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Report

Dynamics of Bromodeoxyuridine Incorporation into DNA of Squamous Carcinoma Cells During Mid and Late Logarithmic Growth

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Head and neck squamous carcinoma cell lines, UM-SCC 1, 5, 9, 11B, and 14B, were exposed *in vitro* to bromodeoxyuridine (BUdR) during logarithmic growth to determine the effects of drug concentration (0.01 to 10 μM) and duration of exposure (3, 7, and 10 days) on cell growth and on incorporation of BUdR into DNA. Concentrations of less than 1.0 μM were not growth inhibitory except with UM-SCC-11B. After 10 days of exposure to 5 μM BUdR, survival fractions for all lines ranged from 2 to 65% of controls. Replacement of thymidine by BUdR in DNA was assessed by gas chromatography/mass spectroscopy. Percentage replacement (% R) was described by the equation $\% R = 100\ (C/t)^s/[(C/t)_{50}^s + (C/t)^s]$, where C is the concentration of BUdR (μM), t is the time in days, s is a constant, and $(C/t)_{50}$ is a constant corresponding to % R = 50%. BUdR incorporation reached a time- and concentration-dependent maximum that, after 3 to 7 days of culture in 10 μM BUdR, ranged from 30 to 60% R. Subsequently, % R declined with time even though the cells were fed daily with fresh BUdR-containing medium.

KEY WORDS: squamous carcinoma cells; bromodeoxyuridine (BUdR) incorporation *in vitro;* thymidine replacement; kinetics of replacement.

INTRODUCTION

The incorporation of 5-halo-deoxyuridine analogues into DNA in place of thymidine increases the sensitivity of mammalian cells to radiation, with a magnitude of sensitization that corresponds to the extent of thymidine replacement (1–5). *In vitro,* 0.1 to 10 μM bromodeoxyuridine (BUdR) can modify radiation dose effects on mammalian cells by factors of 1.5 to 2.2 (2,4,5). To achieve therapeutic advantage *in vivo,* BUdR administration should lead to a greater level of incorporation in tumor tissue than in normal replicating cells. To develop an *in vitro* human cancer model we examined how the concentration and duration of exposure to BUdR affected the level of BUdR incorporation and cell growth in five head and neck squamous carcinoma cell lines.

MATERIALS AND METHODS

Cell Lines

UM-SCC lines characterized for antigenic and morphologic features of human squamous epithelial cells (6) were established in our laboratory (7) and were used between passage 40 and passage 79.

Preparation of Drug Solutions

A stock solution of 0.1 *M* BUdR (5-bromo-2′-deoxyuridine) (P–L Biochemicals, Inc., Milwaukee, Wis.) was prepared in carbonate–bicarbonate buffer solution, pH 9.8 (±0.1), and stored in the dark at 4°C. Dilutions were prepared under low-light conditions and used immediately.

Cell Growth Experiments

The cells were cultured in Dulbecco's minimal essential medium (MEM) and 5% fetal bovine serum. Cells in logarithmic growth were harvested with trypsin–EDTA (6), washed, counted, and delivered to multiple six-well plates at 5×10^4 cells/well. The cell number was confirmed by counts

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of three samples of cell suspension taken at the beginning, middle, and conclusion of the plating procedure. After 3 days the medium was removed and the cultures were fed daily for 10 days with fresh BUdR-containing medium at concentrations of 0.01, 0.1, 1, 5, or 10 μM. Thus, nutrient depletion and drug metabolism were not factors affecting cell growth (6). The plates were kept in UV light-protective bags at 37°C, 5% CO_2, and saturated humidity. All manipulations outside the plastic bags were performed in low light. Cell counts were performed on days 3, 7, 9, and 13 after plating. Three replicates were used for each data point. Cells were trypsinized, centrifuged, resuspended, and diluted in a known volume of trypan blue dye, and viable cells were counted using a hemocytometer. Cell population doubling times were calculated over three doublings during midlogarithmic growth.

BUdR Incorporation

Cells were harvested on culture days 6, 10, and 13 and BUdR incorporation into cellular DNA was determined by means of a gas chromatography–mass spectrophotometry (GC/MS) method developed and validated at our institute (8,9). The results are expressed as the percentage of thymidine replacement by BUdR.

