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Clinical study

The application of 5-bromodeoxyuridine in the management of CNS tumors

Andrew Freese,[1] Donald O'Rourke,[1] Kevin Judy[1] and Michael J. O'Connor[1,2]
[1] *Division of Neurosurgery, Hospital of the University of Pennsylvania, Philadelphia, PA 19104;* [2] *Division of Neurosurgery, Graduate Hospital, Philadelphia, PA 19146, USA*

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Summary

A variety of clinical reports have described the application of the bromodeoxyuridine labeling index as an adjunct to conventional pathological examination of CNS tumors. This index has proven useful in predicting the clinical outcome associated with many such tumors. Furthermore, because of its efficacy as a radiosensitizing agent, bromodeoxyuridine (and the closely related iododeoxyuridine) has been used in combination with radiation therapy for malignant glial neoplasms, with some encouraging results. Although most studies suggest that bromodeoxyuridine is safe, there is evidence that this compound does have potential side-effects, including the observation that it is a mutagen and carcinogen in some experimental systems. A number of new alternative approaches for predicting the clinical outcome of CNS tumors has been developed based on an increased understanding of their molecular biology. However, until such approaches are better characterized, the clinical application of bromodeoxyuridine will continue to play an important role in predicting the clinical behavior of many CNS tumors.

Introduction

To better predict the biological behavior and clinical outcome of CNS tumors, a number of methods have been developed as adjuncts to pathological examination of tissue specimens. Of these methods, perhaps the most studied and accepted at this time is the bromodeoxyuridine (BUdR) labeling index, having replaced the application of tritiated thymidine labeling studies [1–3]. Based on the neurosurgical literature alone, thousands of patients have had studies performed using preoperative administration of BUdR and subsequent analysis of the BUdR labeling index. Bromodeoxyuridine is a DNA base analog, replacing the methyl group of thymidine with the bromide ion [4–7]. Because of its structural similarity to thymidine, it is incorporated into DNA during replication, and thus its labeling of cells is proportional to DNA synthesis during the S phase of the cell cycle [1, 3, 8, 9]. In turn, tissues with a high mitotic and replication rate have a high labeling index.

A breakthrough occurred in 1982 when a monoclonal antibody directed against 5-bromodeoxyuridine was developed by H.G. Gratzner, allowing immunohistochemical staining of cells which have incorporated this compound into their DNA during replication [10]. Based on this technique, accurate measurement of the labeling index in pathological tissue samples may be made. Thus, over the past decade, many studies have focused on the basic science and clinical research applications of this technology for a variety of CNS tumors [3, 11–29]. Furthermore, the significant photosensitivity and radiosensitivity of BUdR incorporated within DNA has led to its application in postoperative radiation therapy and intraoperative ultraviolet tumor bed phototherapy [6, 30–43].

In neurosurgical practice, the virtual abandonment of tritiated thymidine labeling in favor of the

application of BUdR is based on a number of reasons. As a radionuclide, use of tritiated thymidine is fraught with hazard [3], not the least of which is significant patient resistance to receiving a radioactive compound. It is expensive and cumbersome, requiring strict adherence to complicated protocols. Studies suggest the accuracy and reproducibility of results with tritiated thymidine are inferior to those seen with BUdR [1–3, 12, 44, 45]. BUdR has been reported by many authors to be safe, with minimal side effects at the doses used, and efficacious, with a significant predictive value for the biological behavior of tumors.

Unfortunately, there exist a number of concerns about BUdR; perhaps the most relevant is that bromodeoxyuridine is a mutagen and a carcinogen [4, 5, 46–64]. As new alternative approaches towards defining the index of cells undergoing division in pathological specimens continue to develop, they may supplant the BUdR based method in certain patients. Furthermore, additional approaches directed towards identifying genetic fingerprints of tumors may reveal their biological behavior better than the mitotic index alone.

