan, 1985; Caldwell et al., 2005; Nagao et al., 1998; Schubert and Jacob, 1970). Finally, BrdU administration results in the upregulation of several markers of senescence in a variety of primary and transformed mammalian cells (Michishita et al., 1999; Suzuki et al., 2001).

Senescence was originally described by Hayflick and Moorehead (1961) in studies showing that normal human fibroblasts have a finite ability to proliferate. These cells remain viable and bioactive but the culture in its entirety experiences an irreversible loss of the ability to divide. Senescence is characterized most typically by a flat, enlarged cell shape, the induction of senescence markers such as senescence-associated beta-galactosidase (SAβGal), and resistance to apoptosis (Cristofalo and Pignolo, 1993; Dimri et al., 1995;Wang, 1997). Senescence can be induced by a variety of stimuli and is currently understood to be stimulus-, cell-type- and species-specific to varying degrees (reviewed in Campisi and d'Adda di Fagagna, 2007). Further, the initiating events leading to senescence, the resulting molecular signature, and the sequence of altered gene/protein expression is not completely understood. Importantly, despite its ubiquitous

use in stem cell biology, the implications of BrdU incorporation on the long-term function of multipotent stem and progenitor cells has not been systematically investigated. Our studies were designed to test the hypothesis that BrdU induces senescence in neural stem and progenitor cells. We show that BrdU exerts a strong anti-proliferative effect on cultured murine stem/progenitor cells. Reduced proliferation is concurrent with the onset of a senescent phenotype in BrdU-treated cells that alters cell morphology, differentiation potential and susceptibility to apoptosis (unpublished observation).

We show altered proliferation of neurosphere forming cells (NFC) isolated from animals administered an experimentally relevant BrdU treatment regimen. Together, these data uncover a novel and uncharacterized effect of BrdU administration on stem/progenitor cells that has profound implications for the interpretation of results obtained with this thymidine analog. Specifically, long-term *in vivo* BrdU administration may alter the biology of stem and precursor cell pools, severely limiting downstream applications. Conversely, the reliable induction of a senescent-like phenotype in expandable, multipotent neural stem cells may represent a novel platform for molecular studies designed to address questions regarding multiple aspects of neurogenesis and aging.

Finally, we examined the effect of BrdU administration on primary cancer cells with stem-like properties using the NSA. Putative cancer stem cells derived from primary human gliomas were treated with BrdU and the rate of population doubling was compared to that of untreated controls. A single pulse of experimentally relevant BrdU induces a reliable and dramatic reduction in the proliferation of clonally expanded progeny over numerous population doublings. This result suggests the potential of BrdU as a potent therapeutic, targeting cancer stem cells and thereby preventing metastasis.

BrdU Incorporation into Neurosphere-Forming Cells (NFC) Results in the Alteration of Morphology and Growth Rate in Subsequent Neurospheres (NS)

NFC from neonatal C57BL/6 mice (P4 to P8) were plated at clonal density, treated with BrdU (50μM) and propagated in non-adherent conditions until multicellular neurospheres were apparent. In cultures not receiving BrdU treatment, NS appear as cell clusters with a smooth and well-defined border (Figure 3-1A). NS derived from NFC exposed to BrdU, however, are substantially smaller than control NS, and usually have an uneven border (Figure 3-1B-D). Our observations led us to quantify NS proliferation dynamics by measuring both the number and diameter of spheres derived from control and BrdU-treated NFC. These parameters reflect the number of surviving NFC and the amount of proliferation within each NS, respectively. Single NFC were treated with a single pulse of BrdU (0, 10, 50, 100 μM), plated at clonal density and allowed to form NS for 7-10 days. Subsequent analysis reveals no consistent or significant differences in NS number among groups (data not shown). However, there is a significant, dose-responsive decline in NS diameter (Figure 3-2). These data show that *in vitro* administration of BrdU does not inhibit NS formation, but impairs the growth of resulting NS.

In Vivo BrdU Administration Reduces Neurosphere Yield

In order to determine if *in vivo* BrdU administration alters the growth potential of NFC progeny, we administered BrdU [100 mg/kg] or normal saline [0.9%] to young C57BL/6 mice (~P21) via intraperitoneal injections three times during a twenty-four hour period. Two hours following the final injection, animals were sacrificed and tissue harvested for culture of primary NS as well as histology. NS were then plated, counted and measured as in the *in vitro* treatment experiments. NFC obtained from BrdU-treated animals yield fewer primary NS (Figure 3-3) with no significant difference in neurosphere diameter (Figure 3-4) when compared to those obtained from control animals. When cultures are qualitatively considered, it is clear that a wide variation

in NS diameter exists in BrdU-treated cultures. Rather than a population of uniformly smaller NS, there appear to be both larger spheres that resemble those of control cultures, and irregular small spheres that resemble *in vitro*-treated NS. These results confirm an effect on NS growth following *in vivo* BrdU administration.

In Vivo BrdU Administration Increases the Population of SAβGal(+) Cells

We have shown that BrdU administration results in suppressed proliferation of neural stem and progenitor cells. Interestingly, this does not appear to be the result of cell death (unpublished observation). The alteration in proliferation kinetics suggests the possibility that BrdU-treated cells are either in a senescent- or quiescent-like state. Since we did not find any evidence of recovery in treated cells, we argue that the cells are more likely to be in a senescent state. While there is no single definitive marker of senescence, senescence-associated beta-galactosidase (SAβGal) labeling is the most commonly used method for identifying senescent cells.

Animals were treated with BrdU as above and histological samples were collected from the neurogenic regions of the adult brain. The tissue was stained with SAβGal and positive cells were quantified for the various brain regions. There is a baseline amount of senescent cells in the control samples. However, a dose-response can be seen in the BrdU-treated groups where samples from animals receiving three injections show an increase in SAβGal(+) cells compared to animals receiving saline injections or only one BrdU injection (Figure 3-5). Similar results were found in the lateral subventricular zone, dorsal and ventral blades, while the hilus contained significantly more senescent cells overall.

BrdU Inhibits Proliferation of the Putative Cancer Stem Cell of the Brain

The hierarchical model of cancer suggests that a rare subset of cells within the tumor have significant proliferation capacity and the ability to generate new tumors. In this model the remainder of the tumor cells represents differentiating or terminally differentiated cells. Brain

tumor stem cells have been identified and isolated from glioblastomas, medulloblastomas, and ependymomas. These cells share the cardinal characteristics of stem cells and also possess the ability to initiate cancer upon orthotopic implantation (Reya et al., 2001; Vescovi et al, 2006). While the mechanism responsible for the generation of cancer stem cells remains unknown, many believe that disruption of the regulatory mechanisms that control self-renewal are likely involved (Reya et al., 2001).

The actual criteria for identifying brain tumor stem cells remains nebulous; however, to date, all of the reports that describe the isolation and characterization of putative brain tumor stem cells have used the neurosphere assay to help confirm the existence of this specific population of cells (Vescovi et al., 2006). The ability to culture brain tumor stem cells *in vitro* provides both a model system for testing known therapeutic agents and a platform for identifying specific antigenic and molecular markers that might target the tumor-initiating cell.

We examined the effect of BrdU administration on primary cancer cells with stem-like properties using the neurosphere assay (Reynolds and Rietze, 2005; Marshall et al., 2007). Neural stem-like cells capable of forming NS are present in primary gliomas (Ignatova et al., 2002), and represent tumor-initiating cells in serial transplantation paradigms (Piccirillo et al., 2006; Vescovi et al., 2006). NS derived from primary human gliomas and treated with a single pulse of either 5 or 10μM BrdU show a dose-dependent reduction in the rate of population doubling as compared to untreated control cells (Figure 3-6). Cancer stem cells seem particularly susceptible to the anti-proliferative effect of BrdU such that a single pulse of BrdU reliably and dramatically slows the proliferation of clonally expanded progeny over numerous population doublings.

Discussion

We have demonstrated that neural stem and progenitor cells exhibit a dramatic inhibition of proliferative capacity after single-pulse BrdU-administration. *In vitro*, BrdU-treated neurosphere-forming cells (NFC) produce smaller primary neurospheres that also show an impaired capacity for generating secondary neurospheres. These results have profound implications for the interpretation of experimental outcomes based upon BrdU incorporation, and may limit downstream functional assessment of labeled stem/progenitor cells and their progeny since incorporating cells may have radically altered biology. Likewise, quantitative long-term *in vitro* and *in vivo* labeling paradigms can be expected to underestimate stem/progenitor cells and their progeny due to the proliferation suppression following BrdU administration.

In vivo administration of BrdU leads to a reduction in the number of primary NS subsequently obtained from the neural stem cell niche. Because BrdU is known to have a negative effect on repair mechanisms, we can speculate that incorporating cells are less likely to survive the stressful dissociation procedure which combines both enzymatic and mechanical methods for separating cells. Under these circumstances a percentage of incorporating cells may not survive long enough to produce identifiable NS, thus reducing the total yield. The fact that we see a dramatic effect in the neurosphere assay, a surrogate test of stem/progenitor cell presence, suggests that relatively modest BrdU regimens may be a viable method of inducing senescence either in experimental paradigms of aging-associated changes in neural stem cell function and neurogenesis, or in the clinical setting as a potential antineoplastic approach.

Our present data support recent findings suggesting perturbed proliferation following BrdU exposure. Michishita and colleagues (1999) showed that BrdU leads to inconsistent responses of important cell cycle regulatory proteins known to affect senescence and cancer pathways. Slowed proliferation resulting from BrdU incorporation has been described in a number of

cancer cell lines (Minagawa et al., 2005), as well as in thymidine auxotrophic yeast (Fujii et al., 2002), suggesting that the effect may be universal among eukaryotic cells. We have also found that all tested primary and cancer cell lines are susceptible to BrdU-induced senescence, regardless of species, telomerase status, or status of cell cycle proteins such as p16, p21, and p53 (Levkoff et al., 2008).

While BrdU is now most frequently used for birthdating and tracking proliferative cells, it was initially introduced as a mutagen to target rapidly-dividing cancer cells (Djordjevic and Szybalski, 1960; Hakala, 1959; Hakala, 1962). More recently it has been shown that BrdU may not affect all cells in the same way, and is selectively toxic for neural progenitor cells at doses as low as 1μM (Caldwell et al., 2005), which is well below the dose typically used in *in vitro* labeling paradigms. However, because most incorporating cells seem to maintain normal function (Cameron and McKay, 2001), at least in the short term and in the absence of secondary stressors, BrdU is generally regarded as a benign substitute for thymidine.

It may seem surprising that the relationship between BrdU and senescence is only now becoming appreciated given its long history and ubiquitous use; however, this is likely due to the initially subtle, yet progressive, nature of senescence in BrdU incorporating cells. Multiple rounds of replication are required for dramatic effects on proliferation rate to become manifest given the tools typically used to assess normal cellular function. Thus, quantification of BrdU-treated cells shortly after exposure will not reveal the large divergence from normal control cells that is seen with longer post-incorporation intervals. Adult hippocampal neurogenesis, for instance, in which newly-generated granule neurons do not continue dividing after genesis in the subgranular zone would not be expected (again, in the short-term) to show overt perturbation as a result of BrdU administration.

However, such neurogenesis may eventually become impaired over time if the cell cycle kinetics of enough stem/progenitor cells is negatively affected by BrdU incorporation. While there is strong evidence for senescence induction by BrdU, the mechanism of this induction remains enigmatic. Prior microarray studies have identified a number of senescence-associated genes that are upregulated in response to BrdU administration (Suzuki et al., 2001), but their potential causative role has not been established. More recently, chromatin unpacking, regulated by BrdU incorporation into scaffold/nuclear matrix attachment region sequences, has been proposed as an initiating event to senescence induction (Satou et al., 2004; Suzuki, et al., 2002).

BrdU incorporation leads to a delayed but progressive induction of senescence in neural stem/progenitor cells that manifests over multiple rounds of replication, and is accompanied by perturbed differentiation of neural progeny. This effect is likely to be common to all stem cell pools, and emphasizes the need for caution when interpreting results based on long-term BrdU tracking over multiple rounds of replication. The reliable induction of senescence in stem/progenitor cells *in vitro* and *in vivo* may yield a novel platform for molecular studies designed to address multiple aspects of aging, and may also represent a therapeutic approach to slow the growth of cancer cells.

Cancer stem cells seem particularly susceptible to the anti-proliferative effect of BrdU such that a single pulse of BrdU reliably and dramatically slows the proliferation of clonally expanded progeny over numerous population doublings. This result strongly suggests that BrdU may be a potent therapeutic, targeting cancer stem cells and potentially slowing the regrowth of de-bulked primary tumors and/or the metastatic spread of secondary tumors. The wide penetrance of the anti-proliferative effect, combined with the ability for rapid transport across the blood-brain barrier makes BrdU an attractive candidate against all types of cancer. However, these same

attributes also make it likely that indigenous stem cell pools will be adversely affected. Therefore, potential therapeutic BrdU dosing regimens will need to be carefully tested to avoid a permanent depletion of the stem cells and long-term progenitors needed for maintaining tissue homeostasis.

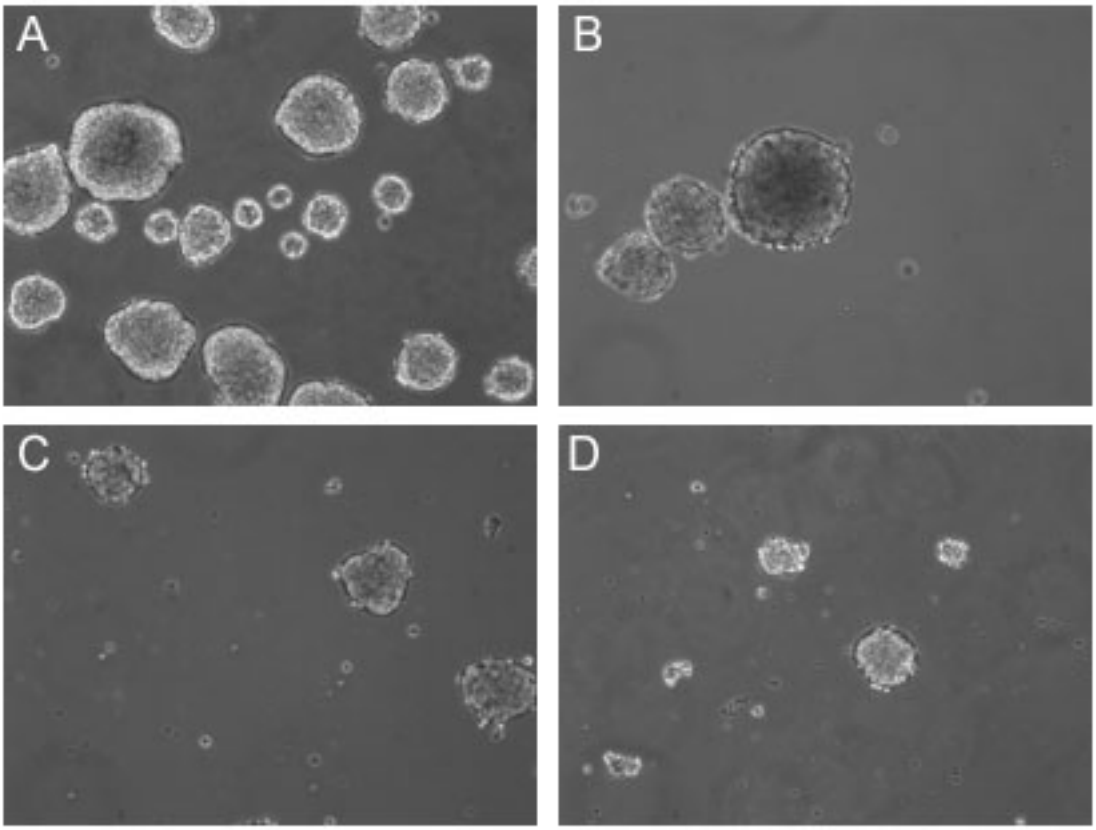


Figure 3-1. Variations in neurosphere morphology following BrdU administration *in vitro*. Representative photomicrographs showing differences in size and border structure between control neurospheres (A) and neurospheres treated with 10, 50, or 100μM BrdU (B-D).