Mathematical Model for BUdR Incorporation

The Hill equation (10) [Eq. (1)] has been proposed by Wagner (11) for relating pharmacodynamic response to drug concentration.

$$R = R_m C^s/[(EC_{50})^s + C^s] \quad (1)$$

In Eq. (1), R is the pharmacodynamic response when the drug concentration is C. R_m is the maximum response when $C = 1$. EC_{50} is the effective concentration 50%, i.e., the concentration when $R = R_m/2$, and s is a constant. If R is the percentage thymidine replacement by BUdR, then % R = % replacement, $R_m = 100$, and Eq. (1) becomes

$$\% \text{ replacement} = 100C^s/[(EC_{50})^s + C^s] \quad (2)$$

A linear transformation of Eq. (2) is Eq. (3), in which s is the

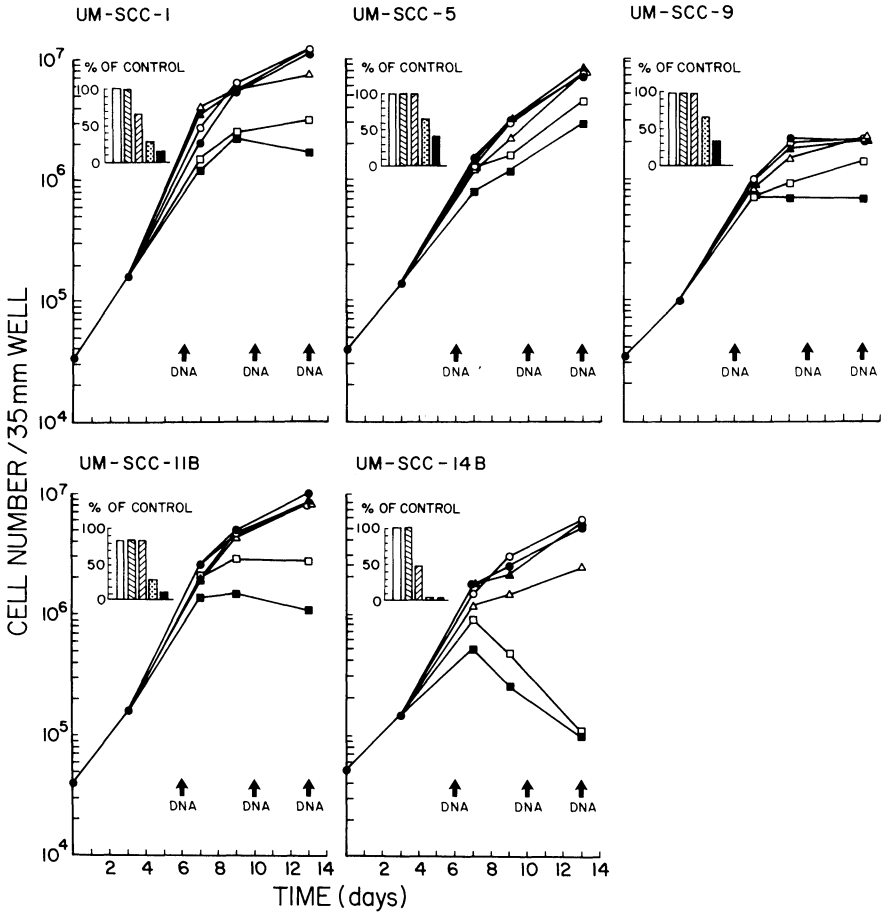

Fig. 1. Effects of BUdR on cell growth. Five (UM-SCC) cell lines were treated daily, from day 3, with BUdR concentrations of 0 (●), 0.01 μM (○), 0.1 μM (▲) 1.0 μM (△), 5.0 μM (□), and 10.0 μM (■). Each point represents the average cell number in replicate wells. Inset: growth inhibition plotted as a percentage of the control cell number in the treated versus untreated cultures on day 13. BUdR concentrations: 0.01 μM (□), 0.1 μM (▨), 1.0 μM (▨), 5.0 μM (▦), and 10.0 μM (■). The arrows indicate the time points at which cells were processed for BUdR incorporation.

slope, ln C is the abscissa, and $-s \ln (EC_{50})$ is the intercept (I).

