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Original Paper

Modulation of [5-^{125}I]Iododeoxyuridine Incorporation into Tumour and Normal Tissue DNA by Methotrexate and Thymidylate Synthase Inhibitors

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A potentially useful method for imaging of micrometastases and *in situ* radiotherapy, would be the incorporation of radioactive labelled iododeoxyuridine (IdU) into tumour DNA. However, there are two main problems: incorporation of the radioactive IdU into normal cells and low incorporation into tumour cells. The aim of this study was to attempt to augment the incorporation of [5-^{125}I]iododeoxyuridine (^{125}IdU) into tumour DNA and to improve the tumour/normal tissue ratio by the use of inhibitors (methotrexate, 5-fluorouracil, AG337, ZD 1694, benzyloxybenzyl uracil) which would prolong the metabolic half-life of the compound. Mammary tumours were induced in GR mice, which were then treated with the inhibitors and the ^{125}IdU. The tumours and representative normal tissue were removed following sacrifice of the animals, and radioactivity within the tissues measured. Pretreatment of mammary carcinoma-bearing GR mice with methotrexate caused approximately a 3-fold increase in the incorporation of ^{125}IdU into tumour DNA, and approximately a ⩾10-fold increase in the tumour/small intestine ratio of incorporated radioactivity. Inhibition of thymidylate synthase, the enzyme involved in IdU dehalogenation, by 5-fluorouracil plus folic acid, or by novel inhibitors AG337 and ZD1694 led to a 3- to 5-fold increase in the ^{125}IdU incorporation. Benzyloxybenzyl uracil, an inhibitor of dihydrouracil dehydrogenase, had little effect. Treatment of tumour-bearing mice with methotrexate plus ZD1694 significantly reduced the rate of tumour growth, but addition of ^{125}IdU (70 μCi/mouse, three daily injections) had no additional antitumour activity. In conclusion, these results do not support the hypothesis that systemic administration of ^{125}IdU can be used for cancer therapy or for imaging purposes unless better methods are found to boost its incorporation into tumour DNA.

Key words: radioactive DNA precursor, thymidylate synthase inhibitors, ZD 1694, AG 337, tumour growth, tumour imaging

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INTRODUCTION

Replication of chromosomal DNA during S-phase in growing cells utilises deoxyribonucleotide triphosphates produced by *de novo* synthesis or by the salvage pathway. These biochemical processes constitute an attractive target for the development of methods of diagnosis and therapy of malignant lesions. The fact that the expression of thymidine kinase, the key enzyme required for the utilisation of thymidine and its analogues, is limited to S-phase cells, is of particular interest as it can allow the discrimination between tissues with a high proportion of growing cells (including tumours) and tissues with low proliferative activities.

A possibility by which the thymidine salvage pathway could be put to use in oncology, is the incorporation of radioactive labelled iododeoxydine (IdU) or similar molecules [1], with the purpose of visualising small (otherwise undetectable) tumour foci, and their eradication by *in situ* radiotherapy. Isotopes ^{123}I, ^{125}I and ^{131}I have been used in several studies [2–4], and ^{211}At (a short-lived artificial halogen) can replace iodine for the purpose [2]. Such precursors will incorporate into tumour cells and can serve for imaging, or, if the incorpor-

