5-Ethynyl-2’-deoxycytidine as a new agent for DNA labeling: Detection of proliferating cells

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**A B S T R A C T**

The labeling of newly synthesized DNA in cells to identify cell proliferation is an important experimental technique. The most accurate methods incorporate \(^{3}H\)thymidine or 5-bromo-2’-deoxyuridine (BrdU)\(^1\) into dividing cells during S phase, which is subsequently detected by autoradiography or immunohistochemistry, directly measuring the newly synthesized DNA. Recently, a novel method was developed to detect DNA synthesis in proliferating cells based on a novel thymidine analog, 5-ethynyl-2’-deoxyuridine (EdU). EdU is incorporated into DNA and subsequently detected with a fluorescent azide via “click” chemistry. This novel technique is highly sensitive and does not require DNA denaturation. However, it was also found that EdU exhibits time-dependent inhibition effects on cell growth. Therefore, here we report a novel deoxycytidine analog, 5-ethynyl-2’-deoxyuridine (EdC), that can be used to detect DNA synthesis in vitro and in vivo at a similar sensitivity level compared with EdU. Furthermore, the EdC-induced cytotoxicity is much less than that of EdU when combined with thymidine. This will be a potential application for the long-term detection of proliferating cells.

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The detection of proliferating cells is a fundamental experimental tool for assaying basic biology, assessing cell health, and evaluating the cytotoxicity of lead compounds. The traditional method of detecting cell proliferation is the incorporation of a thymidine analog, \(^{3}H\)thymidine or 5-bromo-2’-deoxyuridine (BrdU),\(^1\) into duplicated DNA during S phase. Although the classical method of \(^{3}H\)thymidine labeling is very powerful, it exhibits several drawbacks [1]. During the mid-1980s, a method that uses a nonradioactive analog of thymidine, BrdU, to replace \(^{3}H\)thymidine was developed. Similar to \(^{3}H\)thymidine, BrdU can be incorporated into duplicate DNA and subsequently detected with a BrdU-specific antibody to label cell proliferation [2]. Although this BrdU method has gained popularity due to its benefits, there are also a few significant limitations to its overall application. Because the size of the monoclonal antibody is too large to approach the BrdU molecules that are masked within the DNA, this method requires a harsh DNA denaturation step to reveal the epitope for the binding of the anti-BrdU antibody. The denaturation procedure typically includes treatment with hydrochloric acid or heating. Thus, these strong denaturation conditions may destroy tissue structure and other protein epitopes of interest within the tissue and hinder the multiplex analysis of this classical antibody staining technique.

Recently, a novel thymidine analog, 5-ethynyl-2’-deoxyuridine (EdU), was developed as an alternative to \(^{3}H\)thymidine or BrdU for the labeling of DNA in proliferating cells [3,4]. The terminal alkynyl group of EdU can be detected with fluorescent azides via “click” chemistry [5–7]. Because the size of the fluorescent azide reagents is much smaller than that of their corresponding antibodies, the fluorescent azides can easily diffuse through tissues and approach the incorporated EdU without DNA denaturation. The EdU incorporation method has been successfully employed in the detection of cell proliferation in the nervous system [8,9], chick embryos...
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[10], breast cancer cells [11], Escherichia coli [12], mice T cells [13], chick retina [14], avian cochlea [15], plant cells and tissues [16], and human fibroblasts [17,18]. It has been used for tracking of stem cells in vivo [19]. However, the pronounced cytotoxicity of EdU has been reported; cells will eventually die after continuous incubation with EdU [11].

In this study, we developed a novel deoxycytidine analog, 5-ethynyl-2'-deoxycytidine (EdC). Similar to EdU, EdC can be incorporated into DNA and detected with a fluorescent azide dye via click chemistry in vitro and in vivo. We demonstrated that EdC is at least as sensitive as EdU for DNA labeling. In addition, the long-term cytotoxicity of EdC is much less than that of EdU for most cells. The EdC-induced cytotoxicity can be further reduced by the addition of thymidine.