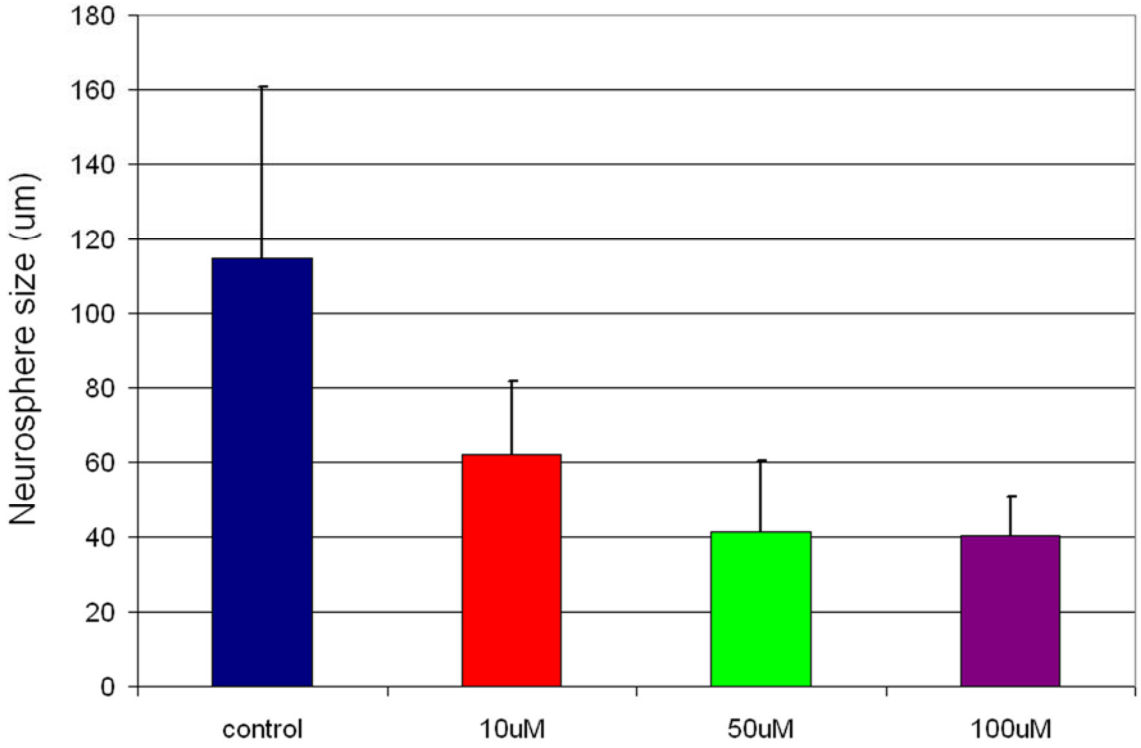


Figure 3-2. BrdU suppresses the growth rate of primary neurospheres. There is a significant dose-responsive reduction in NS diameter in BrdU-treated (10, 50 or 100 μM) NS generated from neonates.(Control v. 10μM $p<0.05$; Control v. 50,100μM $p<0.01$). Data analyzed with one-way ANOVA with the Tukey-Kramer post-hoc analysis; Error bars represent standard deviation.

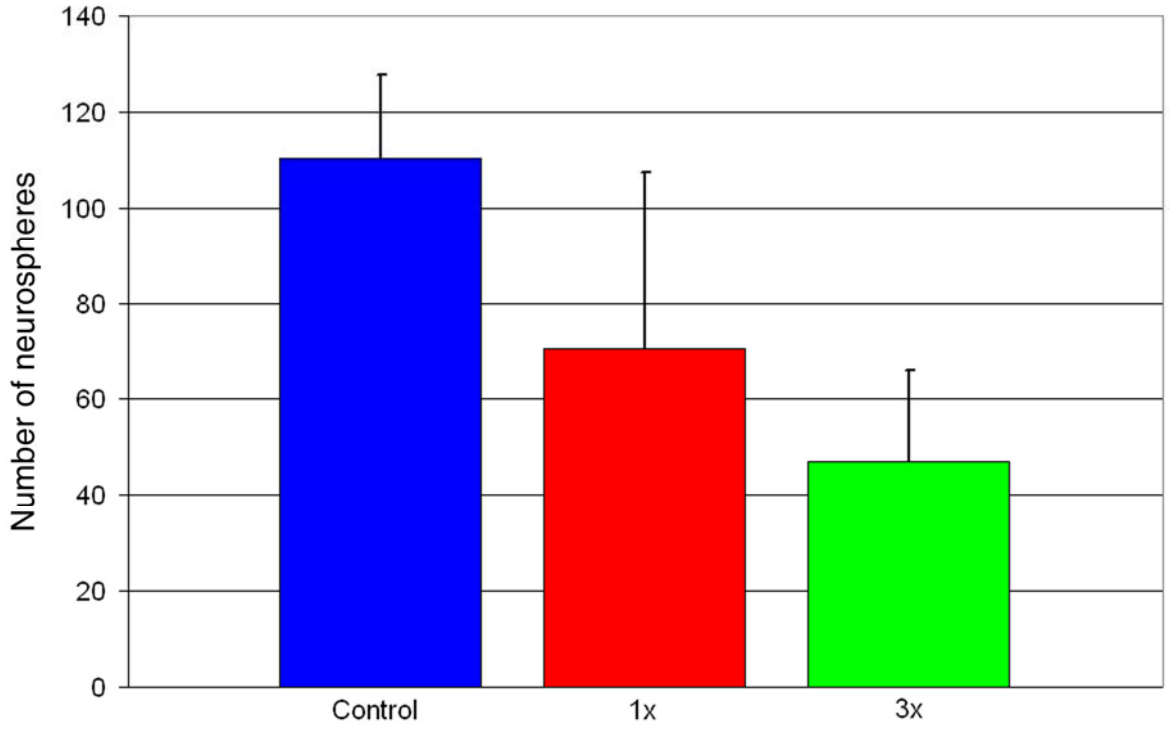


Figure 3-3. In vivo BrdU administration reduces subsequent neurosphere yield. C57/BL6 pups (P21) were given either one (1x) or three (3x) i.p. injections of 100 mg/kg BrdU or 0.9% sterile saline over 24 hours and sacrificed 2 hours after the final injection. BrdU-treated animals yield significantly fewer neurospheres than control animals (Control v. 1x, $p<0.05$; Control v. 3x, $p<0.01$). Data analyzed with one-way ANOVA with Tukey-Kramer post hoc test; Error bars represent standard deviation.

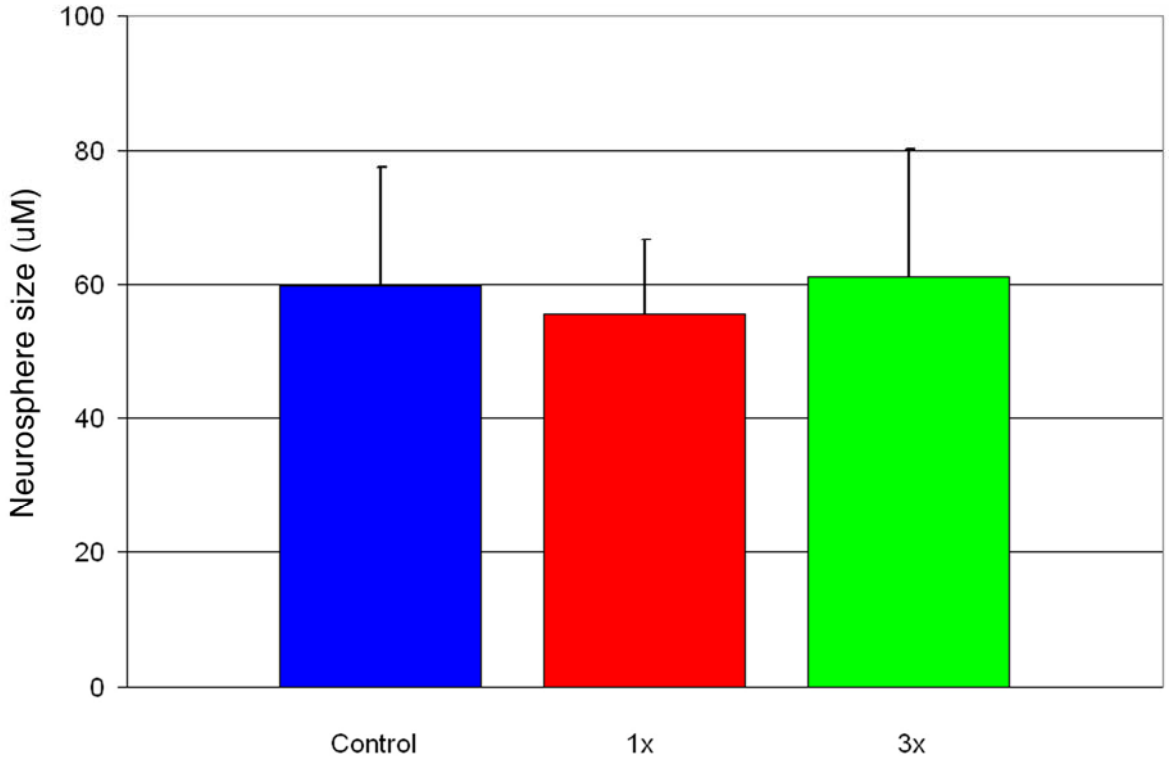


Figure 3-4. In vivo BrdU administration does not influence subsequent neurosphere size. C57/BL6 pups (P21) were given either one (1x) or three (3x) i.p. injections of 100 mg/kg BrdU or 0.9% sterile saline over 24 hours and sacrificed 2 hours after the final injection. Average neurosphere diameter was calculated for the control and experimental groups. There was no significant difference in neurosphere size between BrdU-treated and control animals. Data analyzed with one-way ANOVA with Tukey-Kramer post hoc test; Error bars represent standard deviation.

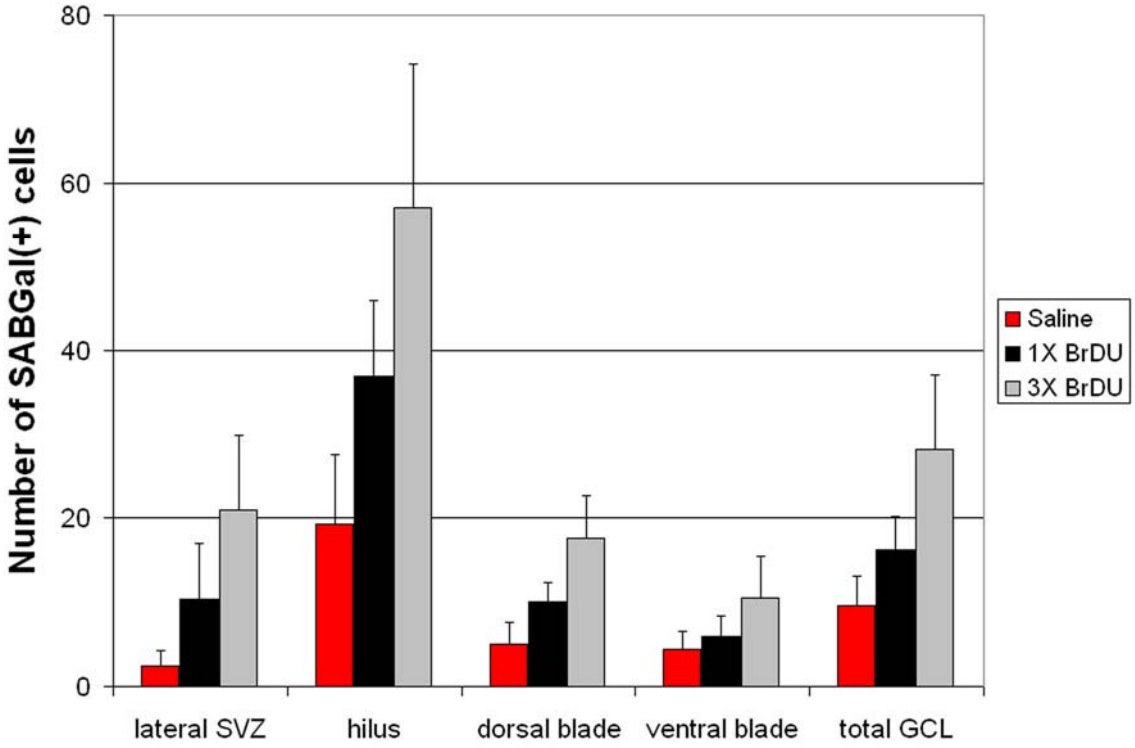


Figure 3-5. In vivo BrdU administration increases the population of senescent cells. C57/BL6 pups (P21) were given either one (1x) or three (3x) i.p. injections of 100 mg/kg BrdU or 0.9% sterile saline over 24 hours and sacrificed 2 hours after the final injection. SAβGal staining was performed on the neurogenic tissues. A dose-response can be seen in the BrdU-treated groups where samples from 3x animals show an increase in SAβGal(+) cells compared to the control or 1x animals. Error bars represent standard deviation.

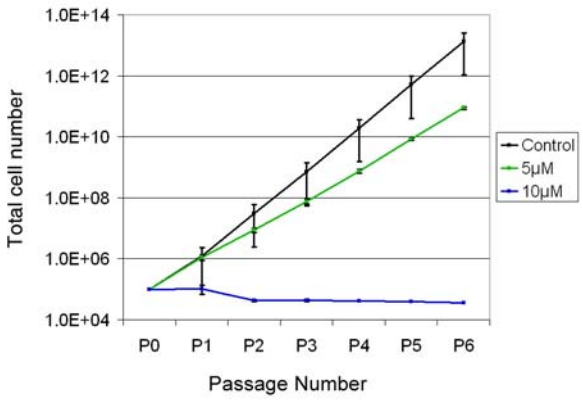


Figure 3-6. BrdU induces a progressive, dose-responsive suppression of cancer stem cell population expansion. Tumor-initiating cancer stem cells isolated from a primary human glioma were treated with a single pulse of 5 or 10μM BrdU and grown in neurosphere cultures. Neurospheres were passaged, quantified, and replated every 4-7 days. Both doses severely suppress cancer stem cell population expansion. Data are represented as mean +/- standard deviation.

CHAPTER 4
BRDU INHIBITS PROLIFERATION OF CANCER CELLS IN VITRO AND IN VIVO

The halogenated thymidine analog 5-bromo-2-deoxyuridine (BrdU) can incorporate into DNA during S-phase of the cell cycle and can therefore be used to detect events including cell division, DNA repair and cell cycle re-entry. BrdU is commonly used to quantitate proliferative index, or to birthdate, identify and track cycling endogenous and transplanted cells. In the previous chapter we reported altered proliferation of neural stem and progenitor cells following BrdU administration *in vitro* or *in vivo*. The reduction in proliferation appears to be indicative of a senescent-like state rather than the result of cell death. Administering BrdU *in vivo* led to an increase in senescent (SAβGal+) cells in neurogenic regions of the murine brain. Finally, we were able to recapitulate the anti-proliferative results from the primary neural stem/progenitor cells in a population of cancer stem cells. These data uncover a novel and uncharacterized effect of BrdU administration on stem/progenitor cells that has profound implications for both the stem cell and cancer fields. In addition to altering the biology of stem and precursor cell pools, BrdU administration may exert its anti-proliferative effect on cancer cells as well.

In this chapter we show that a single, brief exposure to BrdU leads to a reduction in the proliferation rate of all cell lines examined. This anti-proliferative effect follows even brief, low-dose BrdU administration. However, an increase in exposure time and/or a second pulse of BrdU exacerbates the proliferation suppression. Additionally, BrdU cannot be out-competed by the presence of exogenous cytidine or thymidine, suggesting that cells may actually preferentially incorporate the analog. The anti-proliferative effect of BrdU is mirrored in closely related thymidine analogs and appears to be more robust than current anti-cancer analogs. Most importantly, we show that a brief oral or systemic regimen of BrdU leads to significantly delayed tumor progression in a highly aggressive syngeneic rat model of glioma. Our results suggest that

BrdU possesses therapeutic potential as an anti-cancer agent that is independent of its role as a radiosensitizer, and that BrdU should be re-assessed as an adjunctive therapeutic modality based on a new understanding of its anti-proliferative effect.