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ated radioactivity is sufficiently high, for *in situ* radiotherapy. In Chinese hamster ovary cells, incorporation of ^{125}I equivalent to 100 disintegrations per cell has been estimated to be the mean lethal dose; 500 disintegrations per cell lead to death in 99% of cells ([2] and references therein). The efficacy of this approach has been shown in intraperitoneal tumour implants and is recommended for the treatment of brain malignancies [3]. However, this application in systemic disease faces two serious problems. First, the incorporation of radioactivity into DNA of normal cells will have cytotoxic and mutagenic effects. This problem could conceivably be controlled by applying a simultaneous cytostatic chemotherapy which would prevent the proliferation of normal cells, while allowing the incorporation of the radioactive precursors into tumour DNA. Metabolic inhibitors, such as methotrexate and 5-fluorouracil (5-FU), administered together with hydroxyurea have been shown to improve the tumour/normal tissue ratio of incorporation of ^{125}IdU [2]. Second, the incorporation of ^{125}I is low because of its short metabolic half-life which is in the order of minutes [5, 6]. As thymidylate synthase is the enzyme considered to be responsible for IdU dehalogenation ([7] and references therein), administration of inhibitors of thymidylate synthase (including 5-FU) may permit increased ^{125}I incorporation to levels sufficient for imaging of micrometastases, and possibly for killing of tumour cells (the increased rate of death of normal cells would lead to greater side-effects, in principle acceptable in this approach as well as in standard chemotherapy).

In this work, we have explored the possibility for enhancing ^{125}IdU incorporation and improving the tumour/normal tissue ratio of incorporated radioactivity by using methotrexate and candidate inhibitors of IdU metabolism. Our approach was empirical, with the purpose of optimising the conditions for the individual drugs used and then to evaluate their effects in combination.

MATERIALS AND METHODS

Drugs

The novel inhibitors of thymidylate synthase used were AG337 (gift of Agouron Pharmaceuticals, La Jolla, California, U.S.A.; [8]), and ZD 1694 (gift of Zeneca Farma, Ridderkerk, The Netherlands; [9]). Folic acid (Sigma, St Louis, U.S.A.) was used in several experiments, in order to enhance the efficacy of 5-FU (Sigma) as an inhibitor of thymidylate synthase [7, 10, 11]. Propylthiouracil (inhibitor of the Type I iodothyronine 5'-deiodinase; [12]) was obtained from Sigma. 5-Benzyloxybenzyluracil [13, 14] was a gift from Dr Shi-Hsi Chu (Brown University, Providence, U.S.A.).^{125}IdU was obtained from Amersham (U.K.). All other compounds were purchased from usual commercial sources. The doses of drugs administered were chosen so as to ascertain their maximum efficiency in the absence of significant toxicity (see the corresponding references). Toxicity was detected by changes in body weight: under no experimental conditions applied in this work did the weight of the animals decrease by more than 10%.

Animals and tumours

Mammary tumours were induced in GR mice and serially transplanted as described [15]. Only hormone-independent tumours were used for the experiments. The tumours appeared within 1 week after subcutaneous injection of finely minced transplanted tumour tissue. The mice were used for experiments not later than 2 weeks after tumour transplantation.

^{125}IdU incorporation

The ^{125}IdU injection is taken as the time zero point ($t = 0$) unless otherwise indicated. The tumour-bearing mice were given water containing 0.1% KI, starting on the day preceding ^{125}IdU administration, in order to reduce uptake by the thyroid of ^{125}I released by dehalogenation of ^{125}IdU. The ^{125}IdU solution was diluted in normal saline to approximately 10 μCi/0.1 ml and injected intraperitoneally at 0.1 ml/10 g mouse body weight, unless otherwise indicated. In some experiments, intravenous injection of ^{125}IdU was administered to ether-anaesthetised animals via either the tail vein or the penal vein. Other treatments were administered intraperitoneally. After sacrifice by cervical dislocation, the tumour was dissected and samples were removed for determination of the incorporated radioactivity in a Tricarb Cobra γ-spectrometer (counting efficiency 82%). Samples of small intestine, liver and spleen were removed at the same time. DNA isolation was performed by standard procedures [16]. As no radioactivity could be detected apart from that incorporated into DNA, in later experiments the tissues were homogenised in 0.25 M sucrose, 10 mM Tris pH 7.4, 1 mM $MgCl_2$. The homogenate was centrifuged (10 min at 800 ***g***) and the supernatant was discarded. Radioactivity in DNA was then assayed by the diphenylamine method [17] and the ^{125}IdU radioactivity per unit DNA was evaluated in the $HClO_4$ lysate of the nuclear fraction. The data were corrected for decay of ^{125}I. The number of disintegrations per unit time per cell was calculated on the basis of the cellular content of 6 pg of chromosomal DNA [18].