$$\ln [\% R]/[100 - \% R] = s \cdot \ln C - s \cdot \ln (EC_{50}) \quad (3)$$

RESULTS

Effects of BUdR on Cell Growth

The effects of daily (days 3–13) treatments with BUdR on cell growth are shown for the five UM-SCC cell lines in Fig. 1. Percentage growth inhibition by each BUdR concentration is illustrated in the insets. Growth inhibition of UM-SCC 11B was noted with 0.01 to 0.1 μ*M* BUdR. However, at higher concentrations (1, 5, and 10 μ*M*), growth inhibition of all five lines was observed. BUdR cytotoxicity was most apparent with UM-SCC 14B; after 10 days of 5 or 10 μ*M* BUdR, there was a 98% reduction in cell number.

Replacement of Thymidine by BUdR

BUdR incorporation into cellular DNA determined on culture days 6, 10, and 13 (Fig. 1, arrows) is given as % R in Table I. With UM-SCC 9 and 14, treated with 5 and 10 μ*M* BUdR, the peak incorporation was found on day 10 (day 7 of BUdR exposure). In all other cases the % R was highest on day 6 (day 3 of BUdR exposure) and decreased progressively by days 10 and 13 of culture, even though the cell number continued to increase in all cultures treated with less than 5 μ*M* BUdR (Table I and Fig. 1).

Mathematical Relationship Among BUdR Concentration, Time of Exposure, and Incorporation into DNA

Using Eq. (3) and the data in Table I, the slope s, the intercept I; the coefficient of determination r^2, the standard deviation of scatter about the regression line (SD scatter), and the EC_{50} values for the five SCC lines on each of 3 days were calculated. An example of these lines is presented for cell line UM-SCC 11B in Fig. 2. Using the t test it was shown that the slopes for each of the 3 days did not differ significantly. However, analysis of variance of all the slopes indicated that the mean squares for cell lines ($0.05 > P > 0.025$) and for days of observation ($0.025 > P > 0.01$) were both significant. Analysis of variance of the $(EC_{50})^s$ values indicated that for this variable the mean squares for cell lines ($0.005 > P > 0.001$) and for days ($P < 0.001$) were also both highly significant. Therefore, all data for each cell line fell on a single regression line when C/t (where C is the concentration of BUdR in μ*M* and t is the time of exposure to BUdR in days) was used as a variable rather than C alone. This relationship is shown by Eq. (4).

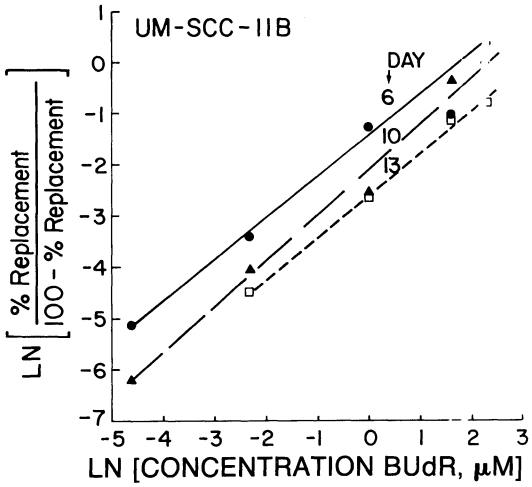

Fig. 2. Plots based on Eq. (3) for cell line UM-SCC-11B for days 6 (●), 10 (▲), and 13 (□) in culture. The parallelism of the lines for different days in these plots suggested that all the data for each cell line could be fitted by Eq. (5) as shown in Fig. 3.

$$\% \text{ replacement} = [100(C/t)^s]/[(EC/t)_{50}^s + (C/t)^s] \quad (4)$$

which has the logarithmic transform shown as Eq. (5).

$$\ln [\% R]/[100 - \% R] = s \cdot \ln(C/t) - s \cdot \ln(EC/t)_{50} \quad (5)$$

Plots of ln [% replacement/(100 − % replacement)] as a function of ln [concentration of BUdR (μ*M*)/time (days)] for the other four cell lines are shown in Fig. 3. The slopes of the regression lines decrease with increasing population doubling time (dt): UM-SCC 11B (s = 0.908; dt = 24 hr), UM-SCC 1 (s = 0.736; dt = 26.9 hr), UM-SCC 14B (s = 0.709; dt = 26.9 hr), UM-SCC 5 (s = 0.658; dt = 29.3 hr), and UM-SCC 9 (s = 0.614; dt = 29.3 hr).