Materials and methods

A549 (human lung cancer cell line) and LLC (mouse Lewis lung cancer cell line) cells were maintained in RPMI 1640 medium, HLF (human embryonic lung fibroblast cell line) and C2C12 (mouse muscle myoblast cell line) cells were maintained in Dulbecco's modified Eagle's medium (DMEM). All media were supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). Cells were grown in an atmosphere of 5% CO2 at 37 °C could be preserved as solid powder in a dark place at 4 °C for 1 year without degeneration or could be prepared to corresponding solutions (20 mM EdU stock solution in H2O, 100 mM EdC stock solution in H2O, and 10 mM Cy3-azide stock solution in dimethyl sulfoxide (DMSO)) and stored keeping out light at 4 °C for 6 months without degeneration.

EdC or EdU labeling of cultured cells

A549 cells were grown on glass coverslips in RPMI 1640 supplemented with 10% FBS. EdU was added to the complete culture medium from a 20-mM stock solution in H2O, and EdC was added to the culture medium at a concentration of 10 μM for 24 h. After labeling, the cells were washed with phosphate-buffered saline (PBS) and fixed by 4% paraformaldehyde in PBS for 30 min at room temperature. The cells were then incubated with glycine at a concentration of 2 mg/ml in PBS for 10 min to quench the fixation effects of paraformaldehyde. Lastly, the cells were permeated by 0.2% Triton X-100 in PBS for 30 min at room temperature. The cells were then incubated with fluorescein isothiocyanate (FITC)- or Cy3-labeled secondary antibodies and evaluated using a fluorescence microscope (Nikon, Tokyo, Japan). The specificity of the 5-iododeoxycytidine (EdC) labeling was determined by staining A549 cells with FITC- or Cy3-labeled secondary antibodies without the addition of EdC.

EdC staining

After paraformaldehyde fixation, the cells were rinsed once with PBS and then stained after a 30-min incubation period with 100 nM Tris (from a 2-M stock solution, pH 8.5), 1 mM CuSO4, ascorbic acid, paraformaldehyde, glycine, and 4% paraformaldehyde, followed by a 24-h incubation in medium supplemented with 10 μM EdC and 10 mM hydroxyurea or 2 mM thymidine, followed by a 24-h incubation in medium supplemented with 10 μM EdC and 10 mM hydroxyurea or 2 mM thymidine. To test the labeling effect of cells incubating with EdU and thymidine or EdC and thymidine, the cells were incubated for 24 h with 10 μM EdU or EdC or the following ratios of EdU/thymidine or EdC/thymidine: 2:1, 1:1, and 1:2. The cells were then fixed and stained with fluorescent Cy3- and DAPI as indicated above.
of ascorbate. After staining, the cells were placed on coverslips, washed two times with PBS and methanol, washed once with PBS, and then stained with DAPI. Lastly, the labeled cells were imaged by fluorescence microscopy.

Continuous cell treatment with EdU and EdC and annexin V/propidium iodide assay

A549 cells were seeded in Petri dishes at a cell density of $1.5 \times 10^5$ on day 1 and treated with 10 µM EdU or EdC on day 3, 4, or 5 for 48, 24, or 4 h, respectively. A Petri dish with A549 cells treated with no EdU or EdC was used as a control. Cells were harvested on day 5 for apoptosis analysis; the culture supernatant of each cell sample was saved and merged with the harvested cells. Then the samples were labeled with annexin V/propidium iodide and measured using a BD FACSCalibur flow cytometer.

High-content screening image analysis

Four cell types (A549, C2C12, HLF, and LLC) were plated into 96-well flat-bottom plates (Greiner Bio-One, Monroe, NC, USA) at 1000 cells per well. The culture medium containing 10 µM EdU or EdC/thymidine ratios (2:1, 1:1, and 1:2) was added to the plate after cell growth for 24, 48, 72, or 96 h. After an incubation period of 24 h, the supplemented medium (plus nucleosides in the case of long-term nucleoside incubation) was replaced in the entire plate and incubated for an additional 24 h. Subsequently, the supernatant was removed and the cells were washed once with PBS. The cells were fixed and stained with fluorescent Cy3-azide and DAPI, as indicated above. Lastly, the cells were imaged on an ArrayScan VTI high-content screening (HCS) reader (Cellomics, Pittsburgh, PA, USA). Data analysis was performed with PanNo (Chi-Square Works, Seabeck, WA, USA), and graphing was conducted using Prism (GraphPad Software; San Diego, CA, USA).
Optimal dose determination of EdC for labeling mouse tissues