Tumour growth

The tumour size was measured by multiplying the three perpendicular dimensions and the factor $\pi/6$ (based on an ellipsoid shape).

RESULTS

^{125}IdU incorporation into tumour versus non-tumour tissue: effects of methotrexate and of inhibitors of thymidylate synthase

As shown in Figure 1, a single dose (50 mg/kg) of methotrexate at −24 h (Group II) caused at most only slight increases in ^{125}IdU incorporation into the tumour as well as in the small intestine. By contrast, a significant increase in ^{125}IdU incorporation was observed when two doses of methotrexate (50 mg/kg each) were given at −24 h and −1 h (Groups II and III, respectively; Figure 1a). With the two doses of methotrexate, the ratio tumour/intestine was also increased (Group III, Figure 1b), suggesting that methotrexate was a more efficient inhibitor of growth in normal cells than in tumour cells. 5-fluorouracil (5-FU) was also effective as an inducer of ^{125}IdU incorporation (Group IV, Figure 1a) but did not improve the tumour/intestine ratio (Group IV, Figure 1b).

When the animals were sacrificed at 4 days instead of 24 h after ^{125}IdU incorporation, the radioactivity per unit weight remaining in both tumour tissue and intestine was reduced by a factor of approximately 2 (Figure 1a) whereas the ratio tumour/intestine was not changed (Figure 1b).

In subsequent experiments, folic acid (50 mg/kg) was administered together with 5-FU, in an attempt to enhance the efficacy of 5-FU as an inhibitor of thymidylate synthase. The combined treatment (Figure 2, Group 2) did not notably