BrdU Administration Reduces Cancer Cell Population Expansion over Time

RG2 rat glioma cells were treated once with 0, 1, 10, or 50μM BrdU for 24 hours and cumulative growth curves were obtained over 18 days (Figure 4-1A). Control and treated cells were quantified and replated at equal densities on Days 5, 12, and 18 post-treatment. At all time points the BrdU-treated cells demonstrate a statistically significant dose-responsive decrease in cell numbers that becomes more pronounced with increasing rounds of replication (data are expressed as population doublings over time, statistical analysis was performed on total cell counts). To more finely analyze the temporal effect of BrdU on cell number we repeated this experiment with non-adherent H9 human lymphoma cells, quantifying at 1, 2, or 4 days after exposure (Figure 4-1B). These results show that there is a delay of about 48 hours before statistically significant differences in cell number are detected. The majority of cell lines examined fail to display any sign of recovery. However, when we followed H9 cells that were treated for 24 hours with 50μM BrdU and quantified periodically over two months, we found the initial, dramatic reduction in the rate of proliferation was followed by a gradual recovery to near normal levels over the course of 61 population doublings (Figure 4-2).

The inhibitory effect of BrdU on proliferation is common to all mammalian cells that we have tested. Figure 4-3 is a graphical representation of BrdU-induced reduction in expansion rate for a number of cell lines. Expansion is expressed as percent of control, and all treated cells show a dramatic, sustained, and statistically significant reduction in the rate of expansion as compared to matched, untreated controls. Since our paradigm includes only a single pulse of BrdU, and since treated cells are affected for numerous population doublings, we reasoned that impaired

proliferation, while requiring initial BrdU incorporation into cellular DNA, is maintained even as the amount of retained BrdU decreases due to dilution with each round of cell division. We exposed MG63 human osteosarcoma cells to 50μM BrdU for 18 hours and assessed BrdU immunolabeling over 2 weeks. At 24 hours after treatment, greater than 95% of cells are BrdU+, and the labeling is characteristically spread over the entire nucleus (Figure 4-4A). By day 6 (Figure 4-4B) most cells are still decorated, but the pattern is patchy and less intense. On day 11 (Figure 4-4C) only about half of all cells still express immunodetectable amounts of BrdU, and the patchy pattern is more pronounced. Finally, by day 13 (Figure 4-4D) only occasional cells are labeled, and these typically show only a single focal point within the nucleus. At later times nuclear BrdU is not detected (data not shown). These data demonstrate that proliferation suppression does not depend on the continued presence of BrdU within the DNA.

Anti-Proliferation Follows Even Brief, Low-Dose BrdU Administration

To determine the lowest effective dose of BrdU for slowing expansion we treated RG2 cells with 0.01, 0.1, 1.0, or 10μM BrdU for 18 hours and quantified after 8 days. A dose response is again apparent, with 10μM eliciting a stronger effect than 1.0μM. However, doses lower than 1.0μM fail to alter expansion (Figure 4-5A). Identical results were also obtained with BJ cells (data not shown). Anti-BrdU labeling reveals that only doses of 1μM or higher result in immunodetectable levels of BrdU within the cell nuclei. At 24 hours post-treatment BrdU is seen in more than 95% of BJ cells treated with 1.0 or 10μM BrdU, while cells treated with 0.1μM BrdU or lower fail to demonstrate any immunolabeling (Figure 4-5B). This finding demonstrates that BrdU must incorporate into cellular DNA at immunodetectable levels in order to elicit the anti-proliferation effect.

BrdU is metabolized through dehalogenation and the resulting uracil residue can be excised from the DNA by the uracil glycosylase repair system (Hume and Saffill, 1986; Kriss et

al., 1963). Various studies report that BrdU is available for labeling anywhere from 2-6 hours following systemic injection *in vivo*. However, the exact half-life of BrdU in culture remains unknown. We next reduced the length of BrdU exposure in order to determine the shortest effective treatment to induce impaired proliferation. In this experiment RG2 cells were treated with 10μM BrdU for 1, 5, 10, or 60 minutes, and the cells were quantified 1 week later. All exposure periods result in statistically significant reductions in proliferation as compared to control, and all exposure times are statistically indistinguishable from one another in terms of efficacy (Figure 4-6A). Similar results were obtained with H9 human lymphoma cells exposed to 1μM BrdU for 5, 10, 20, 30, 60, or 180 minutes. Again, all exposure times were equally effective at eliciting the anti-proliferative effect.

Since we have shown that BrdU incorporation into DNA is required to elicit the anti-proliferative effect, and 60 seconds is far too short an exposure time to hit all asynchronous cells in S-phase, we further analyzed how such a transient BrdU pulse can produce such a profound effect on subsequent replication rate. We exposed both RG2 rat glioma cells and MG63 human osteosarcoma cells to 10μM BrdU for 60 seconds (60") during their log-phase of expansion. After the 60" exposure, cells were either fixed immediately and immunostained for BrdU or replenished with normal medium and fixed 24 hours later for immunolabeling. As expected, none of the cells fixed immediately after the 60" pulse exhibit the typical nuclear staining pattern associated with BrdU incorporation. In marked contrast, however, nearly all of the cells fixed and stained 24 hours after the 60" pulse show an apparently normal distribution of BrdU characteristic of incorporating cells (Figure 4-6B). Finer analysis at higher magnification shows that about 30% of the cells fixed immediately after the 60" pulse do show some punctate nuclear labeling that may be located at replication forks (Figure 4-6B inset), whereas greater than 95% of

the cells fixed 24 hours after the pulse show dramatically more robust immunolabeling (Figure 4-6B).

This finding suggests that, even within 60 seconds of application, BrdU can cross the cell membrane and perhaps enter the nucleus. Furthermore, BrdU that immediately enters the cell must somehow be sequestered in such a way that, during subsequent replication, it can be utilized by the cell for DNA synthesis. For instance, if BrdU is immediately transported into the cytoplasm and remains soluble there, then as cellular division proceeds, it is reasonable to presume that this sequestered BrdU can then be added to replicating DNA chains, thus accounting for the dramatic increase in BrdU immunolabeling seen after 24 hours. That fact that free BrdU sequestered within the cell is not detected in the immediately fixed cells is likely due to BrdU being washed out of the cell during the immunolabeling protocol, which requires both harsh denaturation to form single-stranded DNA and permeabilization to allow antibody penetration into the nucleus.

A Second Pulse of BrdU Exacerbates Proliferation Suppression

BrdU is metabolized quickly both *in vitro* and *in vivo*. The degradation of this analog generally consists of a two-step process that includes dehalogenation followed by the excision of the uracil base by a uracil glycosylase repair system. The window of opportunity for active BrdU to be incorporated into cellular DNA may be limited and, therefore, administering only one BrdU pulse may be insufficient to elicit the maximum benefit. It is important to find the correct balance in drug administration that maximizes efficacy without inducing toxicity. Delivering multiple BrdU pulses may further weaken cells that have already incorporated BrdU in the initial hit while also improving the likelihood of incorporation into cells that may not have been cycling during the initial pulse.

Our previous results demonstrate the impressive anti-proliferative effect of single-pulse BrdU. To determine if a "second hit" of BrdU would further suppress proliferation we treated RG2 cells with a 5 hour pulse of 10μM BrdU (treated). A subset of treated cells received a second 10uM pulse three weeks following the first pulse (re-treated). Cells were quantified and compared to control values at four weeks following the initial pulse (Figure 4-7). Treated cells show significantly suppressed expansion as compared to untreated controls ($p<0.001$), and re-treated cells expand significantly slower than treated cells ($p<0.001$).

BrdU-Mediated Anti-Proliferation is not Blocked by the Addition of Cytosine or Thymidine.

While BrdU normally pairs with adenosine during DNA replication, it is also known to frequently mispair with guanine (Ashman and Davidson, 1981; Kaufman and Davidson, 1978; Meuth and Green, 1974). While the exact cause of BrdU mispairing is unknown, it may be due to perturbation of normal deoxyribonucleoside triphosphate pool sizes (Meuth and Green, 1974). BrdU-triphosphate (BrdUTP) is an inhibitor of ribonucleoside diphosphate reductase, which ultimately leads to a deficiency in the conversion of cytidine diphosphate to deoxycytidine diphosphate (Meuth and Green, 1974; Moore and Hulbert, 1966). High BrdUTP concentrations, therefore, may prevent the formation of dCTP substrate for DNA synthesis. With a decrease in dCTP pools, BrdUTP becomes increasingly competitive for sites opposite template guanines, an effect that can be mitigated by the addition of excess deoxycytidine (Davidson and Kaufman, 1978).

To test whether the anti-proliferative effect of BrdU results from mispairing due to a "deoxycytidineless" state, we followed the expansion rates of cells treated with equimolar BrdU, thymidine, or cytidine, both alone and in combination (Figure 4-8). The reduced cellular expansion produced by 50μM BrdU is not abrogated by the co-administration of equimolar

thymidine or cytidine, nor by a combination of thymidine and cytidine. Furthermore, neither thymidine nor cytidine, alone or in combination, significantly reduces proliferation rate as compared to untreated controls cells. Finally, the anti-proliferative effect of 50μM BrdU is not diminished even when co-administered with up to 250μM cytidine (data not shown). These results suggest that anti-proliferation arises neither from BrdU out-competing cytidine during DNA synthesis, nor as a result of simply altering the intracellular milieu by the addition of excess nucleotides.

Halogenated Pyrimidines Suppress Proliferation More Robustly than Current Anti-Cancer Nucleosides

To determine if halogenated pyrimidines structurally similar to BrdU also perturb proliferation, we analyzed the effect of 5-chloro-2'-deoxyuridine (CldU) and 5-iodo-2'-deoxyuridine (IdU) on H9 cell expansion for three consecutive weeks after an 18 hour exposure to 1, 10, or 50μM of each thymidine analog. All three analogs produce a statistically significant dose-responsive reduction in proliferation that is remarkably similar in degree (Figure 4-9A). Furthermore, this anti-proliferation is non-synergistic, since combinatorial administration does not strengthen the effect (data not shown).

In the same experiment we compared expansion among cells treated with either BrdU or one of two anti-cancer nucleosides, 5-fluorouracil (5-FU) or 5-aza-2'-deoxycytidine (AZA). The chemotherapy agent 5-FU acts in several ways, but principally as a thymidylate synthase inhibitor. Interrupting the action of this enzyme blocks synthesis of the pyrimidine thymidine. 5-FU is transformed inside the cell into different cytotoxic metabolites which are then incorporated into DNA and RNA, finally inducing cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA. It is an S-phase specific drug and only active during certain cell cycles. AZA belongs to a class of cytosine analogues which were developed as inhibitors of

DNA methylation and have shown clinical efficacy in myelodysplastic syndromes (MDS) and acute myelogenous leukemia (Kiziltepe et al., 2007). Despite the widely accepted demethylating activity of AZA, the exact basis of its clinical efficacy and its cytotoxic mechanism still remain unclear.

To compare the efficacy of BrdU to 5-FU and AZA, H9 cells were administered either BrdU, 5-FU (FU), or AZA (A) at 0, 1, 10, or 50μM for 18 hours and cells were quantified weekly for three consecutive weeks (Figure 4-9B). At one week AZA-treated cells are not significantly different from controls, whereas both BrdU- and 5-FU-treated cells are reduced by 50-60%. At two weeks AZA-treated cells still match control levels, while the BrdU and 5-FU groups have dropped to about 10% of control. At week three the BrdU-treated cells are maintained near 10% of control level while the 5-FU treated cells recover to about 35%. AZA showed substantial variability, with some treated cells demonstrating a statistically significant reduction to about 80% of control, but this reduction is not maintained over time (data not shown). These results suggest that the suppressive effect of single-pulse BrdU on cancer cell expansion is more effective than AZA, and more persistent than 5-FU.

Length of BrdU Exposure is Directly Related to the Percentage of Labeled Cells

The efficacy of BrdU treatment depends on widespread penetrance. To determine how well BrdU is incorporated in cancer cells *in vitro*, we compared BrdU labeling in cells treated with BrdU (1, 5, 10 or 50 μM) for 1, 6, or 24 hours. Figure 4-10 shows that the exposure time is more important than dose. The number of labeled cells nearly doubles from one hour to six hours of exposure while the differences between doses are insignificant. These results are consistent with the idea that longer exposure times allow for capturing more cycling cells in an asynchronous population. Interestingly, while the different doses do not alter the number of labeled cells, the

intensity of the BrdU labeling is directly related to dose; where cells treated with higher doses of BrdU display brighter staining (data not shown).

BrdU Administration Slows Glioma Tumor Progression In Vivo

Glioblastoma multiforme (GBM) is the most common primary (i.e., non-metastatic) brain tumor of humans. Despite advances in cytoreductive and cytotoxic therapies, the prognosis for this neoplasm remains dismal, with a median survival time of approximately 12 months (Akman et al., 2002; Basso et al., 2002; Nieder et al., 2000). This has fostered an intense interest in the search for alternative therapeutic modalities that may prove to be more effective or that may augment standard surgical, radiological or chemotherapeutic treatments for these neoplasms (Mariani et al., 2007).

We chose a syngeneic, invasive, non-immunogenic rat glioma model (Aas et al., 1995; Barth, 1998; Ko et al., 1980) to test whether the proliferation suppression of BrdU has a meaningful *in vivo* correlate. The RG2 glioma model has been posited as the equivalent of human GBM, and these tumors are refractory to therapeutic modalities, rendering them nearly impossible to treat efficiently or cure. Their invasive pattern of growth and uniform lethality following an innoculum of relatively few cells make them a particularly attractive model to test new therapeutic modalities (Barth, 1998). First, we injected a bolus of RG2 cells -either untreated or pre-treated in vitro for 24 hours with 50μM BrdU- subcutaneously into Fisher 344 rats and followed tumor progression. All animals eventually develop tumors but the survival time (defined as 3000mm^3 tumor volume) is dramatically delayed in animals receiving pre-treated cells (Figure 4-11A). This result clearly demonstrates that proliferation suppression induced by *in vitro* application of BrdU is maintained in the *in vivo* environment.

In a second set of experiments we tested whether tumor progression can be slowed by *in vivo* administration of BrdU. Animals were inoculated with naïve RG2 cells and then treated

with either i.p. or oral BrdU. After implantation, one group of animals received 6 i.p. injections of BrdU (300mg/kg) over 2 days (Figure 4-11B), while a second group was placed on ad libitum drinking water containing 0.8mg/mL BrdU for 1 week (Figure 4-11C). In both cases there is a statistically significant increase in survival in the animals receiving BrdU, indicating that BrdU is effective at slowing the growth of cancer cells *in vivo* even when it is administered after tumor initiation. Even a modest increase in survival time following a conservative dosing regimen is highly encouraging when one considers the refractory nature of these tumors.

Discussion

Proliferation was suppressed in all of the cells that we examined and prior studies have also demonstrated the ubiquitous susceptibility of mammalian cells to BrdU (Michishita et al., 1999). There is even evidence that BrdU slows replication in thymidine-auxotrophic yeast (unpublished observation, Fujii et al., 2002), suggesting that BrdU-mediated proliferation suppression in all eukaryotic cells may be affected through a common yet still undefined mechanism.

The delayed *in vivo* tumor progression in the extremely aggressive RG2 model is the most important aspect of our study. Despite advances in cytoreductive and cytotoxic therapies, the prognosis for GBM remains dismal, with a median survival time of approximately 12 months (Akman et al., 2002; Basso et al., 2002; Nieder et al., 2000). There is intense interest in finding alternative therapeutic modalities that may prove to be more effective or that may augment standard surgical, radiological or chemotherapeutic treatments for these neoplasms (Mariani et al., 2007). The RG2 glioma model has been posited as the equivalent of human GBM. The fact that brief administration times used in our study result in statistically significant delays in the growth of naïve tumor cells raises the possibility that BrdU alone may be capable of producing biologically significant therapeutic gains under optimized dosing schedules. In addition, the

dramatically delayed progression of BrdU pre-treated cells (treated prior to implantation) suggests that BrdU may prove effective against secondary metastatic tumor formation. Future studies will be directed at examining the homing and invasiveness of BrdU-incorporating cells and their progeny.