Four 3-week-old mice were injected intraperitoneally (i.p.) with 10 μg, 100 μg, 1 mg, or 10 mg of EdC in 0.1 ml of PBS. The control animal was injected i.p. with 0.1 ml of PBS, and the mouse ileums were harvested at 24 h after injection. For the staining of fixed sections, pieces of the tissues were formalin fixed, embedded in paraffin, and sectioned. After paraffin removal, the sections were placed onto glass slides and stained with 10 μM Cy3-azide for 30 min as described except that the reaction and subsequent washes were performed in Coplin jars. The sections were counterstained with DAPI and mounted for fluorescence microscopy.

Fig. 2. Using flow cytometry to test the cytotoxicity of EdC and EdU. A549 cells were incubated with EdU or EdC for 4, 24, or 48 h. Then cells were harvested and measured on a BD FACSCalibur flow cytometer. When the incubation time was ≤24 h, the cytotoxicity of EdC and EdU was little (ii, iii, v, vi) compared with the control live cell ratio (i). A549 cells were highly affected by long-term (48 h) exposure to EdU or EdC, and A549 cells were more sensitive to EdU than to EdC (iv and vii). The data statistics of panels i-vii were summarized in the panel viii.
EdC labeling of mouse tissues

Four 3-week-old mice were injected i.p. with 1 mg of EdC in 0.1 ml of PBS. A control littermate was injected i.p. with 0.1 ml of PBS, and the mouse livers, kidneys, spleens, colons, and ileums were harvested at 24, 48, 72, or 96 h after injection. Pieces of the harvested tissues were embedded in paraffin and sectioned. After paraffin removal and rehydration, the sections were subsequently stained with fluorescent Cy3-azide and DAPI as described above. Lastly, the samples were mounted for fluorescence microscopy.

Results and discussion

Cellular DNA labeling with EdC

To detect DNA synthesis in proliferating cells, we synthesized EdU and EdC (Fig. 1A-i) by a Sonogashira coupling reaction, as described previously [20,21]. For detecting the incorporation of EdU or EdC into the cell, we synthesized a fluorescent azide derivative, Cy3-azide (Fig. 1A-ii). A549 cells were cultured with 10 μM EdC, with 10 μM EdU, or without EdC and EdU for 24 h and then fixed and subsequently stained with fluorescent Cy3-azide and DAPI. As shown in Fig. 1C, the labeling effect of EdC is similar to that of EdU (columns ii and iii). The EdC-labeled cells show strong nuclear staining, and not all nuclei are labeled. In contrast, the cells incubated without EdC and EdU demonstrate no detectable Cy3-azide staining (column i). S phase is the part of the cell cycle during which DNA is replicated, so EdC or EdU could be efficiently incorporated into replicating DNA only during S phase. For reasons given above, not all of the cells could be labeled by EdC or EdU, and the only cells that went through S phase were labeled. DAPI is a fluorescent dye that binds strongly to AT-rich regions in DNA, so it could stain all of the fixed cells. For reasons given above, not all DAPI-positive cells overlap with the Cy3-positive cells.

To demonstrate that EdC labels cellular DNA, A549 cells were incubated overnight with EdC in the presence of hydroxyurea or thymidine, and no detectable Cy3-azide staining was observed in the presence of 10 mM hydroxyurea (Fig. 1C, column iv) or 2 mM thymidine (Fig. 1C, column v). Hydroxyurea is an inhibitor of ribonucleotide reductase that could reduce the production of deoxyribonucleotides via inhibiting ribonucleotide reductase, thereby interfering with the synthesis of DNA and resulting in retardation of the cell cycle traverse during S phase [22]. High-concentration thymidine (2 mM) also could inactivate ribonucleotide reductase and block DNA synthesis in the same way as hydroxyurea [22]. For reasons given above, when cells incubated with 10 μM EdC and 10 mM hydroxyurea or 2 mM thymidine, DNA synthesis would be blocked by 10 mM hydroxyurea or 2 mM thymidine, EdC could not be incorporated into replicating DNA, and EdC labeling signal would be eliminated. These results demonstrate that the nuclear staining in EdC-labeled cells arises from EdC incorporation into the replicating DNA during S phase.