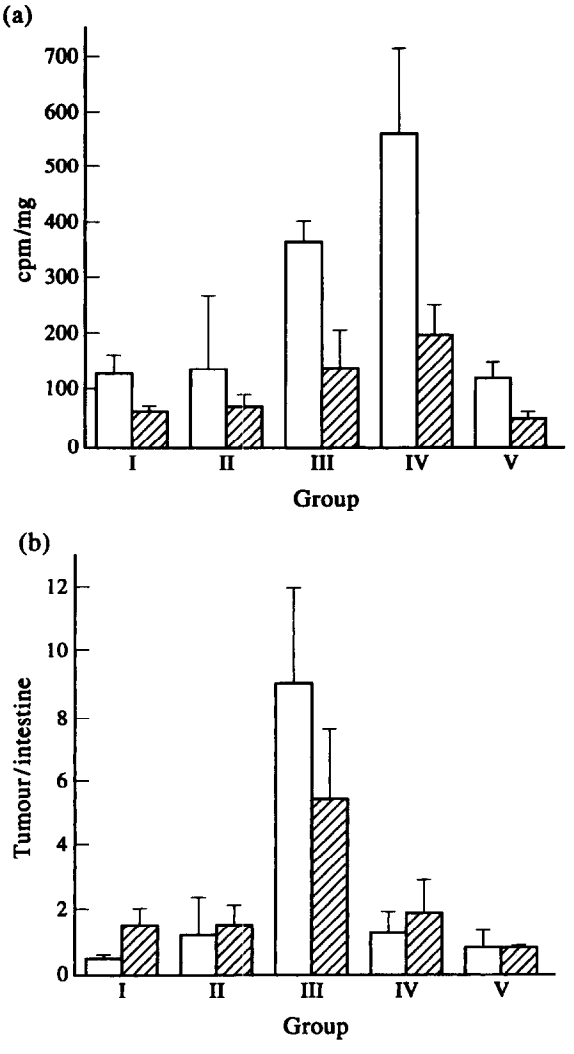

Figure 1. ^{125}IdU incorporation into tumour and small intestine: effects of methotrexate, 5-FU and propylthiouracil. Tumour-bearing mice (six per group) were injected i.p. with the drugs as follows: Group I, untreated control; Group II, methotrexate (50 mg/kg) at −24 h; Group III, methotrexate at −24 h and at −1 h; Group IV, methotrexate at −24 h, 5-FU (70 mg/kg) at −1 h; Group V, methotrexate at −24 h, propylthiouracil (1 mg/kg) at −1 h. At $t = 0$ h, they received i.p. 25 μCi of ^{125}IdU. Three mice of each group were sacrificed at +24 h (open bars), the remaining animals at 4 days (hatched bars), and the radioactivity incorporated into the tumour tissue and the intestine tissue was measured. The radioactivity present in the liver was also checked and found to be negligible (data not shown). (a) Tumour radioactivity; (b) Tumour/intestine radioactivity ratio. The data shown (cpm/mg tissue) are means ± S.E.M.

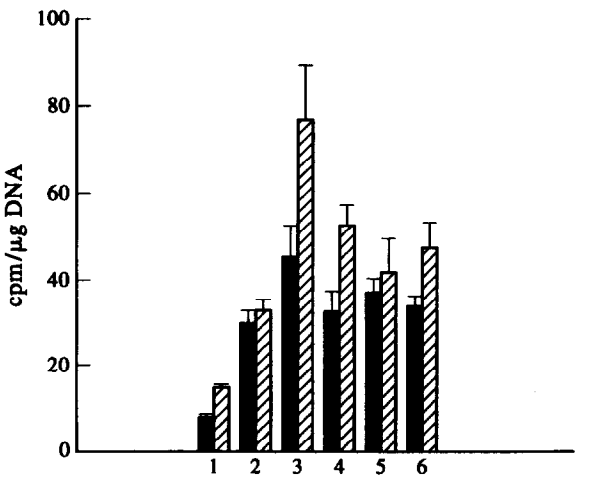

Figure 2. Effect of thymidylate synthase inhibitors on ^{125}IdU incorporation into mouse mammary tumour DNA. Tumour-bearing mice (groups of six) were injected at −1 h with the following drugs: 1, untreated control; 2, 5-FU (70 mg/kg) + folic acid (50 mg/kg); 3, ZD 1694 (10 mg/kg); 4, AG 337 (10 mg/kg); 5, 5-FU + folic acid + ZD 1694; 6, 5-FU + folic acid + AG 337. ^{125}IdU was injected at $t = 0$. The animals were sacrificed 24 h later for the determination of ^{125}IdU incoporation into the tumour (solid bars) and the small intestine DNA (hatched bars). The data shown are means ± S.E.M.

increase the ^{125}IdU incorporation compared with that obtained with 5-FU alone (Figure 1, Group IV).

An additional increase in the rate of incorporation of ^{125}IdU by the tumour tissue was produced by treatment of the animals with other inhibitors of thymidylate synthase. Inhibitors tested, alone or in combination, produced similar effects (Figure 2). The increase in the incorporation was observed both in the tumour and the intestine.

The incorporation of radioactivity into non-proliferating tissues (liver, lung) was negligible, irrespective of treatment with metabolic inhibitors (data not shown).

Treatment with benzyloxybenzyluracil

In spite of the increase resulting from the inhibition of thymidylate synthase, the incorporated radioactivity did not exceed 100 cpm/μg DNA, corresponding to 18 000 disintegrations/μg DNA over 24 h; assuming 10% cells in S-phase at the time of treatment, the radioactivity incorporated per dividing cell corresponds to ≤ 1.1 disintegrations/cell over 24 h. The overall incorporated radioactivity was less than 1% of the injected dose/g of tumour tissue, indicating that the vast majority of the precursor has been rapidly metabolised and/or excreted. A possible metabolic pathway, besides thymidylate synthase, responsible for the elimination of ^{125}IdU is dihydrouracil dehydrogenase [19]. We tested the effect of a recently developed inhibitor of this enzyme, benzyloxybenzyl uracil [13], on the ^{125}IdU incorporation. The result was disappointing as this drug failed to produce the desired effect (Figure 3). When applied simultaneously with ZD 1694, the observed incorporation of ^{125}IdU was in fact slightly lower than with ZD 1694 alone. Similarly, propylthiouracil, inhibitor of the Type I (low-specificity) iodothyronine 5′-deiodinase [12], was without effect on the rate of ^{125}IdU incorporation (Figure 1).