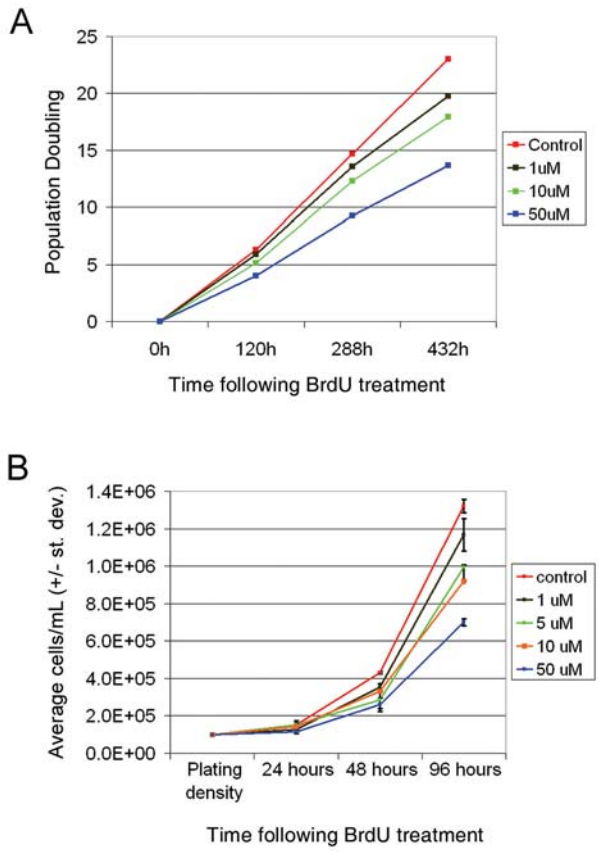


Figure 4-1. BrdU induces a progressive, dose-responsive suppression of cancer cell line population expansion. (A) RG2 rat glioma cells treated for 24 hours with 1, 10, or 50μM BrdU show a dose-responsive reduction in the rate of population doubling over 18 days following removal of BrdU. Cells from all groups were replated at equal density at 120 & 288 hours to prevent overgrowth of the culture vessels. At all time points, BrdU-treated cells lag significantly behind controls, regardless of dose (one-way ANOVA, Tukey-Kramer post-hoc test of significance was performed on the basis of total cell counts at each time point; $p<0.001$, N=3 for all groups). (B) Finer temporal analysis reveals that significantly slowed expansion is apparent as early as 48 hours post-BrdU. H9 human lymphoma cells treated for 24 hours with 1, 5, 10, or 50μM BrdU are not significantly different from control at 24 hours post-BrdU exposure, yet by 48 hours all treated groups lag significantly behind control, and the degree of lag is dose-responsive (one-way ANOVA, Bonferroni post-hoc test of significance; $p<0.001$, N=3 for all groups). Data are represented as mean +/- standard deviation.

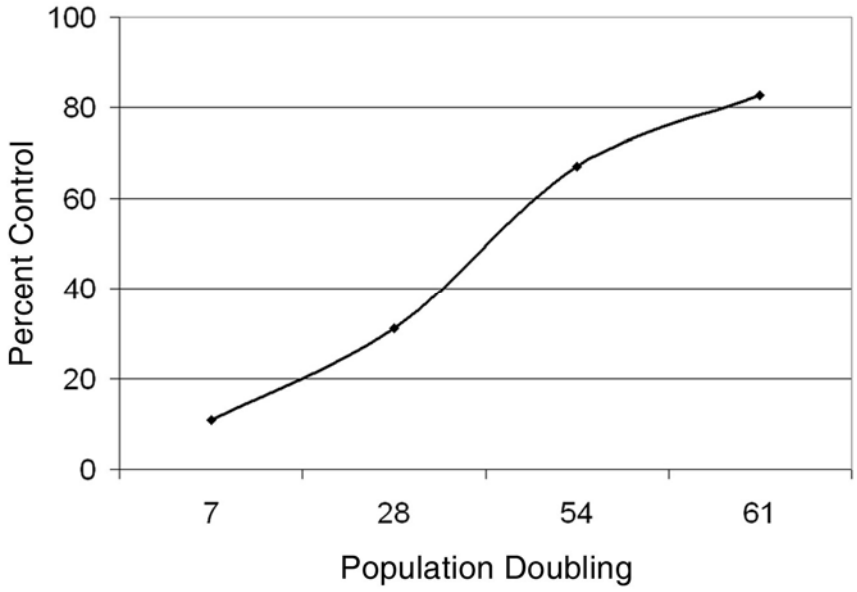
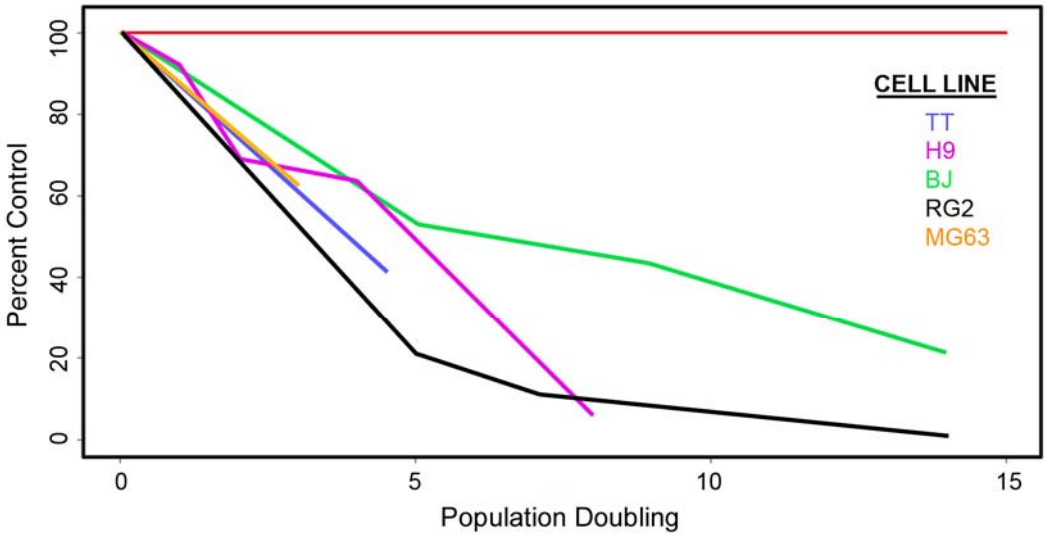


Figure 4-2. Eventual recovery following single-pulse BrdU administration. H9 cells treated for 24 hours with 50μM BrdU and followed for 61 population doublings (approximately $1x10^{16}$ times expansion) show a gradual recovery in expansion rate to near control levels.



line	time	Est. R	s.d. of Est R	z-value	p-value	Significance Using Bonferroni Correction
TT	0	1	0	NA	NA	
	4.5	0.4140	0.0122	48.0434	0.0	*
H9	0	1.0000	0.0000	NA	NA	
	1	0.9213	0.0552	1.4263	0.1538	
	2	0.6913	0.0526	5.8659	4.4663e-9	*
	4	0.6366	0.0184	19.7371	0.0000	*
	8	0.0574	0.0015	643.5753	0.0000	*
BJ	0	1.0000	0.0000	NA	NA	
	5	0.5338	0.0202	23.0678	0.0000	*
	9	0.4328	0.0278	20.3751	0.0000	*
	14	0.2132	0.0032	244.6302	0.0000	*
RG2	0	1.0000	0.0000	NA	NA	
	5	0.2128	0.0106	74.4627	0.0002	*
	7	0.1136	0.0011	831.5313	0.0000	*
	14	0.0074	0.0001	12735.2100	0.0000	*
MG63	0	1.0000	0.0000	NA	NA	
	3	0.6307	0.0425	8.6933	0.0000	*

Figure 4-3. Proliferation suppression is common among all cancer cells examined. Our standard treatment paradigm of 50μM BrdU for 24 hours causes a reliable suppression of expansion in various cell lines. The top panel shows the graphical representation of expansion by BrdU-treated cells (shown as percent control) over a range of 0 - 14 population doublings after removal of BrdU; Statistical analysis of this model is presented in the table (below).

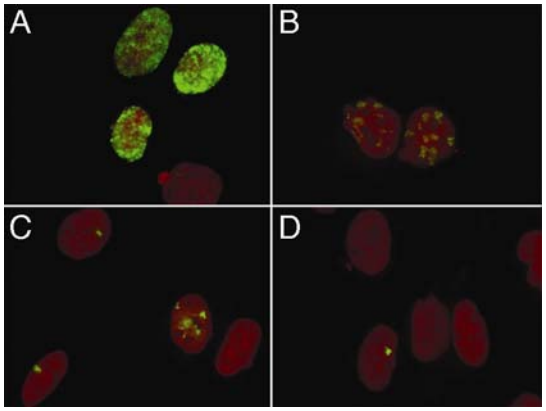


Figure 4-4. Proliferation suppression is independent of BrdU retention. MG63 human osteosarcoma cells were exposed to a single, 18 hour pulse of 50μM BrdU and assessed over time for BrdU retention. At 24 hours post-exposure (A) greater than 95% of all cells show substantial BrdU immunoreactivity (green) within the nucleus. The amount and intensity of BrdU label progressively declines at 6 (B), 11 (C), and 13 (D) days post-exposure. Cell nuclei are counterstained with propidium iodide (red).

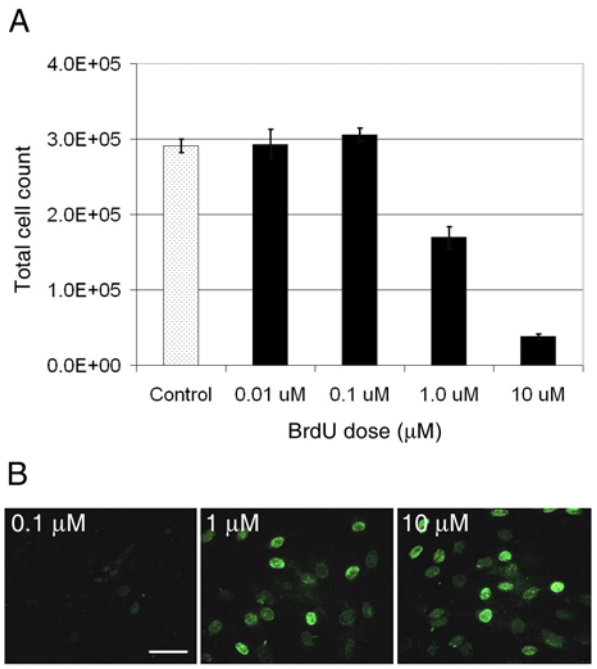


Figure 4-5. Transient, low-dose BrdU suppresses expansion rate. (A) RG2 cells were treated with a single 18 hour pulse of 0.01, 0.1, 1.0, or 10μM BrdU and quantified 8 days later. Cells receiving 0.01 or 0.1μM doses were not significantly different from control at 8 days, whereas expansion of both the 1.0 and 10μM groups was significantly suppressed (one-way ANOVA with a Tukey-Kramer post-hoc test of significance; $p<0.001$. N=3 for each group.). (B) BrdU doses that fail to suppress expansion also fail to immunolabel treated cells. Both the 1μM and 10μM groups demonstrate BrdU immunolabeling (green) of nearly all cells 24 hours after BrdU exposure, whereas the 0.1μM group does not contain immunodetectable BrdU. All nuclei are counterstained with propidium iodide (red). Scale bar = 20μm. Data are represented as mean +/- standard deviation.

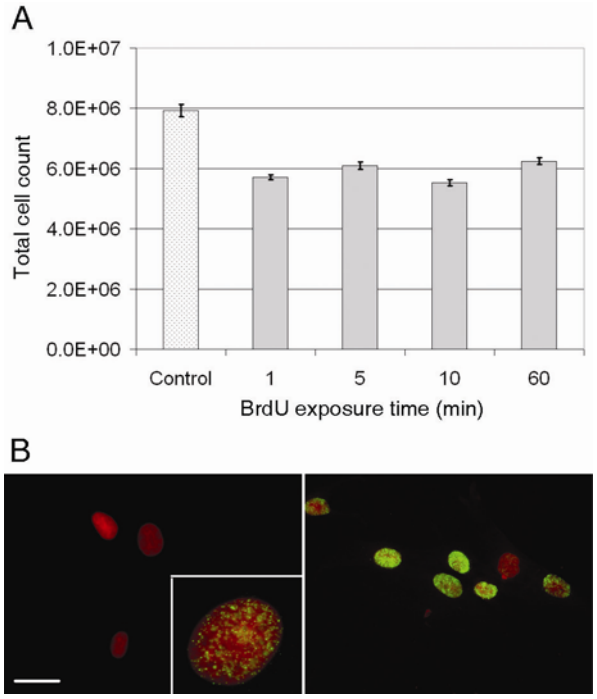


Figure 4-6. BrdU exposure times as short as 1 minute leads to suppressed expansion and delayed labeling of nuclear DNA. (A) RG2 rat glioma cells were exposed to 10μM BrdU for 1, 5, 10, or 60 minutes and quantified after 1 week. All exposure times result in statistically suppressed expansion as compared to control, and there are no significant differences among the treated groups. (B) RG2 cells treated for 1 minute with 10μM BrdU were fixed and immunostained either immediately after removal of BrdU (left panel), or 24 hours after removal of BrdU (right panel). Cells fixed immediately show little or no BrdU immunoreactivity (green), except for a fraction of cells that display subtle nuclear labeling that is apparent only at 100x magnification (inset). In contrast, greater than 95% of the cells fixed 24 hours after the 1 minute pulse show the typical pattern of nuclear immunostaining. All nuclei are counterstained with propidium iodide (red). Scale bar = 20μM.

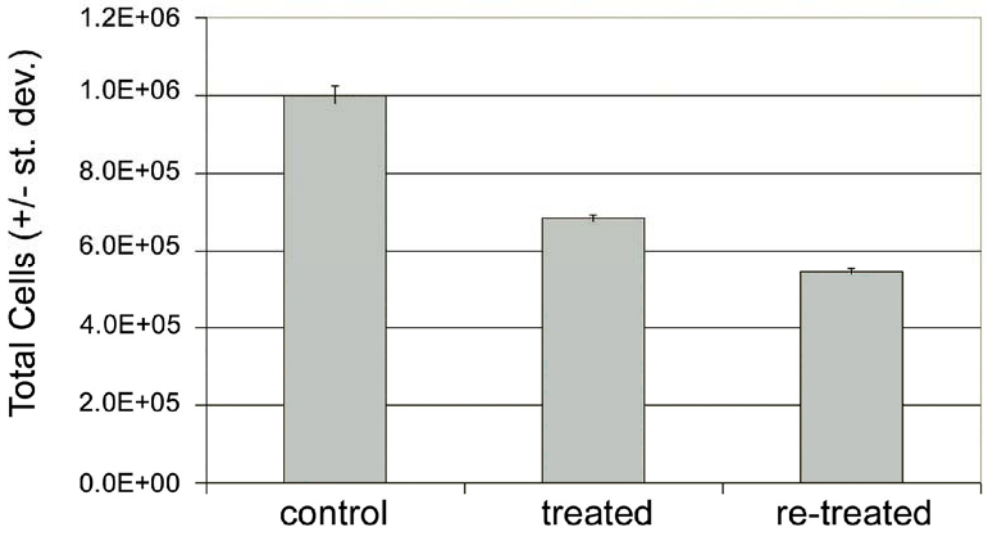


Figure 4-7. A second pulse of BrdU exacerbates expansion suppression. RG2 rat glioma cells received one 5 hour pulse of 10μM BrdU (treated). A subset of treated cells received a second 10uM pulse three weeks following the first pulse (re-treated). Cells were quantified and compared to control values at four weeks following the initial pulse. Treated cells show significantly suppressed expansion as compared to untreated controls ($p<0.001$), and re-treated cells expand significantly slower than treated cells ($p<0.001$). One-way ANOVA with Tukey-Kramer post-hoc test of significance. N=3 for all groups.