Table I. Percentage Replacement of Thymidine in DNA by BUdR as a Function of Time of Exposure and BUdR Concentration

Cell line	Day[a]	BUdR concentration (μ*M*) 10	5	1	0.1	0.01
UM-SCC 1	6	61.3	44.5	17.4	3.2	4.1
	10	45.3	27.6	13.3	2.0	0.3
	13	42.4	31.3	7.7	1.3	0.3
UM-SCC 5	6	32.9	26.0	12.8	3.0	0.0
	10	30.6	17.5	5.8	0.9	0.2
	13	24.1	12.8	3.7	1.1	0.6
UM-SCC 9	6	35.6	32.6	40.3	5.2	2.3
	10	38.2	35.2	11.4	2.3	0.0
	13	30.0	24.1	7.9	1.4	0.0
UM-SCC 11B	6	60.5	47.5	22.7	3.3	0.6
	10	51.1	41.0	9.4	1.7	0.2
	13	32.0	24.5	6.7	1.1	0.0
UM-SCC 14B	6	50.6	49.6	28.1	6.5	1.1
	10	58.1	44.6	24.6	3.1	0.0
	13	34.7	43.1	9.8	1.6	0.7

[a] Exposure to the drug BUdR was begun on day 3.

DISCUSSION

BUdR can have direct growth inhibitory and cytotoxic effects on cells *in vitro*, especially after prolonged exposure to high concentrations during logarithmic growth (2,4,12). In this study the cytotoxic and growth inhibitory effects were greatest in cell line UM-SCC 14B, which exhibited 50% growth inhibition at 1 μ*M* BUdR and progressive cell loss from day 7 to day 13 at 5 and 10 μ*M*. Growth inhibition was also apparent in the other lines after 3 to 5 days in BUdR (approximately three to five doublings). The level of thymidine replacement, ranging from 30 to 60% after 3 days, was striking and, to our knowledge, is the highest reported. In

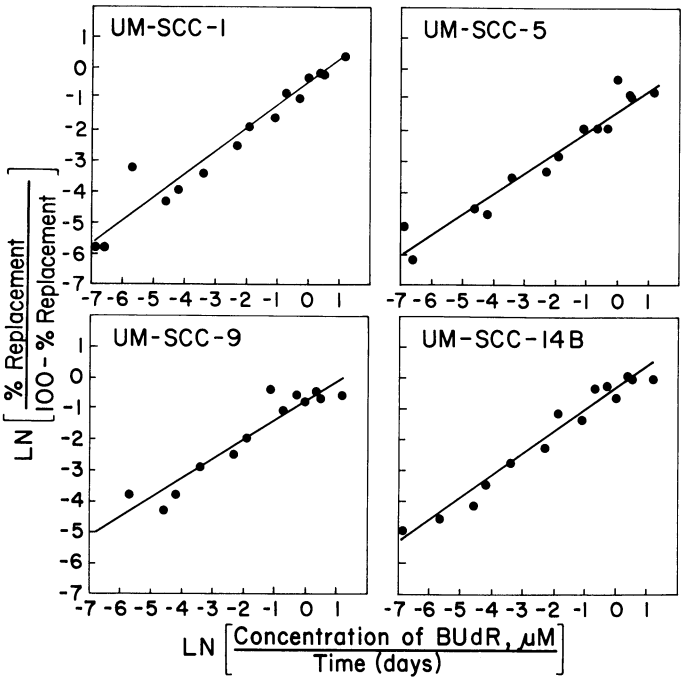

Fig. 3. Plots based on Eq. (5) for each of the UM-SCC cell lines studied.

general, the effects on cell growth correlated with the level of BUdR incorporation. At nearly all time points UM-SCC 1, 11, and 14B incorporated higher levels of BUdR than did UM-SCC 5 and UM-SCC 9, and in the latter pair, there was less effect of BUdR on cell growth. However, for equivalent % R values, 31% R with UM-SCC 1 on day 13 after 5 μ*M* BUdR and 30% R on day 10 with UM-SCC 9 after 10 μ*M* BUdR, the growth inhibitory effect was equivalent (Fig. 1 and Table I). UM-SCC 1, 11B, and 14B exhibited slightly more rapid growth than UM-SCC 5 and UM-SCC 9; therefore, the former three lines may have incorporated more BUdR per unit of time because of more rapid doubling. Differences in incorporation rate and growth inhibition could also result from biochemical differences between lines such as a greater reliance on preformed nucleotides for DNA synthesis or higher thymidine kinase activity in the lines that incorporate the most BUdR (14).