Approaches for BUdR labeling indices

Two approaches have been developed for BUdR labeling indices. The first is to simply evaluate BUdR uptake *in vitro*, in tissue sections derived from operative tumor specimens. However, most studies have used the second approach, based on directly introducing the compound into the blood of a patient for a limited preoperative time period, ranging from at the outset of the case to three days of injections every eight hours preoperatively. The doses range from 200 to 600 mg/m^2; studies suggest that with these doses, the systemic venous concentrations are less than 1.25 μM [3, 6, 20, 24, 65, 66]. The route of administration of BUdR is somewhat controversial, being either intravenous or intrarterial (via the internal carotid artery). Although the majority of clinical studies have used intravenous administration, arguments exist in support of the intra-arterial route [3, 66]. Included among these are the observation that up to 80% of BUdR is debrominated and further metabolized by the liver with the intravenous route [9], rendering its intracranial concentration less effective; the intraarterial approach led to a 11–16 fold higher concentration than with the same dose infused intravenously and reduced the systemic exposure to the compound [3, 60]. A critical argument against the intrarterial route is the observation of increased side effects, including ocular problems such as conjunctivitis and keratitis, probably due to the effective increase in concentration [33, 67].

Once the tumor is excised or a biopsy is obtained in the operating room, the tissue is fixed, dehydrated in chilled 70% ethanol, embedded in paraffin, cut into microsections, and then deparaffinized. Using immunohistochemical techniques with the anti-BUdR antibody, in representative microscopic fields, the ratio of cells with immunostaining to total cells scored is tabulated and termed the 'labeling index'.

Clinical application of BUdR labeling index

The BUdR-labeling index has proven useful in a number of clinical trials. In a variety of CNS tumors, histological and ultrastructural analysis of tissue samples frequently provides suboptimal information about the biological behavior of the tumor, and hence its clinical outcome [68]. This is particularly true of meningiomas and gliomas, and the bulk of clinical trials using bromodeoxyuridine have focused on these two tumor types. In one study, twelve patients with meningiomas had preoperative BUdR labeling; six of the resected tumors were recurrent meningiomas. Of these, four had labeling indices greater than 1.5%, whereas all six of the nonrecurrent tumors had labeling indices less than 1% [14]. In an early study of 20 patients, 6 out of the 9 tumors with a labeling index greater than 1 were recurrent tumors [16]. In another series of 96 patients, meningiomas were resected and analyzed. In all of the patients whose tumors had a labeling index greater than 5%, the meningiomas recurred. Yet only 44% of the tumors recurred when the index was 1% to 5%, and only 6% recurred when the index was less than 1% [21]. Another reported se-

ries evaluated 178 patients with meningiomas; the mean labeling index of recurrent tumors was significantly higher than that of nonrecurrent tumors. The recurrence rate was 100% for tumors with a labeling index of > 5%, 56% with a labeling index of 3–5%, and 31% with an index of 1–3%. In fact, based on regression analysis of these findings, a formula could be constructed to predict the time of reoperation [12, 27].

The data are equally suggestive for gliomas. In an early report, it was found that low grade gliomas had BUdR labeling indices of 2.0–6.7% whereas higher grade gliomas had indices of 9.1–46.5% [3]. In a group of patients limited to a subset of gliomas (fibrillary, protoplasmic, gemistocytic, anaplastic astrocytoma and glioblastoma), there was a close correlation between histological grade and the labeling index, which was stronger than the correlation between the labeling index and age or between grade and age [20]. In another study of 47 patients followed 4 months to 3.5 years, there was a close correlation between the labeling index and clinical outcome [18]. In one series of 50 patients, those patients with an astrocytoma with a labeling index of > 5% had a median recurrence free period of only 9 months, those with an index 3–5% had median recurrence free period of 14.7 months, and in those < 3%, 9 out of 13 patients had no recurrence at 36 months [13]. In another study of 174 gliomas, the correlation between the BUdR labeling index and clinical outcome was very statistically significant for low grade astrocytomas, but not for glioblastomas [45].

The application of the BUdR labeling index has been evaluated for other CNS tumors as well, including meningothelial meningioma [19], pituitary adenomas [3, 25], pediatric astrocytomas [26, 69], meningeal melanocytomas [29], neuroectodermal tumors [17], angiosarcoma [11], thoracic spine teratoma [22], metastases [3] and acoustic neuromas [3]. These results lend support for the validity of the BUdR approach as well.