Effect of liver shunt on ^{125}IdU incorporation into tumour tissue

Since the liver is a major detoxifying organ, we considered the possibility that it may play an important role in the metabolic conversion/dehalogenation of circulating ^{125}IdU. Thus, the effect of liver shunt, clamping the entering blood vessels (vena porta, arteria hepatis) as well as the exiting blood vessel (vena cava) was examined. ^{125}IdU was injected via the penal vein (tail vein blood has to pass through the portal vein and liver before entering the heart) and the animals were maintained for 1 h prior to sacrifice and evaluation of the incorporation of ^{125}IdU into the tumour tissue. The results (Table 1) were the inverse of what was expected: instead of an increase, the liver shunt caused a significant reduction of the

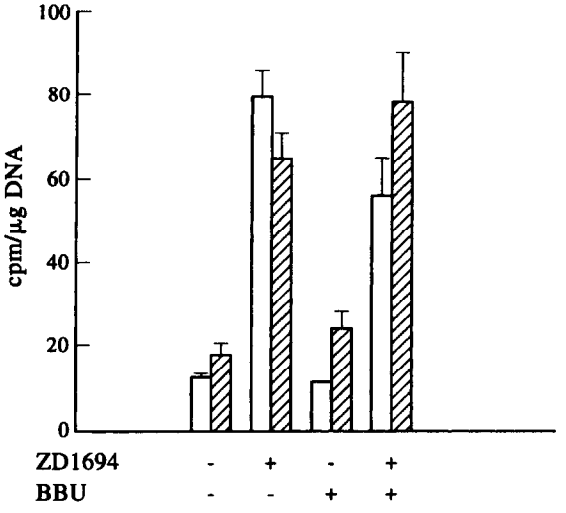

Figure 3. Effect of ZD 1694 and benzyloxybenzyl uracil (BBU) on the incorporation of ^{125}IdU incorporation into mouse mammary tumour DNA. Tumour-bearing mice (groups of 6) were given i.p. ZD 1694 (10 mg/kg) and benzyloxybenzyl uracil (10 mg/kg) as indicated, 1 h prior to ^{125}IdU administration. At 24 h after ^{125}IdU administration, they were sacrificed for the determination of the radioactivity incorporated into the DNA of the tumour (open bars) and of the small intestine (hatched bars). Means ± S.E.M. are shown.

Table 1. Effect of liver shunt on the incorporation of ^{125}IdU by mouse mammary tumour DNA

Mouse	Liver shunt	^{125}IdU incorporation (cpm/μg DNA)
1	No	69.4
2		82.7
3		119
4	Yes	9.6
5		5.3
6		13.7
7		12.8

GR mice (males) were ether-anaesthetised prior to placing the clamps in order to block the blood flow by the arteria hepatis, vena porta and vena cava. The control mice were subjected to identical manipulations except for placing the clamps. The ^{125}IdU-containing solution was then injected via the penal vein (blood collected by the tail vein passes entirely through the liver before entering the heart). The animals were maintained under anaesthesis during 1 h, sacrificed, and the tumours were excised for the determination of the radioactivity incorporated into tumour DNA.

incorporated radioactive precursor. The reasons for this effect are not clear, but may conceivably be due to a slower blood circulation and consequently slower delivery of ^{125}IdU to the tumour.