EdC is a novel deoxycytidine analog and has been previously evaluated as a more selective and less cytotoxic antiviral agent than EdU [23]. Saran investigated the conformational property of EdC by the perturbative configuration interaction using the localized orbital (PCIL0) method and compared the result with that of 2'-deoxycytidine [24]. The results indicated striking similarity in their conformational behavior. Hazra and coworkers demonstrated that human deoxycytidine kinase (dCK) was able to phosphorylate 5-alkyne-substituted deoxycytidine analogs [25]. Therefore, we reasoned that EdC may successfully mimic its parent nucleoside, 2'-deoxycytidine, and function in the enzymatic reactions that induce its biological activity. Our results demonstrated that EdC could be phosphorylated by dCK and then efficiently incorporated into replicating DNA and that its terminal alkyne group could be available to react with Cy3-azide for the labeling of DNA synthesis.

Cytotoxicity of EdC and EdU by flow cytometric analysis

Continuous EdU treatment induces cytostatic or cytotoxic effects [11,24,26], and EdC was less cytotoxic than EdU [23]. To test the cytotoxicity of EdU or EdC, A549 cells were incubated with 10 μM EdU or EdC for 4, 24, or 48 h and then harvested and measured using a BD FACSCalibur flow cytometer. The flow cytometry results are shown in Fig. 2. In comparison with the control cells (Fig. 2-i), pulse labeling for short durations (4 or 24 h) with EdU (Fig. 2-ii and -iii) and EdC (Fig. 2-v and -vi) show little effect...
on the live cell fraction. Long-term (48 h) EdU (Fig. 2-iv) or EdC (Fig. 2-vii) treatment showed cytotoxicity to A549 cells. The proportion of live cells treated with EdC (62.53%) for 48 h is nearly twice that of EdU-treated cells (39.09%). These results indicate that the cytotoxicity of EdU or EdC is time dependent and that the long-term cytotoxicity of EdC is much lower than that of EdU.

In the presence of folate, 5-ethynyl-dUMP (EdUMP), the metabolic product of EdU, caused the time-dependent inhibition of thymidylate (dTMP) synthetase (dUMP → dTMP) \[26\]. Cells could not survive when endogenous thymidine synthesis was inhibited \[27\]. Thus, the cytotoxic properties of EdU are due primarily to the inhibition of the dTMP synthetase, which has been confirmed with cell culture and isolated enzyme systems \[26,28\].

Our experimental results indicate that EdC also induces cytotoxicity with prolonged incubation time. The proportion of live cells treated with EdC for 48 h is 62.53%. Just like EdU, EdC achieves its cytotoxicity as a specific inhibitor of dTMP synthetase. This is due to EdCMP, the phosphorylation product of EdC by dCK, which could be converted to EdUMP by dCMP deaminase \[29\]. EdUMP would cause the time-dependent inactivation of dTMP synthetase.
suppressing cell proliferation [23]. EdUMP is the effector molecule that induces the cytotoxicity of EdC and EdU. To inactivate dTMP synthetase and suppress cell proliferation, EdC requires the assistance of two enzymes (deoxycytidine kinase and dCMP deaminase) compared with one (TK kinase) for EdU. Thus, EdC is a weaker inhibitor of dTMP synthetase than EdU and is less cytotoxic than EdU [23].

Because EdC and EdU owe their cytotoxicity primarily to the specific inhibition of dTMP synthetase and the inhibition of dTMP synthetase is time dependent [23,28], the cytotoxicity induced by EdU or EdC is time dependent as well. As shown in Fig. 2, when the incubation time was short (4 or 24 h), the cytotoxicity of EdU or EdC is not significant (cf. panel i with panels ii, iii, v, and vi). When the incubation time was prolonged to 48 h, the cytotoxicity of EdU or EdC is noteworthy (cf. panel i with panels iv and vii).

Because EdC is less toxic than EdU, the proportion (62.53%) of live cells treated with EdC for 48 h is much larger than that (39.09%) of EdU-treated cells (cf. panel iv with panel vii). Because EdC is less toxic than EdU, the proportion (62.53%) of live cells treated with EdC for 48 h is much larger than that (39.09%) of EdU-treated cells (cf. panel iv with panel vii).