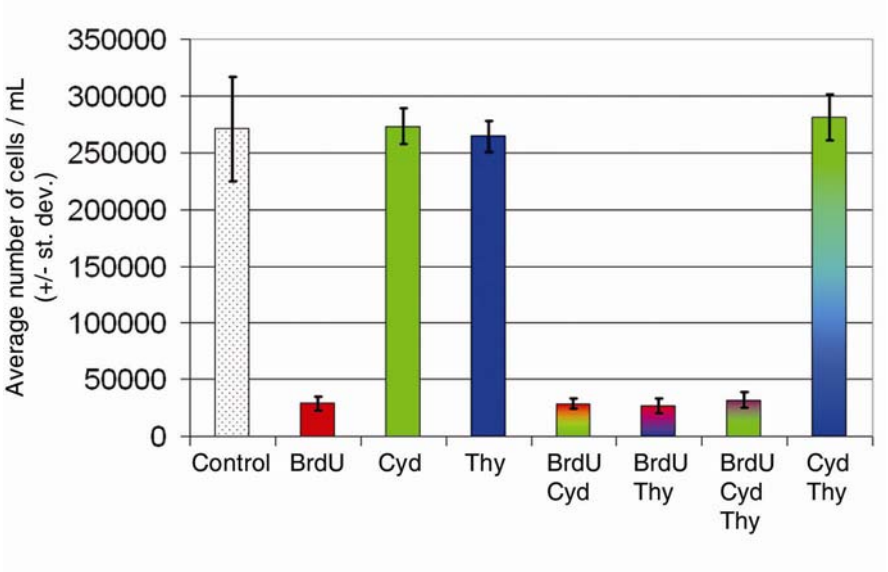


Figure 4-8. BrdU-mediated proliferation suppression is not antagonized by excess cytidine. 50μM BrdU, deoxycytidine (dCyd), and deoxythymidine (dThy) were applied to H9 human lymphoma cells in factorial combinations for 24 hours, and cellular expansion was assessed 1 week later. All of the groups receiving BrdU showed statistically indistinguishable reductions in expansion as compared to control ($p<0.001$). In contrast, groups receiving dCyd, dThy, or dCyd+dThy demonstrated expansion equivalent to control levels. One-way ANOVA with a Tukey-Kramer post-hoc test of significance. N=3 for each group. Error bars represent standard deviation.

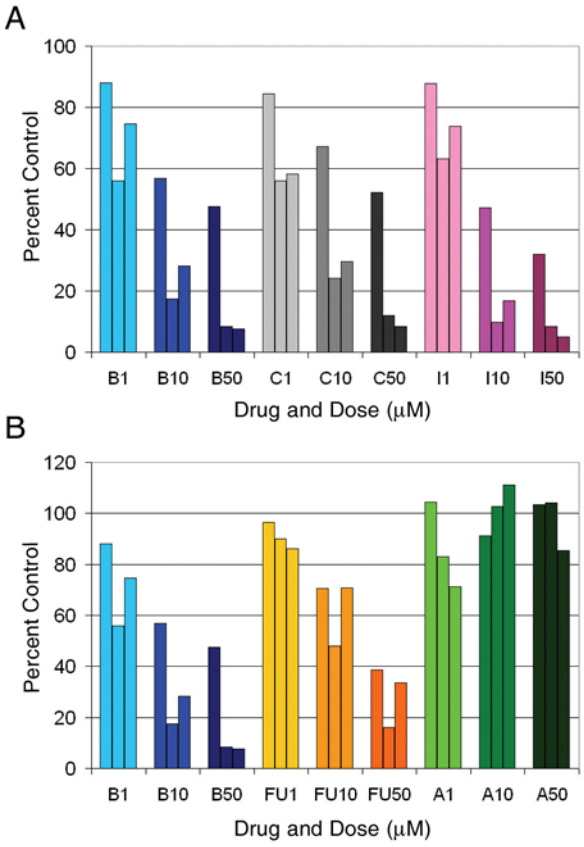


Figure 4-9. BrdU-mediated proliferation suppression is matched by similar halogenated pyrimidines and surpasses current anti-cancer nucleoside analogs. (A) BrdU (B), CldU (C), and IdU (I) were compared for the ability to suppress expansion of H9 human lymphoma cells. Each analog was administered for 18 hours at 1, 10, or 50μM and cells were quantified weekly for three weeks (weekly counts correspond to a left-to-right progression of color-coded triplicates of bars). At all doses and time points the three halogenated pyrimidines produce remarkably similar, statistically significant reductions in cell number as compared to untreated controls. (B) In the same experiment, the therapeutic anti-cancer nucleoside analogs 5-fluorouracil (FU) and 5-azacytidine (A) were also examined for their ability to suppress expansion of H9 cells in the same paradigm. 50μM FU is slightly more effective than 50μM BrdU at week one, but by week two 50μM BrdU is substantially better at suppressing expansion. At week three 50μM BrdU suppression has not changed, while 50μM FU suppression has started to recover toward control. At all other doses and time points BrdU is as effective as or more effective than FU at suppressing expansion, and BrdU suppression persists longer. 5-azacytidine shows variable and transient suppression of expansion to near 80% of control levels. Counts were analyzed with a two-way ANOVA followed by a Tukey-Kramer post-hoc test of significance. N=3 for each group. Data are represented as mean +/- standard deviation.

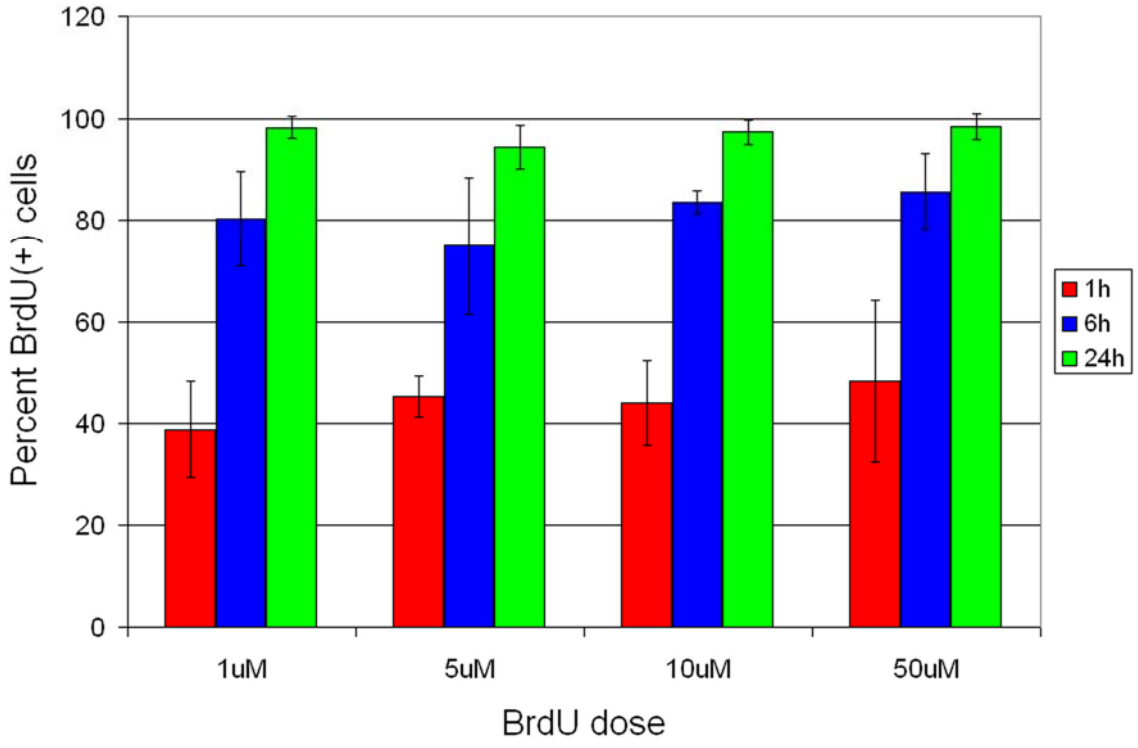


Figure 4-10. Exposure time corresponds with an increased percentage of BrdU-labeled cells. RG2 cells were treated with 0, 5, 10, or 50 μM BrdU for 1, 6, or 24 hours. The percentage of BrdU(+) cells was quantified in control and experimental groups for each dose and exposure time. There is a significant increase in the percentage of BrdU(+) cells exposed for 6 or 24 hours compared to 1 hour ($p<0.001$); however, there were no significant differences between doses. Counts were analyzed with a two-way ANOVA followed by a Tukey-Kramer post-hoc test of significance. N=3 for each group. Data are represented as mean +/- standard deviation.

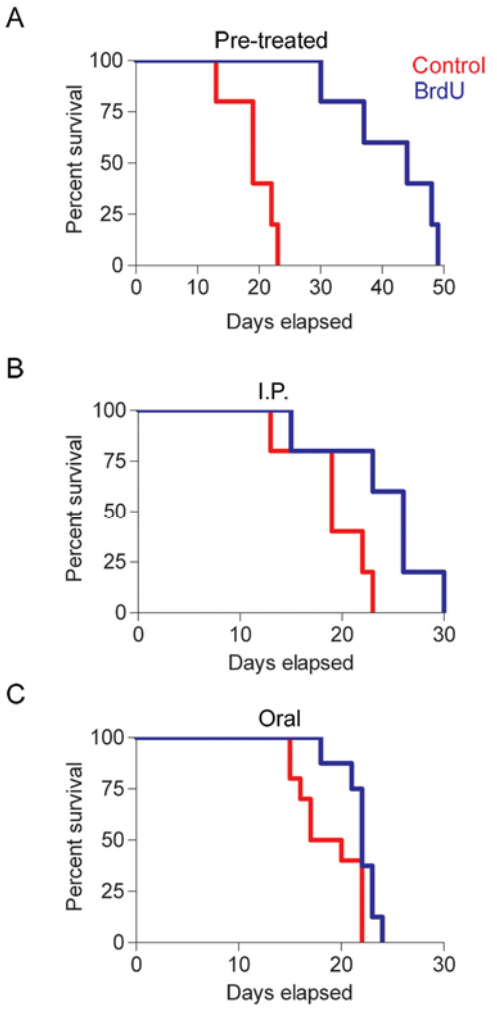


Figure 4-11. BrdU administration slows tumor progression in a syngeneic in vivo glioma model. RG2 rat glioma cells, either untreated or pre-treated with 50µM BrdU, were injected subcutaneously in Fisher 344 rats. Tumor progression was monitored by taking measurements every other day and calculating tumor volume. Animals were euthanized when the tumor volume reached the critical end point (3000mm^3). (A) Animals that received the RG2 glioma cells that had been pre-treated with 50µM BrdU for 24 hours show a significant delay in tumor progression when compared to the control animals ($p = 0.002$). (B) Animals that received subcutaneous injections of untreated RG2 cells but were administered BrdU by i.p. injections (300mg/kg x 3 / 2days) also show significant delays in tumor progression ($p = 0.02$). Additionally, (C) animals that received subcutaneous injections of untreated RG2 cells and were administered BrdU in their drinking water (0.8mg/ml for 7d) show a significant delay in tumor progression ($p = 0.04$). Kaplan-Meier survival curves (Chi-square test with associated p-value). N=10 for each group.

CHAPTER 5
CHARACTERIZING THE MECHANISM OF ACTION FOR THE ANTI-PROLIFERATIVE EFFECT OF BRDU

Despite its extensive history there is no accepted consensus mechanism of action for BrdU. It has been suggested that BrdU alters the stability of DNA thereby increasing the risk of sister-chromatid exchanges, mutations, and double-strand breaks (reviewed in Taupin, 2006). However, most of these effects are found only when BrdU incorporation is combined with secondary stressors. Early toxicity studies showed that BrdU can induce chromosomal breakage and increase the sensitivity of treated cells to ionizing radiation (Djordjevic and Szybalski, 1960; Erickson and Szybalski, 1963; Hsu and Somers, 1961), and this radiosensitizing effect has continued to be pursued as an adjunctive therapy in the treatment of a variety of cancers. BrdU readily crosses the blood-brain barrier, and has been combined with conventional chemotherapy and radiation treatment in several clinical trials (Kinsella et al., 1984, Phillips et al., 1991, Robertson et al., 1997a, Robertson et al., 1997b, Groves et al., 1999, Prados et al., 2004). While the clinical benefits of including BrdU as a radiosensitizer have been disappointing –showing, at best, modest improvements for some outcome measurements- it is possible that other therapeutic effects of BrdU were not appreciated, either because of interference by the other treatment modalities in these studies, or because finer analytical resolution is required to discern them.

Surprisingly little attention has been focused on examining the influence that BrdU alone may exert on cellular function. In the present study we show that a single, brief exposure to BrdU leads to a progressive and sustained impairment of cell-cycle progression in all examined cancer cells *in vitro*. Treated cells do not die, but gradually accumulate in the G_1 phase of the cell cycle while showing a variable upregulation of some senescence-associated proteins. DNA damage is one of the primary causes for the changes we see in the cell cycle kinetics and subsequent senescent phenotype in BrdU-treated cells. Interestingly, BrdU treatment does not

appear to cause DNA damage. It is known that senescent cells can influence their local environment through the secretion of various factors, altering growth signals for neighboring cells. However, BrdU-treated cells do not promote or inhibit the proliferation of untreated co-cultured cells.

Single-Pulse BrdU Does Not Result in Increased DNA Damage or Apoptosis

Reports associating BrdU with DNA damage generally involve the use of a secondary stressor such as irradiation, rendering it difficult to discern whether BrdU alone has an effect. It is plausible that BrdU exposure induces DNA damage which can cause stress in other cellular components, leading to amplification of apoptosis (Lin et al., 2007). H2A.X, a member of the histone H2A family, is synthesized in the G1/S phases and is involved in chromatin organization. In response to DNA damage, H2A.X is phosphorylated at the Serine 139 residue and referred to as γH2A.X. We labeled control and BrdU-treated cells with γH2A.X at various time points following BrdU administration to determine if DNA damage, specifically double-strand breaks (DSB), is induced. We found with BJ and MG63 cells that there are no consistent differences in the number of γH2A.X positive cells between treated and control groups during the first 4 days after exposure to BrdU. At no time did the percentage of positive cells in either control or treated groups exceed 2% of the total population, and in some instances the percentage was higher in the controls (Figure 5-1).

Previous studies reveal the sensitivity of BrdU-substituted DNA to visible light irradiation. This combination results in DNA lesions, specifically sister-chromatid exchanges. It is suggested that the debromination of 5-bromouracil after visible light irradiation results in the production of uracil residues (Maldonado et al., 1985; Hutchinson and Kohnlein, 1980). To determine if the anti-proliferative effect of BrdU is related to photosensitivity, we examined increased BrdU-mediated sensitivity to photolysis, as described by Michishita and colleagues (2002). Treated

cells that were protected from ambient light for five days still show profound expansion suppression, demonstrating that DNA damage due to irradiation does not account for the observed effect (Figure 5-2).

The relationship between cellular metabolism and cell cycle control is not well understood. However, it has been reported that energy deprivation can prevent passage through the G_1-S cell cycle checkpoint (Mandal et al., 2005). While BrdU can incorporate into mitochondrial DNA (Davis and Clayton, 1996), little is known about how it affects mitochondrial health and/or function. Mitochondrial membrane potential is a key indicator of cellular viability and is critical for ATP production. Additionally, the collapse of the electrochemical gradient across the mitochondrial membrane is an early event in the apoptotic cascade. We compared the mitochondrial membrane potential of treated and control MG63 cells at 1 and 7 days after a 24 hour pulse of 50μM BrdU. These results fail to reveal differences in mitochondrial membrane potential between control and BrdU-treated cells (Figure 5-3). Identical results were obtained with both H9 and BJ cells (data not shown).

Activation of cleaved caspase 3 is another early event in cellular apoptosis. We examined BJ and MG63 cells for caspase 3 expression over 4 days after a 24 hour pulse of BrdU (Figure 5-4). The percentage of cells expressing caspase in all groups was very low, never exceeding 1% of the total. Additionally, there were no consistent differences between treated cells and matched controls in either group.