Effect of methotrexate, ZD 1694 and ^{125}IdU on tumour growth

Although the ^{125}IdU incorporation achieved in the preceding experiments was far below that reported to induce death of Chinese hamster fibroblasts in culture [2], a lower proportion of double-strand DNA breaks may be sufficient to cause the elimination of damaged cells *in vivo*. This could be the case if, for instance, alterations in the cell phenotype resulting from DNA damage facilitated the recognition of tumour cells by macrophages or natural killer cells. In this context, increased cytotoxicity of the cells of the immune system after exposure of tumour cells to DNA-damaging agents has been reported in several studies ([20] and references therein). To test the effect of ^{125}IdU on tumour growth, we treated the mice daily with methotrexate in order to favour the incorporation of the radioactive precursor into the tumour rather than normal tissues, and with ZD 1694 in order to obtain the optimal rate of incorporation. Three daily injections of ^{125}IdU, at an increased dose intensity (70 μCi/mouse per day) were administered intraperitoneally. Two experiments were carried out and yielded very similar results; one of the two experiments is shown in Figure 4. The combination of methotrexate and ZD 1694 caused a lasting reduction in the rate of tumour growth (Figure 4a). There was a tendency for a small and transient additional decrease in the tumour growth when ^{125}IdU was added to the cytostatics (three injections on days 1, 2 and 3, 70 μCi/mouse). At the end of the experiment (9 days after the last injection of ^{125}IdU), the animals were sacrificed, the tumours were weighed and the residual incorporated radioactivity in the tumour, intestine and spleen was determined. Tumour weight was lower in the treated ^{125}IdU animals than in controls (Figure 4b). In the small intestine and spleen, tissues known for their high cell proliferative activity, small amount of ^{125}I were present whereas the tumour tissue contained a several times higher radioactivity (Figure 4b, lower panel). The treatment was characterised by a low apparent toxicity. The body weight of the animals in the groups which received methotrexate and ZD 1694 was decreased by 8.1 ± 5.0% on day 5 (2 days after the end of treatment) and by 9.1 ± 8% on day 8; for the animals given ^{125}IdU along with the cytostatics, the weight decreases were 8.1 ± 4.1% on day 5 and 5.1 ± 3.1% on day 8.

DISCUSSION

The absolute necessity for dividing cells to incorporate deoxyribonucleotides is an attractive target for cancer diagnosis and therapy. Antimetabolites which inhibit *de novo* synthesis of such precursors have limited anticancer activity, often due to intrinsic or acquired resistance which allows the cells to overcome the inhibition by overexpression of key enzymes involved. Another way of exploiting the tumour DNA synthesis is to supply an abnormal variant of a precursor molecule. IdU is incorporated in the place of thymidine, and although not lethal in itself, it enhances the efficiency of cell killing by ionising radiation. In order to render IdU more effective as a radiosensitiser, and to reduce its undesirable effects on normal tissues, a treatment which favours the incorporation into tumour versus normal cells is needed.

An even more attractive alternative is the use of a radioactive DNA precursor (IdU labelled with ^{125}I, or with a shorter half-life iodine isotope such as ^{123}I, ^{124}I or ^{131}I) which would kill the cell by producing double-strand DNA breaks at each disintegration (cf. [1] and references therein). This sort of cytotoxic therapy would circumvent the most common types of drug resistance, and in order to acquire a resistance to a toxic thymidine analogue, the tumour cells would have to lose two copies of a gene required for either its entry (membrane nucleoside transporter) or utilisation (thymidine kinase). Selectivity of cell killing can also be imagined if one considers that the cell cycle in normal cells can always be blocked by a