EdC-induced cell toxicity reduction after thymidine incubation

EdC and EdU owe their suppressive effects on cell growth primarily to the specific inhibition of dTMP synthetase; the blockage would be more readily reversed by incubating cells with thymidine than with deoxuryridine or deoxyutidine [23,26,28]. For this reason, we first examined whether cells incubated with EdU or EdC in the presence of thymidine would influence their incorporation. Thus, A549 cells were incubated with 10 μM EdU or EdC in the presence of 5, 10, or 20 μM thymidine for 24 h. The cells were then fixed and stained with fluorescent Cy3-azide and DAPI. Because thymidine is an inhibitor of ribonucleotide reductase, cell growth would be suppressed at high thymidine concentrations. Therefore, the maximum concentration employed for thymidine is 20 μM, which is not inhibitory to cell growth [22,23,27].

As shown in Fig. 3A, the intensity of the EdU stain and the EdU-positive cells are inversely proportional to the concentration of thymidine. When cells were incubated with EdU and an increased thymidine concentration, the EdU incorporation into cells was pronouncedly inhibited and the intensity of EdU staining was significantly decreased (Fig. 3A). As shown in Fig. 3C, along with the increasing thymidine concentration, the ratios of the number of proliferating cells labeled with EdU to the number of total cells labeled with DAPI decreased significantly [from 75.85% [incubated with 10 μM EdU only] to 57.85% [incubated with 10 μM EdU and 5 μM thymidine], to 37.28% [incubated with 10 μM EdU and 10 μM thymidine], to 23.91% [incubated with 10 μM EdU and 20 μM thymidine]]. These findings are not atypical because thymidine is a competitive substrate of thymidine kinase and would competitively inhibit the phosphorylation of EdU by thymidine kinase, subsequently blocking the incorporation of the 5’-triphosphate of EdU into the DNA. As shown in Fig. 3B, the EdC stain intensity slightly decreased proportionally to the thymidine concentration, which was much less than that of the EdU staining (Fig. 3A). See Fig. 3C compared with EdU; as the concentration of thymidine increased, the ratios of the number of proliferating cells labeled with EdC to the number of total cells labeled with DAPI decreased slightly [from 88.89% [incubated with 10 μM EdC only] to 84.48% [incubated with 10 μM EdC and 5 μM thymidine], to 75.63% [incubated with 10 μM EdC and 10 μM thymidine], to 63.85% [incubated with 10 μM EdC and 20 μM thymidine]].

Because the effect of thymidine on the EdC stain is quite finite, we next investigated whether incubation with thymidine would reduce the cytotoxicity of continuous cell labeling by EdC. To test the impact of continuous cell labeling by EdU, EdC, or EdC and thymidine on cell proliferation, four cell types (A549, C2C12, HLF, and LLC) were plated into 96-well flat-bottom plates for 24 h and then treated with 10 μM EdU or EdC or EdC/thymidine ratios (2:1, 1:1, and 1:2) for 48 h. Then cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, and subsequently stained with fluorescent Cy3-azide and DAPI. The harvested cells were imaged using an HCS system, and the image data were statistically analyzed. The surviving number of cells per field was counted based on DAPI staining and revealed EdC- or EdC-induced cell toxicity. The results of HCS images and image analyses are shown in Fig. 4. The surviving numbers of cells descended proportionally to the incubation time with the EdU or EdC treatment for all four cell lines (A549, LLC, C2C12, and HLF) (Fig. 4A). However, the surviving numbers of C2C12 and HLF cells declined quite slowly and were close to a straight line proportional to the incubation time in the presence of EdC by the addition of 10 or 20 μM thymidine (Fig. 4A-iii and A-iv). This result indicates that the EdC-induced cytotoxicity for C2C12 and HLF cells is abated to a fairly low level by incubation with thymidine (Fig. 4B).