We also examined Annexin-V binding which reveals the loss of plasma membrane asymmetry that allows phosphatidylserine, normally located in the inner layer, to be exposed on the cell surface. Such loss of asymmetry is thought to be associated with cells that will eventually execute an apoptotic program. H9, Saos-2, and BJ cells were examined after a single

24 hour exposure to BrdU (50μM). In addition, a dose response study was carried out with MG63 cells. The results with Annexin-V are highly variable and somewhat confusing (Figure 5-5). There is a dose responsive increase in the percentage of MG63 cells labeled with Annexin-V with greater than 60% of all cells either dead or Annexin-V(+) after exposure to 50μM BrdU for 24 hours (Figure 5-5A). The results with the other cell lines are highly variable (Figure 5-5B). Treated H9 cells seem to show a slight increase in the Annexin-V(+) population, while there is no increase in treated Saos-2 cells. BJ cells also fail to reveal differences in Annexin-V levels between treated and control groups, but the base level of expression is around 50% even in the untreated controls. Because the Annexin-V results are so dramatically different from the results obtained with other markers of apoptosis, and because there is large intra-cell line variability in both baseline Annexin-V expression and degree of change after BrdU exposure, we believe that these results do not accurately reflect cell death in our culture paradigm. This is supported by reports in the literature demonstrating that Annexin-V labeling can be transient and reversible (Hammill et al., 1999), and that Annexin-V labeling can increase even without eventual cell death (Holder et al., 2006)

Finally, in order to assess late-stage apoptosis, we examined cells with the Terminal Uridine Deoxynucleotidyl Transferase dUTP nick end labeling (TUNEL) assay which detects DNA fragmentation characteristic of apoptotic cells. As with the γH2A.X labeling, we found only negligible increases in TUNEL(+) treated cells that cannot account for the profound reduction in expansion rate, since TUNEL+ cells never accounted for more than 0.5% of the total population (Figure 5-6). Thus, three methods for detecting apoptosis indicate that the level of cell death in BrdU-treated cultures is very low and not significantly different from the control level.

BrdU Alters the Cell-Cycle Profile

The cell cycle is regulated by the sequential activation and inactivation of cyclin-dependent kinases (CDKs), through the periodic synthesis and destruction of cyclins. Understanding cell cycle control is a major focus of cancer research because it provides information on both the process of tumorigenesis as well as potential therapeutic targets. During the first gap phase (G_1) a cell prepares for DNA replication and determines its fate. Mitogenic and growth inhibitory signals are integrated and the cell uses this information to make the ultimate decision to proceed, pause, or exit the cell cycle (Johnson and Walker, 1999). The Restriction Point within G_1 represents a critical checkpoint in determining the viability of the cell. Events like DNA damage signal changes in the molecular network at the Restriction Point that influence the cell to either pause for repair and/or (permanently) exit the cell cycle.

Since BrdU leads to a reduced cellular expansion over time that is not the result of increased cell death, we hypothesize that there must be an alteration in the cell cycle profile of treated cells. We treated asynchronous BJ fibroblasts with 50μM BrdU for 24 hours and compared their cell cycle profile to control cells after one week. As expected, there is a statistically significant reduction in the proportion of BrdU treated cells in S-phase that is offset by an increase in the fraction of treated cells in G_0/G_1 (Figure 5-7). Finer analysis with similarly treated RG2 cells reveals that as early as 6 hours after BrdU administration there is a statistically significant reduction in the proportion of cells in S-phase in treated groups (Figure 5-7). The ratio of treated to control cells in S-phase varies, but at all times over 1 week post-exposure there are fewer BrdU-treated cells in S-phase. H9 cells treated for 24 hours with 50μM BrdU also show a reduction in the proportion of cells in S-phase by 24 hours post-BrdU exposure, and this reduction remains relatively stable over the next two days (data not shown). These findings

demonstrate that BrdU exposure leads to a rapid alteration in cell cycle distribution that precedes a detectable delay in expansion rate as measured by total cell quantification.

The increase in the population of BrdU-treated cells in G_1 suggests the possibility that these cells are either exiting the cell cycle or are unable to traverse the Restriction Point. The control of cellular proliferation is tightly regulated by various intrinsic and extrinsic factors. However, the retinoblastoma protein (pRb) is recognized as a guardian of the Restriction Point and cell cycle progress (Weinberg, 1995). To test the effect of BrdU on pRb phosphorylation, RG2 and BJ cells were administered BrdU (50μM) at 0 hours and cell lysates were collected at various post-administration time points (1 hour, 6 hours, 96 hours, and 7 days) for Western blot analysis. At 24 hours, the BrdU-containing media in the 96 hour and 7 day culture flasks was replaced with BrdU-free media. The level of phosphorylated pRb in BrdU-treated RG2 cells is negligible at 6 hours post-administration and there is no detectable expression at 96 hours or 7 days (Figure 5-8 A&C). Additionally, phosphorylated pRb expression is no longer detectable in BrdU-treated BJ cells by 96 hours post-administration (Figure 5-8 B&D). The time at which the level of phosphorylated pRb declines corresponds with the G_1 accumulation (see Figure 5-7). The decrease in expression level appears to be phosphorylation-specific as the levels of total pRb are comparable between control and BrdU-treated cells, particularly in the BJ cells, at these time points (Figure 5-8 E&F).

The two most commonly mutated genes in cancer are p53 and p16. These two prominent proteins can greatly influence cell cycle kinetics and can function dependently or independently of each other. Both the INK4 (p15, p16, p18, p19) and Cip/Kip (p21, p27, p57) families of cyclin-dependent kinase inhibitors (CDKIs) regulate cell progression through G_1. Interestingly, the majority of the cell lines we employ have abnormal expression patterns for many of the

primary markers related to G_1 arrest (see Table 5-1), making it difficult to assign the effect of BrdU to any of the prominent cell cycle and/or senescence pathways. However, the expression profile of a key cell cycle protein, p21, has been reported as normal in both the RG2 and BJ cell lines. The induction of p21 can prevent cell cycle progression by inhibiting a variety of cyclin/CDK complexes and/or by inhibiting DNA synthesis through PCNA binding (Johnson and Walker, 1999). Interestingly, the level of p21 does not change following BrdU treatment (Figure 5-8 E&F). This result is not necessarily surprising knowing that both H9 and Saos-2 cells do not express p21 yet are still sensitive to BrdU treatment.

The slowed expansion and altered cell cycle profile of BrdU-treated cells resembles a senescent-like phenotype and there is evidence that halogenated pyrimidines can induce senescence in a variety of cell types (Michishita et al., 1999; Suzuki et al., 2001; Michishita et al., 2002; Minagawa et al., 2005). However, the expression levels of known senescence-associated proteins are not consistently altered by BrdU exposure. For instance, senescence-associated β-galactosidase (SAβGal) activity (Dimri et al., 1995) is upregulated in RG2 cells 24 hours after exposure to 10μM BrdU (Figure 5-9). In contrast, there is no detectable SAβGal activity in severely suppressed MG63 cells (data not shown). Similar ambiguous results were obtained in relation to telomerase activity and telomere maintenance. Telomere erosion during cellular replication has been shown to activate DNA damage signaling pathways that can inhibit subsequent cell cycle progression and induce senescence (de Lange, 2006). To examine BrdU-mediated perturbation of telomerase activity as a mechanism of slowed cell cycle progression, we performed Telomeric Repeat Amplification Protocol (TRAP) analysis on control and BrdU-treated RG2 cells. TRAP analysis performed 24 and 48 hours after treatment reveals strongly reduced telomerase activity (Figure 5-10A). Again, though, this reduction is variable and cell-

line specific, as MG63 cells fail to demonstrate a reduction in telomerase activity, even three weeks post-exposure (Figure 5-10A). Furthermore, the telomerase-negative BJ and Saos-2 cell lines also demonstrates BrdU-mediated proliferation suppression, suggesting that telomerase activity is not directly involved in BrdU-mediated anti-proliferation, but may itself be reduced in telomerase-positive cells subsequent to cell cycle alterations.

Under some circumstances telomeres can be maintained by a telomerase-independent mechanism referred to as Alternative Lengthening of Telomeres, or ALT (Dunham et al., 2000). Since telomere length can be maintained in the absence of telomerase activity, and since BrdU induces slowed proliferation in cells regardless of telomerase activity, we looked for evidence of telomere shortening through terminal restriction fragment (TRF) analysis in H9 cells, but failed to detect differences in average telomere length between treated and control cells (Figure 5-10B).

BrdU-Incorporating Cells Do Not Influence the Proliferation of Neighboring Cells

Senescent cells possess an unusual phenotype that maintains metabolic activity while preventing either cell division or cell death. This level of activity allows senescent cells to secrete various factors that influence the microenvironment and neighboring cells. Inducing senescence in cancer cells has been the focus of many groups looking for novel cancer therapies. However, it remains unknown exactly how senescent cells may affect nearby cancer cells. Senescent fibroblasts have been shown to stimulate the proliferation of co-cultured cells, specifically neoplastic cells. Interestingly, the presence of senescent cancer cells also increases the proliferation of co-cultured cells *in vitro*, though less significantly than that seen with senescent fibroblasts. This “bystander effect” is believed to be a result of secreted factors including extracellular matrix proteins, growth factors and cytokines. However, senescent cells also show different secretion patterns and expression levels of these factors as compared to

actively replicating cells (reviewed in Ewald et al., 2008). These differences may allow for the specific targeting of senescent cancer cells.

The idea that senescent cells can influence neighboring cells is well supported *in vitro.* The downstream effects caused by secreted factors from senescent cells likely depend on the recipient cell type. However, the *in vivo* correlate does not appear as strong. Ewald et al. (2008) show that the transplantation of senescent cancer cells failed to increase the establishment, growth, or proliferation of non-senescent cancer cells in a xenograft model. They argue that proliferative bystander effect of senescent cancer cells are negligible and support further development of senescence induction therapy.

We have shown that BrdU administration leads to a senescent-like state in various cancer cell lines. However, it remains unclear how BrdU-treated cells may influence neighboring cells, particularly untreated cells. Our data thus far indicate that BrdU must be incorporated into a cellular DNA to alter proliferation rate. An alternative hypothesis that could account for the suppression of population doubling time is the suppression of cell cycle progression by a fraction of cells that incorporate BrdU. For example, if BrdU-incorporating cells secrete a growth-inhibitory factor, then the proliferation kinetics of a population of cells might be suppressed by a minority of BrdU-incorporating cells.

We co-cultured BrdU-treated (1μM, 3 hour exposure time) and control (untreated) H9 cells in varying combinations to determine if BrdU-treated cells affect the untreated cells. Control cells, BrdU-treated cells, a 1:1 control:treated mixture, a 9:1 control:treated mixture, and a 1:9 control:treated mixture were compared after 1 week (Figure 5-11). As expected, expansion of the BrdU-treated cells is dramatically impaired, representing only 18% of the control value. Interestingly, there is a near linear relationship among the following control:treated mixtures: the

9:1 ratio consists of 90% control cells, and their expansion is 92% of control; the 1:1 mixture falls near the midway between treated and control, at 62% of the control value; and the 1:9 mixture, containing 95% treated cells shows exactly 95% of the anti-proliferative effect with expansion that is 23% of control. These data strongly demonstrate that the anti-proliferative effect of BrdU is intrinsic to only those cells exposed directly, and there is no proliferation suppression by treated cells. BrdU does not appear to cause a bystander effect on the untreated co-cultured cells; therefore, the presence of BrdU-treated cells neither promotes nor inhibits the proliferation activity of neighboring cells.

Discussion

BrdU has a long history as a potential anti-cancer drug, and it is known that at high doses and in combination with secondary stressors, such as ionizing radiation, BrdU can have lethal consequences for incorporating cells. The present findings are surprising in that our BrdU regimen is exceedingly mild, even by current experimental pulse-chase birthdating paradigms in which cells incorporate BrdU and continue to function in an apparently normal manner. Furthermore, the suppressed proliferation that is described occurs in the absence of secondary insults that would stress the cells ability to maintain homeostasis or undergo DNA repair.

A role for BrdU in senescence-induction of mammalian cells has recently been described in the gerontology field (see Minagawa et al., 2005), but this role is not yet widely appreciated by the larger scientific community, and has not previously been applied as an approach to slow tumor progression. Emerging *in vivo* studies underscore the importance of cellular senescence in altering the growth properties of tumors in humans and rodents (Campisi, 1995; Liu and Hornsby, 2007), and we show here that cancer cells treated with single-pulse BrdU show some signs consistent with senescence. Both the altered cell-cycle profile and the upregulation of SAβGal are common manifestations of a senescent phenotype; however, SAβGal upregulation

varies widely by cell line, with some showing no enzyme activity even during severe suppression of proliferation rate, and there is evidence suggesting that SAβGal is not necessarily a selective marker of senescence (Severino et al., 2000).

While the control of cellular proliferation is tightly regulated by various intrinsic and extrinsic factors, pRb is generally regarded as the master regulator of the Restriction Point and is now implicated in cellular senescence. Rb is believed to control a senescence-initiating pathway that may synergize with, but is distinct from, telomere loss (Thomas et al., 2003; Weinberg, 1995). Our result, showing perturbed proliferation that corresponds with hypo-/un-phosphorylated pRb yet appears to be independent of telomere maintenance, supports this theory. The post-administration times at which the levels of phosphorylated pRb become undetectable in BrdU-treated RG2 and BJ cells almost perfectly correspond to the accumulation of these cells in G_1. However, results showing similar proliferation suppression in pRb null Saos-2 human osteosarcoma cells (data not shown) again indicate that pRb is not required for BrdU effect.

It seems, then, that BrdU exposure does not lead to classically defined "senescence" but rather to a generalized slowing of proliferation and does not, in our experimental paradigm, lead to crisis or subsequent cell death. Furthermore, the battery of cell lines we have examined all show similar proliferation suppression following BrdU exposure, yet differ widely in the status of quintessential senescence markers (see Table 5-1). This variability makes it difficult to define a "universal" mechanism of action and suggests that these molecular players, while possibly involved in a cell type dependent manner, are neither required nor causative for the suppressive effect of BrdU.

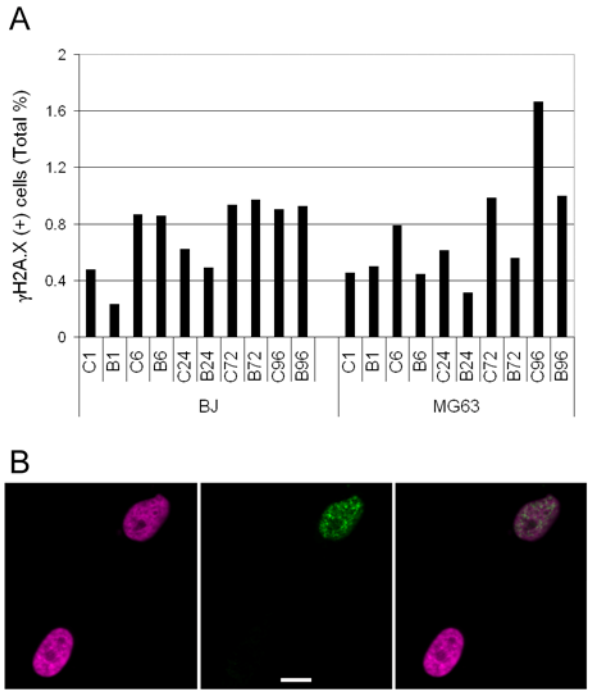


Figure 5-1. BrdU does not lead to increased γH2A.X immunoreactivity. (A) BrdU-treated (B) and matched control (C) BJ or MG63 immunostained for γH2A.X at 1, 6, 24, 72, and 96 hours after a single 24 hour pulse of BrdU, and positive cells are expressed as the percentage of the total cell population. While there is a trend toward greater γH2A.X expression in both control and treated cells with increasing time in culture, there are no consistent differences between matched control and treated samples in either cell type. (B) Representative photomicrographs showing examples of γH2A.X(+) and γH2A.X(-) MG63 cells 96 hours after a single 24 hour pulse of 50μM BrdU. The panel on the left shows DAPI staining of two nuclei (pseudocolored magenta). The middle panel shows γH2AX immunolabeling (green) of the same field of view. The right-hand panel is an overlay of two panels. Characteristic γH2AX(+) foci are present in the upper nucleus, indicating double-strand DNA breaks. Scale bar = 10μM.