Other application of bromodeoxyuridine

Recent advances have suggested that a double labeling protocol involving bromodeoxyuridine and the closely related iododeoxyuridine (IUdR) might be employed, allowing the determination of not only the duration of the S phase but also the potential doubling time for these cells [44, 70–72]. This experimental protocol involves the *in vitro* application to glioma cell lines of IUdR for 1–3 hours, followed by BUdR for 30 minutes. Thereafter using immunocytochemical techniques with an antibody (Br-3) directed against only BUdR and another antibody (IU-4) directed against both BUdR and IUdR, the labeling index for cells in the S phase can be determined as well as the length of the S phase in the cells which take up IUdR [72].

In addition, further developments in flow cytometry have been applied to assessing the BUdR labeling index from operative brain tumor specimens. Naturally, if perfected this approach would allow more rapid and efficient evaluation of the labeling index, even intraoperatively [24, 73, 74]. In one study, 256 patients received intraoperative BUdR, and flow cytometry analysis of cells derived from the tumor specimen was performed. This approach was useful for detecting the heterogeneity of cells in the tumor which was felt by the authors to potentially be of value in designing an appropriate chemotherapy protocol, but did not show a direct correlation with the rate of tumor growth [73]. In another study, 16 patients with tumors underwent perioperative BUdR intravenous injections. Thereafter, tumor specimens were divided into two sections, one half subjected to standard labeling index analysis and the other half subjected to flow cytometry. The authors in this study felt that flow cytometry provided results that correlated well with the standard BUdR labeling index [24]. Another study corroborated these findings in 22 patients with benign or malignant tumors [74]. Another technique allowed rapid evaluation of BUdR labelling in glioblastoma tissue using an alternative 'glitter drop' technique, based on random sample analysis of the BUdR index in pathological specimens [75].

As mentioned above, some authors have advocated the use of bromodeoxyuridine in an *in vitro* setting; the compound is introduced into the removed tissue specimen rather than the patient [9,

76]. In one study of a variety of human brain tumors, a resemblance of the labeling index *in vitro* was suggested to that seen from *in vivo* administration of BUdR in other studies [9]. Another study examined the predictive value for biological aggressiveness using BUdR in primary explant cultures derived from human meningiomas [77].

The BUdR labeling technique has also been used for a variety of experimental manipulations, in particular to examine the effects of therapeutic approaches in both tissue culture and animal models of CNS tumors [78–84]. In C6 rat glioma cells, examination of perturbation of the cell cycle by nitrosurea was performed using the BUdR labeling technique [78]. The effects of interferon-beta on U-251 M6 cells and the effects of cis platinum on murine AC and human GB-1 and GB-2 cell lines were evaluated using this labeling index [79, 82]. In rats, the biological behavior of induced brain tumors was also followed with BUdR labeling indices [80, 81, 84]. These experimental results lend further support to the application of the BUdR technique for predicting the biological behavior of a variety of brain tumors.

Bromodeoxyuridine and radiation therapy

In addition to studies examining the prognostic value of the BUdR labeling index, some investigations have focused on the use of BUdR and other halogenated pyrimidines (iododeoyuridine and fluorodeoxyuridine) as a photosensitizing and/or radiosensitizing agent in adjunct therapy for gliomas. These studies are based on the initial findings made by W. Szybalzski that halopyrimidines sensitize cells to the cytotoxic effects of radiation therapy [7, 30, 85, 86]. In particular, radiation therapy alone may leave behind tumor 'clonogens', which are cell populations that escape the radiation damage and then rapidly proliferate [35, 87, 88]. Thus, the combination of radiation therapy with a radiosensitizing agent appears to address these cell populations in particular [35]. Since radiation damages cellular DNA, and halopyrimidines affect normal DNA repais and synthesis, there could be a synergistic effect.