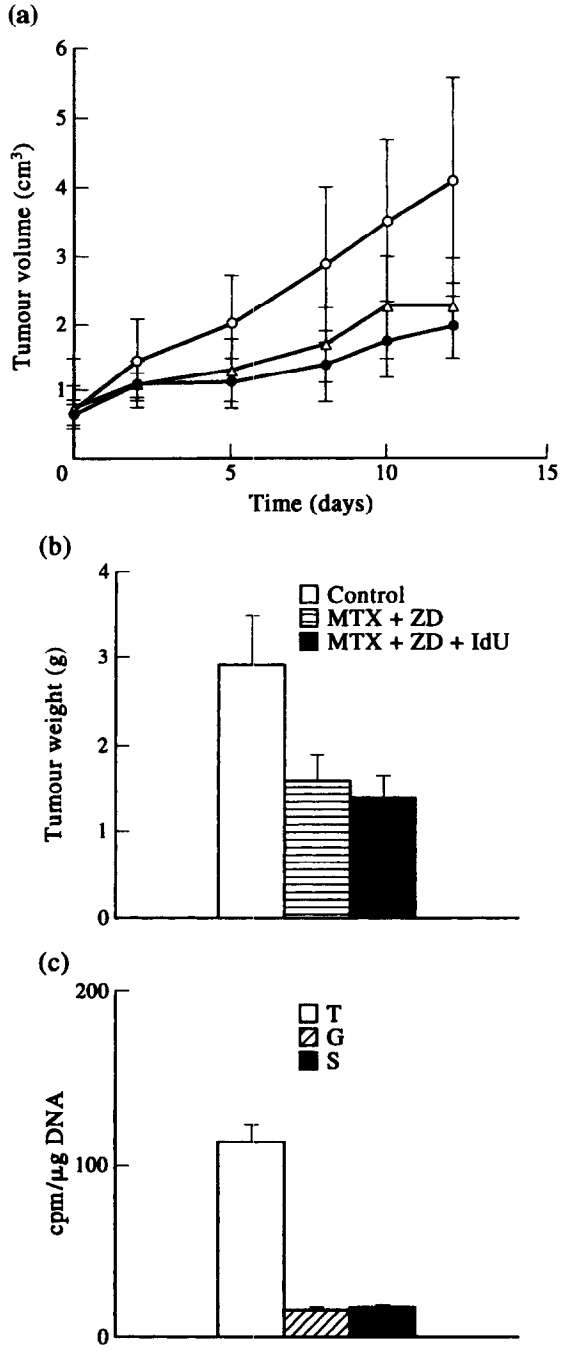

Figure 4. Effect of methotrexate, ZD 1694 and ^{125}IdU on tumour growth. Tumour-bearing mice (four per group) were given methotrexate (50 mg/kg) on days 0, 1, 2 and 3 and ZD 1694 (10 mg/kg) on days 1, 2 and 3 (triangles). Another group of mice received the same drugs plus ^{125}IdU (70 μCi/animal) on days 1, 2 and 3, always 1 h after the injection of the cytostatics (solid circles). Control animals were untreated (open circles). (a)Tumour volume; (b) tumour weights. The animals were sacrificed on day 12, and the weights of the tumours were determined. (c) The incorporation of ^{125}I into the DNA of different tissues of the mice treated with methotrexate, ZD 1694 and ^{125}IdU was evaluated on day 12.T, tumour; G, small intestine; S, spleen. All data shown are means ± S.E.M.

cytostatic drug such as hydroxyurea [4] or methotrexate [2 and this work] to which tumour cells will have developed resistance.