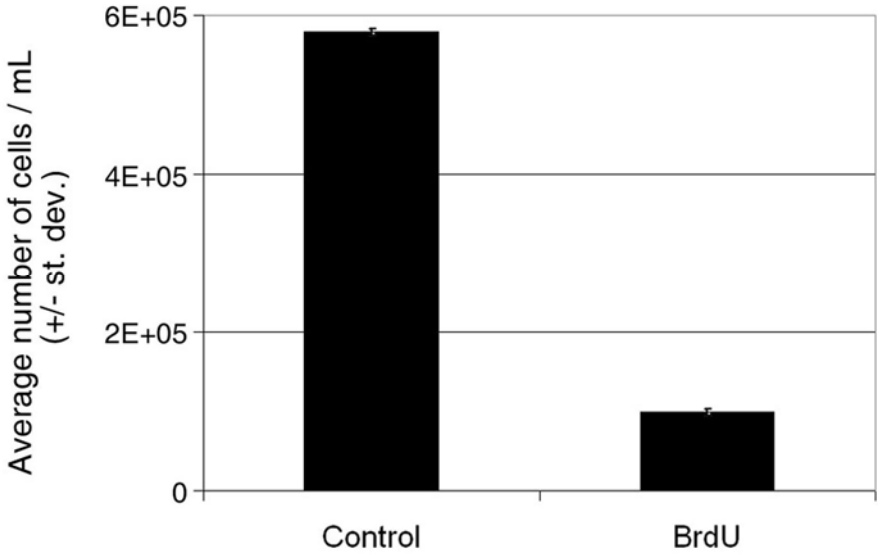


Figure 5-2. Expansion suppression is not due to BrdU-mediated photolysis. Since BrdU has been shown to increase the sensitivity of cells to irradiation, including light, we exposed MG63 human osteosarcoma cells to 50μM BrdU for 24 hours and cultured them under light protection for 5 days. Even under these conditions BrdU-treated cells expanded at a significantly slower rate than controls (unpaired t-test, Welch-corrected, $p<0.0001$). N=3 for each group; Error bars represent standard deviation.

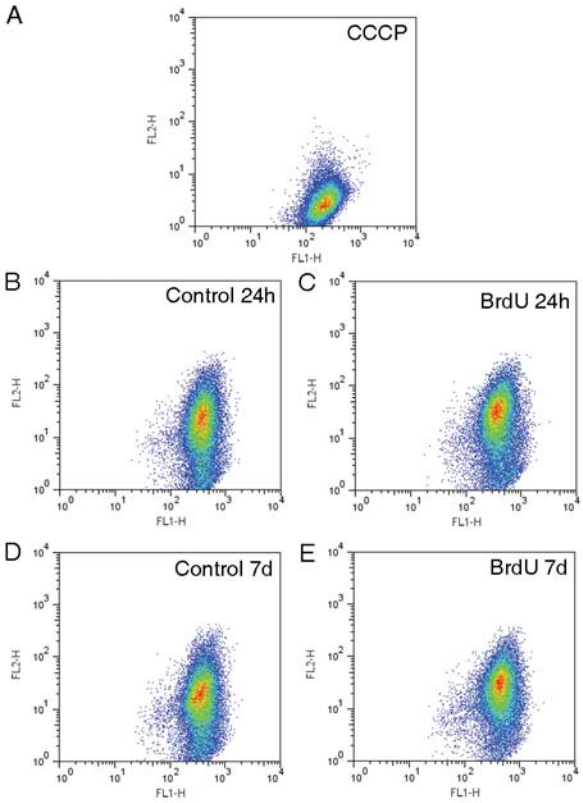


Figure 5-3. BrdU does not perturb mitochondrial membrane physiology. MG63 human osteosarcoma cells received a 24 hour pulse of 50μM BrdU and mitochondrial membrane physiology was assessed via the JC-1 potentiometric dye. Mitochondrial membrane depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. (A) Control MG63 cells were treated with CCCP (a mitochondrial membrane disrupter) as a positive control for depolarization. At both 24 hours (B&C) and 7 days (D&E) post-BrdU exposure control and treated cells display equivalent membrane potentials.

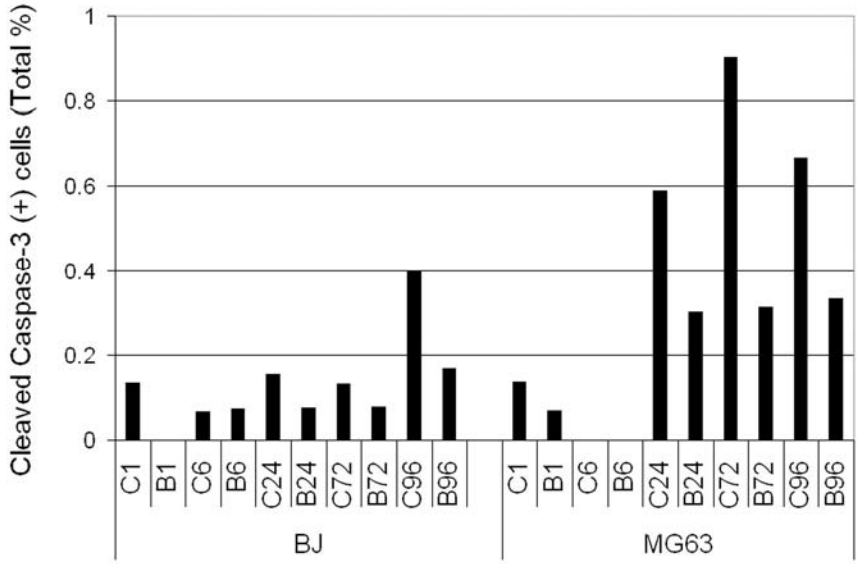


Figure 5-4. BrdU does not induce cleavage of caspase-3 in treated cells. Cleaved caspase-3 was assessed in control (C) and BrdU-treated (B) BJ and MG63 cells at 1, 6, 24, 72, and 96 hours after a single 24 hour exposure to 50μM BrdU. In none of the groups does the percentage of caspase 3+ cells exceed 1% of the total population, and there are no consistent differences between treated and control groups in either cell line.

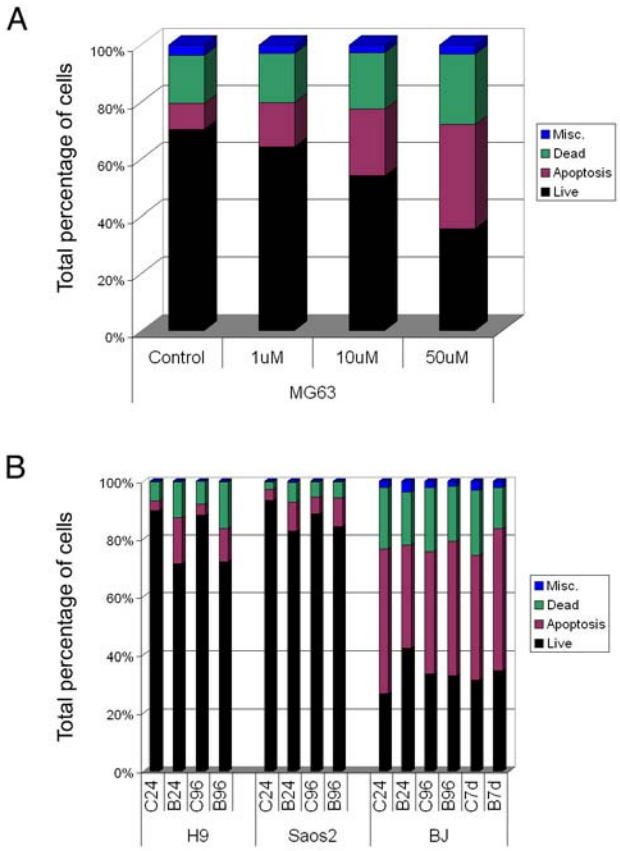


Figure 5-5. BrdU treatment causes variable, cell line-specific Annexin V assay results. (A) Annexin-V labeling (purple) shows a dose-responsive increase in MG63 cells exposed for 24 hours to 1, 10, or 50 μM BrdU. In addition, there is a slight increase in dead cells (green) with increasing concentration of BrdU. (B) Annexin-V labeling was assessed in control (C) and treated (B) H9, Saos-2, and BJ cells at various times after a single 24 hour exposure to 50μM BrdU. H9, but not Saos-2 or BJ cells show an increase in Annexin-V label (purple) by treated cells. Additionally, untreated control cells show wide variability in the constitutive level of Annexin-V labeling, with approximately 50% of control BJ cells labeled positive.

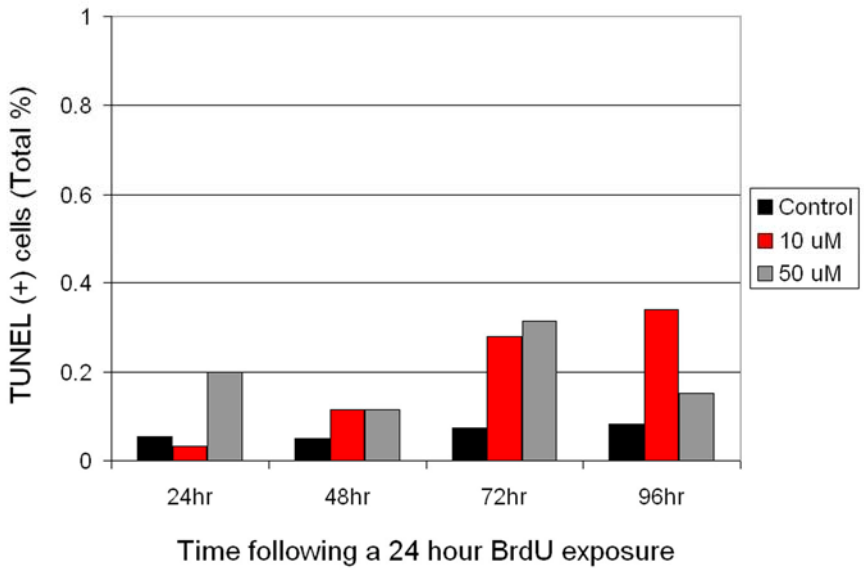
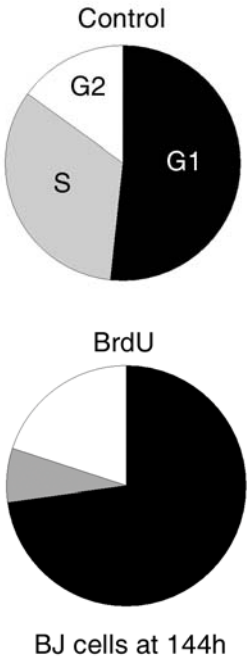


Figure 5-6. BrdU induces a negligible increase in late-stage apoptotic cell death. Apoptosis was assessed with the TUNEL assay in RG2 rat glioma cells that received a 24 hour pulse of either 10 or 50 µM BrdU. At all time points following BrdU exposure treated groups show significant increases in TUNEL+ cells as compared to control. However, even the highest rate of apoptosis represents less than 0.5% of the total cell number.



Cell line	Time (h) Post-BrdU	Treatment	%G1	%G2	%S
H9					
	24	Control	50.1	1.1	48.8
	24	BrdU	44.2	19.2	36.5
	48	Control	43.4	0	56.6
	48	BrdU	49.3	9.1	41.6
	72	Control	43.5	0.2	56.3
	72	BrdU	50.2	19.6	30.1
RG2					
	6	Control	32.6	11.5	42.0
	6	BrdU	44.6	9.7	34.4
	12	Control	34.0	9.4	44.2
	12	BrdU	38.9	9.0	38.7
	24	Control	32.6	12.8	42.7
	24	BrdU	51.1	13.1	24.2
	48	Control	39.8	8.5	38.4
	48	BrdU	51.0	12.8	22.5
	168 (7d)	Control	27.2	11.6	46.9
	168 (7d)	BrdU	31.2	15.9	37.6

Figure 5-7. BrdU alters the cell cycle profile of treated cells. Cell cycle kinetics of asynchronous control and treated cells were assessed via flow cytometry (propidium iodide staining) after a 24 hour pulse of 50μM BrdU. 144 hours after exposure, treated BJ cells (pie charts) show a substantial increase in the percentage of cells in G_1/ G_2 with a corresponding reduction in S-phase. The table on the right shows the results of finer temporal analysis with H9 and RG2 cells. Even as early as 6 hours after BrdU exposure there is a reduction in the percentage of cells in S-phase that persists over time.

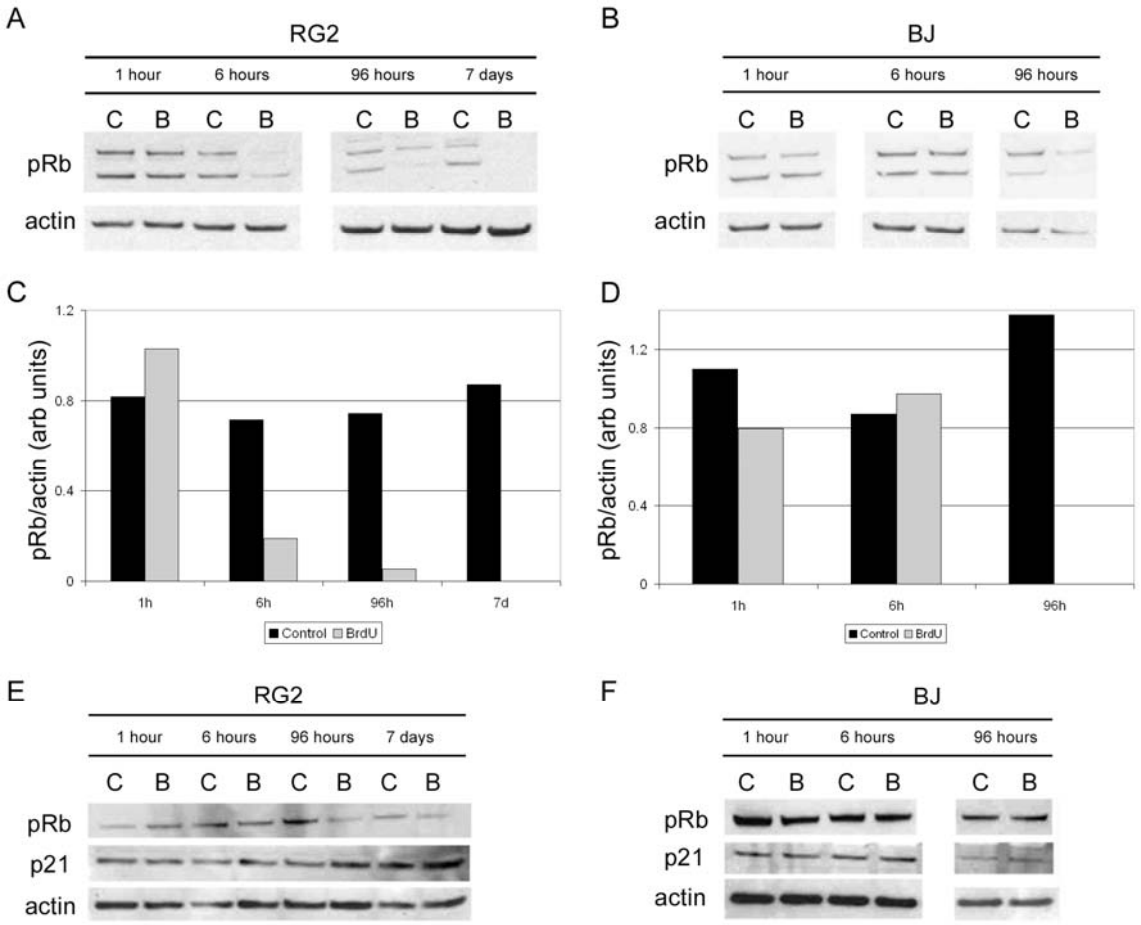


Figure 5-8. Phosphorylation of pRb is reduced in some cell types following BrdU exposure, while total pRb and p21 remain unchanged. (A&B) RG2 and BJ cells were exposed to a single 24 hour pulse of 50µM BrdU and analyzed for phosphorylated pRb (Ser249,Thr252) by Western blot analysis at 1, 6, 96 hours, and 7 days post-administration. (C&D) Densitometric analysis was performed to quantitate the changes in phosphorylated pRb protein levels in RG2 and BJ samples normalized to actin. There is a dramatic reduction in phospho-Rb beginning at 6 hours in the RG2 cells, and at 96 hours in the BJ cells. (E&F) Similarly treated RG2 and BJ cells were analyzed for total Rb and p21 protein expression by Western blot analysis. Total Rb decreases slightly in RG2 cells at one week post-administration, but is not altered in BJ cells. In neither cell type is p21 expression altered as a result of BrdU exposure. (amount of protein loaded/well: (A&B) RG2 1h, 6h: 17µg; RG2 96h, 7d: 20µg; BJ 1h, 6h: 15µg; BJ 96h: 17µg; (C&D) RG2: 15µg; BJ: 17µg).