One *in vitro* experimental model using either radiation or ultraviolet light therapy demonstrated an increase of 1.8 fold and 3.8 fold, respectively, in cell death in the presence of BUdR [89]. Ultraviolet light combined with BUdR induces single strand chromosomal breaks in 9L rat brain tumor monolayer cultures, whereas ultraviolet light alone did not. There was also a two fold increase in single strand chromosomal breaks in these cultures in the presence of bromodeoxyuridine and radiation, compared to radiation alone [89]. However, at this point, it is not known to what extent chromosomal strand breaks might contribute to radiation-mediated cell death. Some authors feel that halogenated pyrimidines induce radiosensitization by altering DNA precursor pools and inhibiting enzymes, such as thymidylate synthase, involved in DNA synthesis and repair [90]. There may also be differences in the mechanism of bromodeoxyuridine, iododeoxyuridine, and fluorodeoxyuridine; the first two appear to require incorporation directly into DNA, where they cause their effect, whereas fluorodeoxyuridine appears to affect cell cycle synchronization and enzyme activity indirectly [90–95]. Examination of the effects of ionizing radiation on the bromine atom incorporated into DNA using BUdR demonstrated that its cellular effects on yeast cells (*Sacchoromyces cerevisiae*) were dependent on the bromine selective monochromatic K absorption edge [96]. This finding suggested that the incorporation of bromine into DNA might account in part for the increased susceptibility of cells to radiation-induced injury [96].

Based on such suggestive data, a number of clinical trials have used BUdR as an adjunct to conventional postoperative radiation therapy and have served as a rationale for combining perioperative intravenous or intraarterial introduction of BUdR and tumor bed ultraviolet light irradiation. Throughout radiation therapy, the doses of BUdR administered typically range between 400 and 600 mg/m^2 day. In an initial Japanese trial [36, 43, 97, 98] of 107 non-randomized patients, 48 patients with high grade gliomas underwent combination intra-arterial BUdR infusion with conventional postoperative radiotherapy. Of these patients, survival was 68%, 44%, and 18% at one, two and three years,

respectively. Unfortunately, there was significant morbidity associated with the intra-arterial [internal carotid] infusion.

Another series reported 60 patients with high grade gliomas who were treated with either BUdR or iododeoxyuridine in combination with radiotherapy. The median survival was only 13 months and no patients survived longer than 36 months [40]. Iododeoxyuridine infusion was combined with hyperfractionated radiotherapy in another series of 47 patients with glioblastomas: the mean survival was 11 months with only 14% surviving beyond 2 years [41]. In 1990, another report [42] examined the survival of 23 patients with malignant gliomas treated with intra-arterial BUdR and external beam radiotherapy. The median survival was 20 months for the entire group, but only 14 months for patients with glioblastoma. In one recent study [6], BUdR was infused intraarterially before and during conventional external beam radiation therapy for patients with pathologically verified Grade III and Grade IV gliomas. In this study of 18 patients, the median survival was 22 months, compared to a matched population, treated with conventional radiation therapy, survival of only 9 months. The addition of chemotherapy (BCNU) to radiation therapy alone only increased the median survival to 12 months [6]. In yet another study, patients with gliomas received BUdR, 800–1000 mg/m^2, five days a week for 5–6 weeks, during radiation therapy, but the small number of patients, their variable presentation and other methodological issues render the data difficult to interpret [31].

Finally, in 1991, a report indicated that in a group of 160 patients with glioblastoma who received BUdR and radiotherapy, followed by chemotherapy (procarbazine, CCNU, and vincristine), the median survival was 56 weeks [37, 38, 43]. A similar protocol used for patients with anaplastic astrocytoma [BUdR, radiotherapy and chemotherapy] yielded better results with a median survival in excess of 5 years [39, 43]. Thus, there are some encouraging data to suggest that the application of bromodeoxyuridine (and other halogenated pyrimidines) in combination with radiation therapy may lead to improved survival; unfortunately to date the benefit is not dramatic.

Problems with the bromodeoxyuridine approach

Although there is much encouraging evidence for the utility of the BUdR labeling index in clinical neurosurgical practice, there are a number of potential pitfalls. Included among these are i) the finding of significant variability of the labeling index within a tumor in some studies, ii) an inexact correlation between the labeling index and the clinical outcome in certain studies, iii) the presence of side effects of the administration of the drug, particularly at the higher doses used for radiosensitization studies, and iv) evidence that bromodeoxyuridine is a mutagen and a carcinogen. Finally, with the advent of new techniques that obviate introduction of BUdR (or any other substance) into patients, there now exist attractive potential alternative approaches, some of which are based on direct analysis of cell specific and tumor specific genetic markers which may correlate tumor biology and clinical outcome better than the BUdR labeling index.