An unexpected finding in this study was that the extent of thymidine replacement reached a maximum after 3 to 6 days and then decreased with further exposure to BUdR (Table I). Although this decline corresponded to the beginning of the plateau phase of growth, the cell number continued to increase after day 10 in all cell lines except UM-SCC 9 and in the UM-SCC 1, 11B, and 14B cultures fed with the highest BUdR concentrations (5 or 10 μ*M*). Since incorporated BUdR can be cytotoxic (2,4,12), degradation of highly substituted DNA from dying cells may result in a decrease in the average degree of substitution in the population. Cell death may also release a pool of thymine/thymidine that can compete with BUdR for incorporation into the remaining viable tumor cells. Another possibility is that thymidine kinase activity in the population declines; in fact it has been shown previously that thymidine kinase activity in cells exposed to 5-iodo-2′-deoxyuridine decreased with a half-life of 8.5 days (13). Either or both of these mechanisms may account for our observations.

Prolonged BUdR infusion schedules are currently in use in experimental clinical trials. If the kinetics of incorporation into tumor cell DNA *in vivo* are similar to those we observed *in vitro,* then it might be more effective to use shorter BUdR infusions followed immediately by radiation treatments. It is unlikely that replacement levels of the magnitude we found (up to 61%) will be reached in nonlogarithmically growing populations *in vivo;* nevertheless, large radiation enhancement factors have been demonstrated with thymidine replacement values of 23% and less (5). Furthermore, since incorporation of BUdR into normal tissues is a dose-limiting factor after prolonged infusions (7–14 days) (14,15), shorter infusions that favor incorporation into tumor but limit normal cell incorporation should increase therapeutic advantage.

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REFERENCES

1. B. Djordjevic and W. Szybalski. *J. Exp. Med.* 112:509–531 (1960).
2. W. C. Mohler and M. M. Elkind. *Exp. Cell Res.* 30:481–491 (1963).

3. W. C. Dewey and R. M. Humphrey. *Radiat. Res.* **26**:538–553 (1965).
4. S. Wakisaka. *Cancer Res.* **39**:244–249 (1979).
5. T. J. Kinsella, P. P. Dobson, J. B. Mitchell, *et al. Int. J. Radiat. Oncol. Biol. Phys.* **13**:733–739 (1987).
6. A. Shapira, E. Virolainen, J. J. Jameson, *et al. Arch. Otolaryngol.* **112**:1151–1158 (1986).
7. T. E. Carey, K. A. Kimmel, D. R. Schwartz, *et al. Otolaryngol. Head Neck Surg.* **91**:482–491 (1983).
8. P. L. Stetson, J. Maybaum, U. A. Shukla, and W. D. Ensminger. *J. Chromatogr.* **375**:1–9 (1986).
9. J. Maybaum, M. G. Kott, N. K. Johnson, *et al. Anal. Biochem.* **61**:164–171 (1987).
10. A. V. Hill. *J. Physiol.* **40**:IV–VII (1910).
11. J. G. Wagner. *J. Theor. Biol.* **20**:171–201 (1968).
12. J. M. Kim, A. S. Gelbard, A. G. Perez, *et al. Biochim. Biophys. Acta* **134**:388–394 (1967).
13. R. M. Humphrey and T. C. Hsu. *Tex. Rep. Biol. Med.* **23**:334–336 (1965).
14. G. Morstyn, T. Kinsella, S.-M. Hsu, *et al. Int. J. Radiat. Oncol. Biol. Phys.* **10**:1441–1445 (1984).
15. T. J. Kinsella, A. Russo, J. B. Mitchell, *et al. Int. J. Radiat. Oncol. Biol. Phys.* **10**:69–76 (1984).