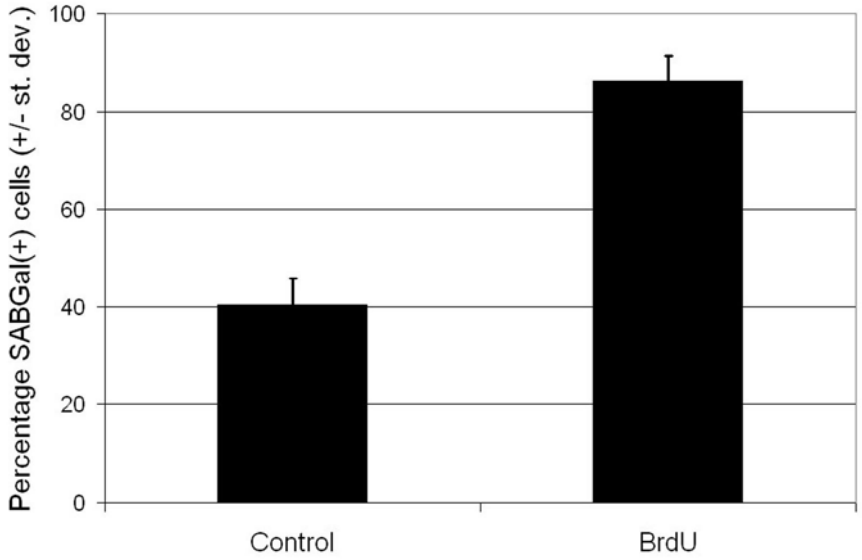


Figure 5-9. BrdU induces an increase in SAβGal activity. RG2 rat glioma cells treated with 50μM BrdU for 24 hours show an increase in the percentage of SAβGal+ cells within 24 hours. Error bars represent standard deviation.

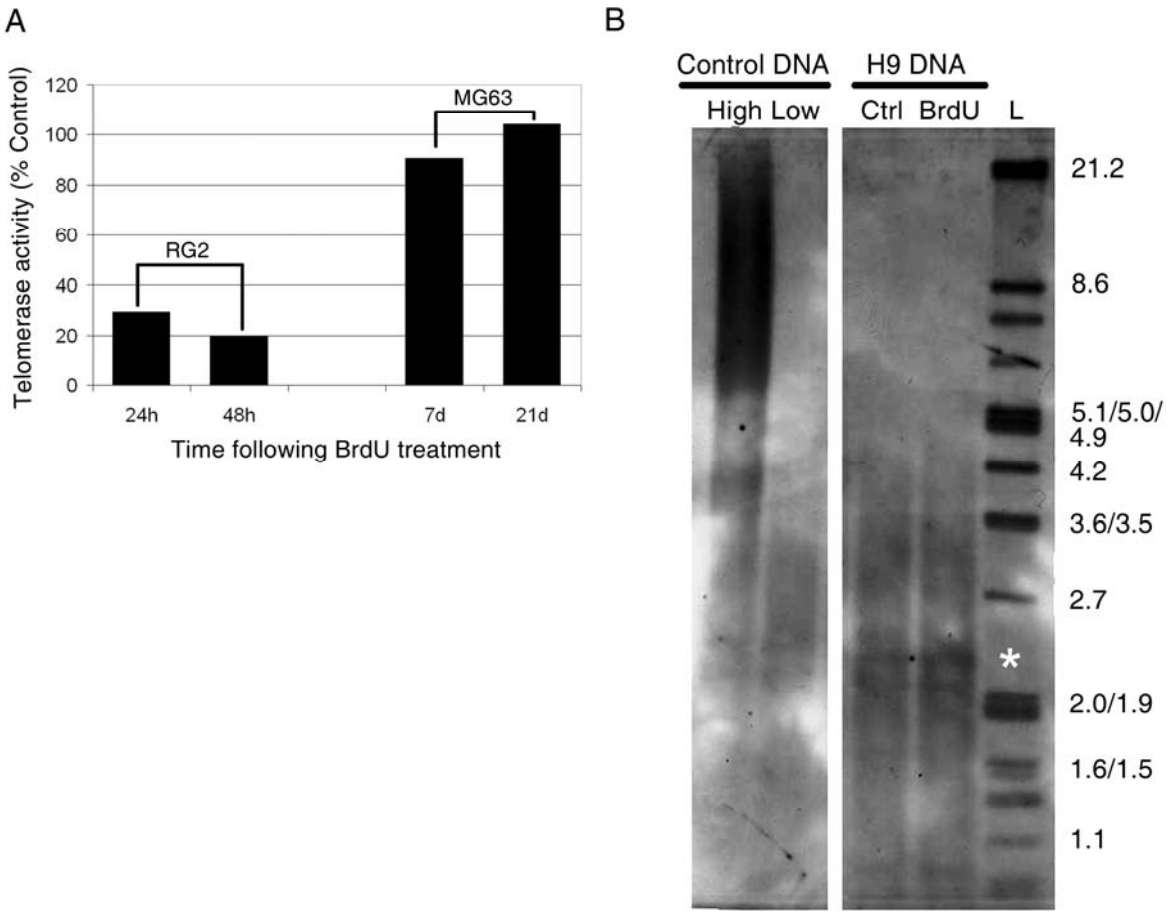


Figure 5-10. BrdU has varying effects on telomerase expression but does not alter telomere length. (A) RG2 rat glioma and MG63 human osteosarcoma cells were assayed for telomerase expression via TRAP analysis after a 24 hour pulse of 50μM BrdU. RG2 cells show a dramatic and statistically significant reduction ($p<0.001$ at 24 and 48 hours) in telomerase activity within 24 hours of BrdU exposure, while telomerase in treated MG63 cells does not vary from control levels even at 7 or 21 days post-BrdU when proliferation is severely suppressed. (B) TRF was performed on H9 human lymphoma cells 7 days following a 24 hour pulse of 50uM BrdU. There is no discernible difference in telomere length between the control and BrdU-treated cells.

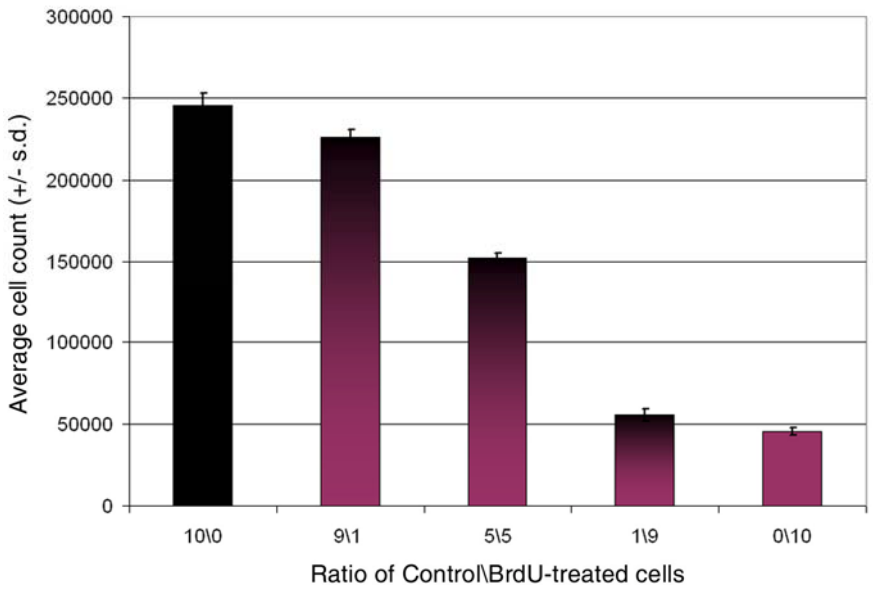


Figure 5-11. The BrdU-treated cells do not inhibit expansion of untreated cells. Control H9 human lymphoma cells were mixed in varying ratios with H9 cells that had been exposed 1 week earlier to 1μM BrdU for 3 hours. Cells were quantified after 6 days of co-culture. The results show that there is a near-linear reduction in expansion as the ratio of BrdU-exposed cells increases. The 9\1 mixture (90% untreated cells) is reduced from control (10\0 ratio) by approximately 10%. Likewise, the 1\9 ratio (90% treated cells) shows approximately 10% greater expansion than the 0\10 (100% treated cells) ratio. The equal mixture of control-to-treated cells (5\5) expanded at 62% of the control rate. These findings show that treated cells do not exert a suppressive effect on the expansion of untreated cells. One-way ANOVA with Student-Newman-Keuls post-hoc test of significance. All columns are significantly different with $p<0.001$, except 1\9 vs. 0\10 where $p<0.05$; $N=3$ for all groups.

Table 5-1. Reported statuses of prominent senescence-related markers for all cell lines tested

Cell line	Description	p53	P16	p21	pRb	Telomerase
H9	Human lymphoma	Mutant	-/-	Not expressed	Normal	Positive
RG2	Rat glioma	-/-	-/-	Normal	n/a	Positive
MG63	Human osteosarcoma	-/-	-/-	Normal	Normal	Positive
BJ	Human immortalized fibroblasts	Normal	Normal	Normal	Normal	Negative
Saos-2	Human osteosarcoma	-/-	Normal	Not expressed	-/-	Negative
TT	Human thyroid cancer	Mutant	n/a	n/a	n/a	Positive

CHAPTER 6
CONCLUSIONS AND SIGNIFICANCE

BrdU has a long history as a potential anti-cancer drug, and it is known that at high doses and in combination with secondary stressors, such as ionizing radiation, BrdU can have lethal consequences for incorporating cells. The present findings are surprising in that the BrdU regimen is exceedingly mild, even by current experimental pulse-chase birthdating paradigms in which cells incorporate BrdU and continue to function in an apparently normal manner. Furthermore, the suppressed proliferation we describe occurs in the absence of secondary insults that would stress the cells ability to maintain homeostasis or undergo DNA repair.

A role for BrdU in senescence-induction of mammalian cells has recently been described in the gerontology field, but this role is not yet widely appreciated by the larger scientific community, and has not previously been applied as an approach to slow tumor progression. Emerging *in vivo* studies underscore the importance of cellular senescence in altering the growth properties of tumors in humans and rodents, and we show here that cancer cells treated with single-pulse BrdU show some signs consistent with senescence. Both the altered cell-cycle profile and the upregulation of SAβGal are common manifestations of a senescent phenotype; however, SAβGal upregulation varies widely by cell line, with some showing no enzyme activity even during severe suppression of proliferation rate, and there is evidence suggesting that SAβGal is not necessarily a selective marker of senescence. It seems, then, that BrdU exposure does not lead to classically defined "senescence" but rather to a generalized slowing of proliferation and does not, in our experimental paradigm, lead to crisis or subsequent cell death. Furthermore, the battery of cell lines we have examined all show similar proliferation suppression following BrdU exposure, yet differ widely in the status of quintessential senescence markers. This variability makes it difficult to define a "universal" mechanism of action and

suggests that these molecular players, while possibly involved in a cell type dependent manner, are neither required nor causative for the suppressive effect of BrdU. The cell cycle data indicate that BrdU-treated cells accumulate in G_1 suggesting that these cells may either exit the cell cycle or are unable to traverse the Restriction Point. While the control of cellular proliferation is tightly regulated by various intrinsic and extrinsic factors, pRb is generally regarded as the master regulator of the Restriction Point and is now implicated in cellular senescence. Rb is believed to control a senescence-initiating pathway that may synergize with, but is distinct from, telomere loss. Results showing perturbed proliferation that corresponds with hypo-/un-phosphorylated pRb yet appears to be independent of telomere maintenance support this theory. The post-administration times at which the levels of phosphorylated pRb become undetectable in BrdU-treated RG2 and BJ cells almost perfectly correspond to the accumulation of these cells in G_1. However, a result showing similar proliferation suppression in pRb null Saos-2 human osteosarcoma cells (data not shown) again indicates that pRb is not required for BrdU effect.

Proliferation was suppressed in all of the examined cells and prior studies have also demonstrated the ubiquitous susceptibility of mammalian cells to BrdU. There is even evidence that BrdU slows replication in thymidine-auxotrophic yeast, suggesting that BrdU-mediated proliferation suppression in all eukaryotic cells may be affected through a common yet still undefined mechanism. Cancer stem cells seem particularly susceptible to the anti-proliferative effect of BrdU. The neurosphere-forming assay allows us to study the behavior of stem cell-like tumor-initiating cells, and a single pulse of BrdU reliably and dramatically slows the proliferation of clonally expanded progeny over numerous population doublings. This result strongly suggests that BrdU may be a potent therapeutic, targeting cancer stem cells and potentially slowing the regrowth of de-bulked primary tumors and/or the metastatic spread of

secondary tumors. The wide penetrance of the anti-proliferative effect, combined with the ability for rapid transport across the blood-brain barrier makes BrdU an attractive candidate against all types of cancer. However, these same attributes also make it likely that indigenous stem cell pools will be adversely affected. Therefore, potential therapeutic BrdU dosing regimens will need to be carefully tested to avoid a permanent depletion of the stem cells and long-term progenitors needed for maintaining tissue homeostasis.

Delayed *in vivo* tumor progression in the extremely aggressive RG2 model is the most important aspect of our study. Glioblastoma multiforme (GBM) is the most common primary (i.e., non-metastatic) brain tumor of humans. Despite advances in cytoreductive and cytotoxic therapies, the prognosis for this neoplasm remains dismal, with a median survival time of approximately 12 months. This has fostered an intense interest in the search for alternative therapeutic modalities that may prove to be more effective or that may augment standard surgical, radiological or chemotherapeutic treatments for these neoplasms. The RG2 glioma model has been posited as the equivalent of human GBM. The fact that brief administration times used in this study result in statistically significant delays in the growth of naïve tumor cells raises the possibility that BrdU alone may be capable of producing biologically significant therapeutic gains under optimized dosing schedules. In addition, the dramatically delayed progression of BrdU pre-treated cells (treated prior to implantation) suggests that BrdU may prove effective against secondary metastatic tumor formation. Future studies will be directed at examining the homing and invasiveness of BrdU-incorporating cells and their progeny.

In re-assessing BrdU as a potential anti-cancer drug it is important to ask why the clinical trials of BrdU as a radiosensitizer were relatively ineffective at extending survival. Even though these previous studies were concerned only with the extent to which BrdU augmented the

standard chemical and radiation therapies, one would expect that the anti-proliferative effect of BrdU should still have been evident. Based upon the present findings I can offer reasonable speculation as to why results from these earlier trials were so ambiguous. First, these clinical studies were performed solely on the basis of *in vitro* evidence of BrdU radiosensitization; thus, the study designs did not have the benefit of *in vivo* models that may have revealed dosing schedules and drug interaction effects that either optimize or attenuate BrdU incorporation. Second, in all of the clinical trials BrdU was added to an existing, multimodal therapy that involved both chemotherapeutics and fractionated irradiation. Under these circumstances it isn't clear to what extent BrdU was systemically available during stages of active tumor cell division. Related to this is the question of dose and treatment length optimization; without the benefit of animal models it simply was not possible to determine sufficient dosing schedules to maximize therapeutic outcome. Even the present results with *in vivo* BrdU administration do not necessarily represent an optimized treatment paradigm, as I was intentionally conservative in my approach, and was able to elicit a therapeutic effect while staying well below side effect limitations. It remains for future studies to extend these results to define the most effective treatment regimen from a cost/benefit perspective.

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BIOGRAPHICAL SKETCH

Lindsay Harris Levkoff was born and raised in Sarasota, Florida. She graduated from Pine View School in June 1999. Lindsay attended the University of Florida from 1999 to 2001 and then transferred to the University of Colorado, Boulder, where she graduated with her Bachelor of Arts degree in kinesiology and applied physiology in May 2003. During college, Lindsay was awarded a Howard Hughes Medical Institute undergraduate research opportunity program (UROP) award to conduct research in the laboratory of Dr. Linda Watkins. Lindsay's research in behavioral neuroscience culminated in multiple presentations at scientific conferences as well as authorship on two publications (February 2005 issue of *Behavioral Neuroscience* and December 2006 issue of *Pain*). In 2004, Lindsay enrolled in the Interdisciplinary Program in Biomedical Sciences (IDP) at the University of Florida College of Medicine and received the Presidential Fellowship and Grinter Scholarship upon admission. She began her doctoral study under the guidance of Dr. Eric Laywell, in the Department of Anatomy and Cell Biology. Lindsay's graduate research characterizing the anti-proliferative effect of BrdU on highly proliferative cell populations was published in the August 2008 issue of *Neoplasia*. Upon completion of her Ph.D. in December 2008, Lindsay plans to pursue a career dedicated to the study of cancer biology.