Variability of the labeling index

The labeling index has been found to be somewhat variable in some studies, depending on the specific tissue section location in pathological specimens, as well as the cell type and culture conditions in tissue cultures. In one clinical study, significant intra-specimen variability of the labeling index was found in tissue sections obtained from 16 patients with tumors and treated with perioperative BUdR [24]. In an experimental paradigm, the labeling index in cultured cells was variable, depending on their location on the dish and the media substrates [99]. There was also significant variability in BUdR uptake in a number of different cultured human glioma cell lines, including U-251, U-118, and D-54, and BUdR uptake could be modulated by a thymidylate synthase inhibitor (fluoropyrimidine) [100]. In another study, the BUdR labeling index in cultured glioblastoma cells was dependent on media TTP pools [101].

Inexact correlation between the BUdR labeling index and clinical outcome in some studies

Some studies have suggested an inexact correlation between the BUdR labeling index and tumor growth rates. In one study [28], two meningiomas treated with radiation therapy and re-excised had low labeling indices using BUdR, but were found to rapidly recur clinically. In another study, out of 6 recurrent meningiomas, although 4 had a BUdR labeling index greater than 1.5%, two had a labeling index less than 1%; all non-recurrent tumors had a labeling index less than 1% [14]. Furthermore, Yoshii, *et al.* [3], have pointed out that the actual rate of proliferation of a tumor depends on a variety of factors, including the proportion of cells in the S phase or the growth fraction, the cell cycle time, and the rate of cell loss from the tumor cell population. In addition, other determinants of tumor growth include the availability of trophic factors, the presence and expression of certain genes, and the potential for clonal deletion [102–107]. The BUdR labeling index only represents the growth fraction; however the argument can be made that the growth fraction is most closely correlated to tumor growth clinically [3].

Side effects of bromodeoxyuridine

Most investigators consider the clinical application of BUdR as safe with minimal side effects. In fact, it was in part the concern over the safety of tritiated thymidine and the purported safety of BUdR that prompted the abandonment of the tritiated thymidine labeling index. However, there are reports of significant side-effects associated with BUdR, particularly at the higher doses initially used in its Phase I clinical trials and in studies evaluating BUdR as a radiosensitizing agent. At the outset of clinical application of bromodeoxyuridine, during its Phase I trials with high doses, there was significant bone marrow suppression, prompting the reduction to the dosage used in later trials [23, 24, 65]. When given at the doses required for radiosensitization studies (400 to 800 mg/m^2), multiple side effects were seen in several patients in one study, including ocular exposure keratitis and mucopurulent conjunctivitis, and in one patient, a spontaneous corneal perforation [33]. In other studies, intraarterial infusion of BUdR before and after radiation therapy resulted in numerous side effects, including fatigue, anorexia, dermatitis, blepharitis, mucositis, transient hepatitis, iritis, nail ridging, myelosuppression and, in one case, Steven's Johnson syndrome [6, 67]. These reports indicate the potential toxicity of BUdR to rapidly dividing cells in tissues throughout the body, particularly using intraarterial injection.

Nishizaki pointed out that BUdR might prove to be particularly toxic to patients with hepatic disorders since it is largely debrominated in the liver within twenty minutes [9]. Transient elevations in hepatic enzymes have been detected [67]. Furthermore, in cultured human meningioma (MG-1) cells, there was significant enhancement by BUdR of latent virus synthesis [108]; similar results in other cell lines raises the concern that latent viruses, such as Herpes Simplex Virus One (HSV-1), in humans might be activated by administration of BUdR [109]. The application of novel drug delivery systems for localized delivery of BUdR into a tumor might obviate some of these concerns [1, 110].

Bromodeoxyuridine is a mutagen and a carcinogen

In the late 1950s, an understanding of the molecular basis of mutagenesis and carcinogenesis was initiated using a select group of potent mutagens [4, 5, 49, 50, 111]. One of these mutagens was 5-bromodeoxyuridine. In 1959, E. Freese first characterized a novel type of mutation, termed a transition mutation, in phage T4, using bromodeoxyuridine as the prototypic agent [4, 5]. Since then, a large number of studies have investigated and confirmed the finding that bromodeoxyuridine is in fact a mutagen and a carcinogen. In fact, because of its potency and molecular specificity, BUdR has been instrumental over the past three decades in further elucidating the molecular basis of mutagenesis.