Fig. 4B shows the cell images of the four cell types (A549, C2C12, HLF, and LLC) plated into 96-well flat-bottom plates for 24 h and then treated with 10 μM EdC or EdC/thymidine ratios (2:1, 1:1, and 1:2) for 48 h. Thymidine slowed down the decline of the surviving cell counts of all four cell lines to some extent, proportional to the added thymidine concentration. The numbers of surviving cells with these cell lines treated with 10 μM EdC in the presence of 20 μM thymidine increased remarkably in comparison with 10 μM EdC only (Fig. 4B, column v). The surviving cell numbers of C2C12 and HLF cell lines treated with 10 μM EdC and 20 μM thymidine are the same with the cell lines without any treatment.

When cells are incubated with EdC and thymidine, thymidine can be phosphorylated by thymidine kinase and the dTMP synthetase reaction (dUMP → dTMP) is circumvented in the pathway that leads to endogenous thymidine synthesis [23,28]. Thus, the EdC-induced inhibition of cell growth would be more readily reversed by thymidine. Because deoxyxycytidylic acid (dCMP) deaminase, which catalyzes the hydrolytic deamination of dCMP to dUMP and ammonia, also acts on some 5-substituted deoxyxycytidylic acids [29], EdC may exhibit two deoxyribonucleotide salvage pathways to follow in cellular metabolism. In one pathway, EdC would be phosphorylated to 5’-mono-, 5’-di-, and 5’-triphosphate (EdC → EdCDP → EdCTP), successively, by cellular kinase and then incorporated into cellular DNA. In the other pathway, EdC is first monophosphorylated to EdCMP by dCK. Then EdCMP would be converted to EdUMP by dCMP deaminase, and EdUMP would be subsequently phosphorylated into EdCTP.
phosphorylated to 5'-di- and 5'-triphosphate (EdC → EdCMP → EdUMP → EdUTP) and then eventually incorporated into DNA. For this reason, EdC would be incorporated into DNA in two triphosphate forms (EdCTP and EdUTP).

It has been reported that, in primary rabbit kidney (PRK) cells, the dose inhibiting the incorporation of [methyl-3H]dThd by 50% of EdC is 120 μg/ml, and the corresponding dose of EdC to inhibit the incorporation of [5-3H]dCyd by 50% is only 3 μg/ml [23]. The data that EdC inhibited [5-3H]dCyd incorporation at a concentration that was quite a bit lower than the inhibitory concentration for [methyl-3H]dThd incorporation may suggest that EdC follows the EdC → EdCMP → EdCTP route as the major metabolic pathway and the EdC → EdCMP → EdUMP → EdUTP route is the minor metabolic pathway of EdC. As shown in Fig. 3C, a much greater inhibitory effect of thymidine on EdU than on EdC incorporation into DNA also could be interpreted as evidence for EdC following EdC → EdCMP → EdCTP as the major metabolic pathway.

In the two salvage pathways, the first phosphorylation step catalyzed by dCK is considered as rate limiting [30]. Thymidine is not a substrate for dCK and cannot competitively inhibit the phosphorylation of EdC [25]. However, dTMP, which is the product of the

![Fig. 6. Use of EdC to assay DNA synthesis in different tissues and for different times. Mice were injected i.p. with 1 mg of EdC. Organs were harvested after 24, 48, 72, or 96 h. The sections of organs were subsequently stained with Cy3-azide and DAPI. (A) Only feeble fluorescent signals of EdC labeling on liver sections could be detected after 48 and 72 h, and there were no detectable EdC labeling signals after 24 or 96 h. (B) The vast majority of nuclei on kidney sections were not labeled with EdC throughout the incubation time. (C) The intensity of Cy3-azide on spleen sections was increased from 24 to 72 h and then began to decrease. (D) Strong specific Cy3-azide stain could be detected on colon sections from beginning to end. (E) Strong EdC labeling signal could be seen on ileum sections after 24 and 48 h, and the intensity of Cy3-azide was decreased sharply after 72 and 96 h.](image-url)
phosphorylated thymidine by thymidine kinase, is a competitive inhibitor for the further phosphorylation of EdUMP. Thus, it would suppress EdC incorporation into DNA in the EdUTP form. In the above-mentioned metabolism mechanisms of EdC and thymidine, thymidine would decrease the intensity of the EdC stain; however, the reduction would be limited.