Unfortunately, whereas *in vitro* the phenomenon of radioactive suicide is easily manifested, in the living organism the metabolic half-life of ^{125}IdU is too short to allow a sufficient rate of incorporation for cell kill; the amount of the radioactive precursor incorporated into tumour cells *in vivo* is several orders of magnitude lower than under cell culture conditions. The current hypothesis is that the metabolic elimination of ^{125}IdU is due to its dehalogenation by thymidylate synthase. However, our results indicate that this is not the case. Although inhibition of thymidylate synthase led to an increase in the rate of ^{125}IdU incorporation, irrespective of the inhibitor used, the gain of incorporated radioactivity was relatively small (5- to 10-fold) and may have been due, at least in part, to the enhanced entry of ^{125}IdU into the cells via the nucleoside transporter [21]. The incorporation of the radioactive precursor was accomplished within a very short time following its administration; the incorporated radioactivity at 1 h after injection was not lower than after 24 h and decreased progressively during subsequent days (cf. [2, 22]). Interestingly, treatment with a combination of methotrexate and ZD 1694 for 4 days, along with a high dose of ^{125}IdU, strongly increased the tumour/normal tissue ^{125}IdU incorporation ratio measured 6 days after treatment (Figure 4c). A possible mechanism involved may be inhibition of the cell cycle in normal cells; alternatively, those non-tumoral cells which were labeled following ^{125}IdU injection, may have been at the end of their lifetime 9 days later.

We have explored other possible ways of reducing the loss of ^{125}IdU from the circulation. Halogenated pyrimidines including IdU have been shown to be better substrates of dihydrouracil dehydrogenase than the naturally occurring pyrimidine nucleobases uracil and thymine [13, 14, 19], and therefore inhibition of this enzyme could prolong the metabolic half-life of ^{125}IdU. However, benzyloxybenzyluracil, an inhibitor of dihydrouracil dehydrogenase [13], had little effect on the rate of ^{125}IdU incorporation, and similarly, propylthiouracil, an inhibitor of Type I iodothyronine 5'-deiodinase [12], was without effect on ^{125}IdU incorporation. Finally, we considered the possibility that metabolic elimination of ^{125}IdU may take place predominantly in the liver, the organ responsible for most of the detoxifying processes in the body. However, attempts to protect ^{125}IdU by clamping the liver during the time allowed for incorporation of the precursor were ineffective, and, in fact, led to decreased incorporation, possibly due to a slower access of the precursor to the tumour as a consequence of the obstructed blood flow.

In a patient study, the incorporation of radioactive IdU(^{131}I-labelled) has been used for detection of malignant lesions by scintigraphy [4]. The patients were pretreated for 2–3 days by hydroxyurea, which blocks DNA replication in sensitive cells, with the intention of producing hydroxyurea-resistance of the neoplastic cells. When ^{131}IdU was given subsequently, together with hydroxyurea, ^{131}IdU uptake by the tumour cells was favoured. However, certain (known) tumour lesions were not visualised by this approach; the authors attributed this failure to the possibility that resistance to hydroxyurea may not have been uniformly achieved.

A fortuitous finding of our study is the demonstrated antitumour activity of the combination of methotrexate and ZD 1694, at doses which caused no detectable toxicity. In the animals treated with methotrexate and ZD 1694, the additional administration of a high dose of ^{125}IdU caused a small additional delay in tumour growth. We presume that the lack of notable antitumour activity of ^{125}IdU is due to insufficient incorporation of the radioactive DNA precursor.

In conclusion, we have shown that inhibition of thymidylate synthase leads only to a limited increase in the uptake of ^{125}IdU by tumour as well as by normal proliferating cells. It is unlikely that a considerably higher incorporation of the radioactive precursor could be obtained in the human under similar conditions, although circulating thymidine concentration in rodents is considerably higher. According to a report of Dethlefsen [23], when ^{125}IdU is injected together with unlabelled IdU, the incorporation of the label into tumour DNA is proportional to the injected dose up to 5 mg/mouse, indicating that the level of unlabelled precursor is not a limiting factor. The addition of methotrexate improves the tumour/ normal tissue ratio of the incorporated radioactivity. The use of radiolabelled IdU for tumour imaging, aimed at the visualisation of lesions too small to be detectable by standard methods, might be further developed, possibly in combination with better methods of delivery such as isolated organ perfusion [24]. Early detection of such micrometastases may be crucial for at least some patients. Better methods to protect the radioactive precursor against metabolic conversion and/or elimination are needed for imaging and for possible therapeutic applications under conditions of systemic administration of radioactive IdU.

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