A number of *in vitro* experiments have confirmed the mutagenicity of bromodeoxyuridine. Included among these is the finding that BUdR in-

duced a high frequency of transient trifluorothymidine (F3TdR)-resistant variants in TK6 human lymphoblast cell lines [47]. Mutagenic effects of BUdR were also demonstrated in diploid human fibroblasts and lymphoblasts [62, 112]. In addition, BUdR was found to markedly affect the mutagenic profile of fusion Chinese hamster ovary (CHO) cells and Syrian hamster melanoma cells (ZE) [53]. In other studies, 5-BUdR was used to evaluate reversion mutations in cells containing multiple copies of a mutated target gene, confirming the sequence specificity of BUdR-induced mutations and demonstrating that they do not occur randomly or spontaneously [113]. The incorporation-dependent reversion mutagenesis was analyzed in murine A9 cells possessing a single copy of the *E. coli* gpt gene integrated into chromosomal DNA. In this assay, > 90% of the revertants were due to GC-AT transitions, indicating precise sites in the gene for the mutagenic action of BUdR [48, 55, 64]. The mutagenic effects of BUdR were also found to be cell cycle dependent, occurring only during the S phase in cell cycle synchronized cells of the human lymphoblast lines WI-0L2 and TK-2 [8].

Another study revealed that a brief exposure and low dose of BUdR could induce mutagenic effects. Mouse FM3A cells exposed to BUdR at concentrations greater than 100 µM for one to two hours showed a significantly increased mutation rate as determined by the number of colonies resistant to ouabain. Cells incubated for 12 or more hours in 50 µM BUdR also showed a significantly increased mutation rate. These findings demonstrate that at an *in vitro* concentration within one to two log levels of those *in vivo* plasma concentrations achieved with human clinical trials, a significant mutational rate could be seen [6, 56].

In another study, an analogue of BUdR, 5—BVDU (bromovinyl-deoxyuridine), was a mutagen in a variety of *in vitro* assays, including the L5178Y TK +/– mouse lymphoma mutation assay. In addition, BVDU induced micronuclei in Chinese Hamster Ovary (CHO) and mouse lymphoma L5178Y cells [114].

Of concern, BUdR at a concentration of 10 µM induced increased radiosensitization in C3H 10T1/2 cells within one cell doubling time. The transformation rate increased significantly when these cells were exposed to BUdR for more than one cell doubling time. Radiation induction of transformation was also synergistically elevated by BUdR above levels following radiation therapy or BUdR therapy alone [115]. These results raise concern that BUdR could enhance radiation-induced transformation of cells; thus its clinical application as a radiosensitizing agent may have risks [115].

A standard assay for mutagens which also act as carcinogens is the analysis of sister chromatid exchanges in cell culture systems. Well known carcinogens had additive effects on sister chromatid exchanges when treated with BUdR vs control, indicating that BUdR itself has relevant carcinogenic potential [116, 117]. In another study, BUdR modulated the ability of camptothecin (a DNA topoisomerase I inhibitor) to induce sister-chromatid exchanges (SCE) in human lymphoblastoid cells (NC3) [118]. Furthermore, BUdR also induced an increase in sister chromatid exchanges and chromosomal fragility in a human mutant B-lymphoblastoid cell line (CCRF-SB) in a dose-dependent manner [63] and it directly increased the sister chromatid exchange rate in a mutant cell line (AsHA) derived from a patient with ataxia telangectasia, in Bloom syndrome cells, and in rat 9L gliosarcoma cells [61, 119].

There are two postulated steps at which BUdR can induce its mutagenic effects. The first is due to base pairing errors during replication of DNA containing BUdR [51, 52, 60, 111, 120–123]. The second is due to the change in nucleotide triphosphate pools (in particular dCTP and dUTP) relative to the triphosphate pool of BUdR, and thus increasing the relative frequency of errors in nucleotide incorporation during DNA synthesis [51–54, 60, 120]. Support for the latter mechanism was found in a study examining the effects of altering the dNTP pools on the induction of sister chromatid exchanges by BUdR in a variety of cell lines and protocols [54].

Of concern, in one *in vitro* study, there were long-term, delayed carcinogenic effects of exposure to BUdR. Cultured Syrian hamster embryo cells were treated for one hour with BUdR, followed by 5 minutes of irradiation with ultraviolet light. Thereafter, these cells were subcultured and tested for both

growth in culture and in newborn hamsters. Some microscopic changes were observed within a few cell divisions; however, 100–200 population doublings were required for the emergence of a neoplastic subpopulation [124]. This raises concern about the possibility that BUdR uptake into human cells might actually induce novel tumor formation months or even years after initial exposure.

A number of *in vivo* studies have also demonstrated the mutagenic and carcinogenic potential of BUdR. When male and female BALB/c mice were exposed to 1 mg BUdR subcutaneous injections at day 1, 3, and 7 after birth, and then given intraperitoneal injections of urethane every third day, there was a synergistic increase in the incidence of tumors, including lung adenoma, adenocarcinoma, and hemoblastic disorders [46]. In female rats treated with BUdR for four days, there was an increased frequency of ovarian tumors and uterine polyps, and in male rats, there was an increased frequency of testicular seminomas [58]. In another study, in conjunction with the classical tumor promoter, TPA (12-O-tetradecanoyl-phorbol-13-acetate), nude mice given BUdR had significantly reduced G1 transit times and increased delay in the progression through the S phase compared to control or TPA alone [125, 126].

Of particular importance was the finding that BUdR can persist for long periods of time in animal tissues. Outbred L10 rats had four subcutaneous injections of 3.2 mg of BUdR at days 1, 3, 7, and 21 following birth. The animals were sacrificed at approximately one hour, one month and one year after the final injection. Using immunocytochemical staining of BUdR, although there were fewer cells demonstrating labeling at one year than at earlier times, many cells were still immunoreactive, particularly in those tissues with low cell turnover [127, 128]. This raises concern that the introduction of BUdR into humans might allow its persistence for extended periods of time. This would be of relevance for germ line cells in which mutations might be passed on to offspring.

These findings suggest a potential flaw in the experimental protocol applying BUdR labeling in a clinical setting [116]. Because BUdR might itself induce neoplasia, it is possible that tumor cells remaining in the unresected margins of those debulked tumors with the highest labeling index (and thus, the highest uptake and incorporation of BUdR) are subsequently induced by this compound to undergo a higher rate of malignant transformation. Thus, although unlikely, it is conceivable that the statistically relevant correlation between BUdR uptake and tumor recurrence or regrowth rates clinically is a self-fulfilling prophecy. Unfortunately, in most clinical and laboratory studies, appropriate controls to exclude this possibility have not been performed.

Alternative approaches

As a result of the many new advances made in understanding the molecular biology and immunocytochemistry of CNS tumors, there is an increasing variety of alternative approaches to the use of BUdR labeling indices as a predictor of tumor growth and clinical outcome. In particular, recent advances in molecular biology have led to insights into the interactions among oncogenes, tumor suppressor genes, and other cellular genes in the regulation of normal cellular function and tumorogenesis [129]. Many human cancers, including CNS tumors, appear to result from aberrant regulation and/or expression of distinct functional categories of genes at particular times during the evolution of a neoplasm. There is growing support in CNS tumor biology for a model originally advanced for colorectal carcinoma (reviewed in 130) which views tumor progression as a stochastic set of genetic events, each of which confers a progressively more malignant phenotype [104]. Although distinct genetic events have been correlated with a variety of CNS tumors, the progression of astrocytomas has been best characterized, as described below.

It appears that, as an early event in astrocytoma progression, sequences are deleted on the short arm of chromosome 17, since this change is observed in all histological grades of astrocytoma [103]. In turn, interferon alpha and interferon beta-1 gene deletions from the short arm of chromosome 9 are observed in a significant number of anaplastic astrocytomas and glioblastomas, but not in low grade as-

trocytomas [106]. Furthermore, loss of heterozygosity for loci on chromosome 10 and epidermal growth factor receptor (EGFR) gene amplification appear to be restricted to the most malignant of human gliomas, particularly glioblastoma [103, 105]. Distinctly aberrant EGFR transcripts are often coamplified and coexpressed with normal EGFR transcripts in human glioblastoma [107, 108]. Finally, expression of the vascular permeability factor/vascular endothelial growth factor (VEGPF) gene, encoding a well characterized mitogen for endothelial cells, appears to be overexpressed in those glioblastomas particularly associated with increased vascularity and edema [131]. The identification of such sequential, genetic alterations in operative specimens may eventually permit a close correlation between these genetic changes and biological behavior and provide a biological basis for a gene therapy based approach to such tumors.

Other novel approaches correlating immunocytochemistry and the biological behavior of CNS tumors include the application of an antibody directed against DNA polymerase alpha expressed in both human glioma cell lines and human brain tumors. Since this enzyme is particularly expressed in cells undergoing DNA replication, immunohistochemical localization of this enzyme might prove to be a good marker for the number of cells undergoing mitosis in a tumor specimen [132]. In a similar approach, a newly developed antibody directed against thymidylate synthase correlates closely to the early S phase (as well as the G1 phase) in the cell cycle in some cell lines [133]. Analogous results were found in 16 human brain tumors using immunostaining for the proliferating cell nuclear antigen (PCNA) with an anti-PCNA antibody; a close correlation was seen between the PCNA labeling index and the BuDR labeling index [134]. PCNA is believed to be an auxiliary protein of a DNA polymerase [134–136].

An alternate marker, staining for nucleoloar organizer region associated argyrophilic proteins (AgNORs), was found to correlate with tumor grade and clinical behavior in 78 cases of meningiomas [137]. This marker also correlated well with the BuDR labeling index in C6 glioma cell lines and glioma-derived tissue sections [138–140].

Another intriguing observation was recently made, offering a potential approach towards defining those tumor cells which have greatest growth rates. In a variety of neuroepithelial tumor cell lines, including medulloblastoma D283 Med, retinoblastoma WERI-ORb1 and astrocytic glioma U-251MG cells, there was a significant loss in S1 intermediate filament proteins [141]. Examination of the number of cells lacking such proteins in pathological specimens might provide an estimate of the mitototic index, although this approach may prove to be too laborious.

In cryostat sections of 12 gliomas, examination of a variety of cell surface receptors on monocytes, including RM3/1, Cr3(CD11b) and Ki67, was found to correlate with the proliferative rate [142–144]. In one study of 48 brain tumors, the Ki-67 labeling index was found to correlate well with both the BUdR labeling index as well as tumor histology [144]. In another study of meningioma derived tissue, probes for insulin-like growth factor II (IGF-II) mRNA were studied; these might prove useful as markers for tumor growth rates since IGF-II might play a role in tumor growth [76, 145]. In a number of reports, attempts were made to correlate cytogenetic analysis of the chromosomal changes in meningioma specimens and the clinical outcome, with variable results [131, 146–149].

Since all of these novel tumor marker based approaches are performed on operative pathological specimens, there is no requirement for introduction of a potentially toxic substance into patients. If these markers provide reliability and accuracy comparable to the BUdR labeling index, they may supplant or supplement the BUdR-based approach.

Conclusions

The application of bromodeoxyuridine labeling indices has played a significant role in understanding and predicting the clinical outcome of a variety of CNS tumors [150]. In addition, application of BUdR or other halogenated pyrimidines as an adjuvant to radiation therapy may be of value. In the evaluation of malignant tumors, the benefits of *in vivo* use of BUdR may outweigh its existing and po-

tential risks. However, justification of its application to predominantly benign tumors, such as acoustic neurinomas and pituitary adenomas is less clear. In addition, given the potential of BUdR to cause mutations in the germ line, application to pediatric patients and young adults of reproductive age is also questionable. Furthermore, over the past several years, a number of new molecular biology-based approaches towards defining the mitotic labeling index and aggressiveness of tumors in pathological specimens have been developed. Additional refinement of these approaches may someday obviate the need for introducing a potentially toxic compound into patients. Until then, application of the BUdR technique is likely to provide important prognostic information for a number of patients with CNS tumors.

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This article is dedicated to Ernst Freese, 1925–1990, a pioneer in the understanding of the molecular basis of mutagenesis.

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Address for offprints: A. Freese, Division of Neurosurgery, Hospital of the University of Pennsylvania, Silverstein Pavilion 5, 3400 Spruce St., Philadelphia, PA 19104, USA