When cells were incubated with EdC and thymidine, thymidine could be phosphorylated by thymidine kinase and the endogenous thymidine synthesis could be carried through, inducing cell proliferation. With continued DNA replication, more and more EdC was incorporated into the cells. Thus, the mean fluorescence intensity increased proportionally to the incubation time. When EdC incorporation approached saturation, the mean fluorescence intensity increased slightly. When cells were incubated with EdC or EdU alone, EdUMP, a metabolite of EdC and EdU, would block the dTMP synthetase reaction. Then DNA replication would be inhibited by the lack of endogenous thymidine synthesis, inducing cell necrosis. The inhibition of dTMP synthetase by EdUMP is time dependent, and different cells exhibit different sensitivities to EdC or EdU.

Use of EdC to assay DNA synthesis in animals

To determine the dosage of EdC for assaying DNA synthesis in animals, four 3-week-old mice were injected i.p. with 10 μg, 100 μg, 1 mg, or 10 mg of EdC in PBS. The un.injected littersmates were used as the control. The tissues were harvested and fixed 24 h after injection. Paraffin sections were subsequently stained with fluorescent Cy3-azide and DAPI, washed, and then imaged by fluorescence microscopy. Compared with the control mouse, which was injected i.p. with 0.1 ml of PBS, no changes were detected on the experimental mouse in weight gain, food use efficiency, directed behavior, or tissue sections. As shown in Fig. 5, the ileum surveyed showed low background staining in the control section (Fig. 5-i). An obvious Cy3-azide signal was observed in the ileum of the EdC-injected mouse, and the intensity of Cy3-azide increased proportionally to the dosage of EdC (Fig. 5-ii–v). When the dosage was larger than 1 mg, the fluorescence intensity rarely increased (Fig. 5-iv and -v). We ascertained that the optimal dosage of EdC to assay DNA synthesis in mice is 1 mg.

To assay the DNA synthesis in different tissues and times using EdC, 1 mg of EdC was injected i.p. into four 3-week-old mice, and the organs were harvested 24, 48, 72, or 96 h later. An un injected littermate was used as a control. The organs were fixed, embedded in paraffin, sectioned, and stained with Cy3-azide and DAPI. The staining results of five different organs (liver, kidney, spleen, colon, and ileum) are shown in Fig. 6. In the liver sections (Fig. 6A), hardly any EdC staining signal was detected after 24 h (Fig. 6A-i), the fluorescence intensity became strong after 48 h (Fig. 6A-ii), and the intensity of Cy3-azide decreased with incubation time. After 96 h, only a few EdC-labeled hepatocytes were detected (Fig. 6A-v). In sections throughout the kidney cortex, no obvious EdC-labeled cells were found for all incubation times (Fig. 6B). In the sections of spleen (Fig. 6C), the intensity of EdC staining increased with an increase in incubation time from 24 to 72 h (Fig. 6C-ii–iv) and the strongest EdC labeling effect appeared after 72 h (Fig. 6C-iv). After 72 h, the intensity of Cy3-azide began to decrease (Fig. 6C-v). In the sections of colon, EdC staining was very intense throughout all incubation times (Fig. 6D). Lastly, in the sections of ileum (Fig. 6E), EdC strongly labeled proliferating cells after 24 and 48 h (Fig. 6E-ii and E-iii) and the intensity of EdC staining decreased sharply after 72 and 96 h (Fig. 6E-iv and E-v).

All five organs surveyed showed low background staining in the control sections, whereas very intensely stained cells were seen in four organs (liver, spleen, colon, and ileum) in the EdC-injected mouse. Understanding the physiological basis for those histological observations requires further study. We conclude that EdC is well suited for detecting cell proliferation, not only in cultured cells but also in tissues. The low cytotoxicity of EdC can produce highly incorporated EdC DNA, which can be used to track the cells in vivo.

Conclusion

We have developed a novel deoxycytidine analog, EdC, which can be used to detect DNA synthesis in vitro and in vivo via a Cu(I)-catalyzed click reaction. EdC demonstrates similar sensitivity in comparison with EdU, and its cytotoxicity is much less than that of EdU for most cell lines. For some cell types, EdC-induced cytotoxicity can be reduced after the addition of thymidine with obvious fluorescence intensity loss. We conclude that, for some cell lines, EdC can be used as a less toxic nucleoside analog than EdU, not only for pulse but also for continuous cell labeling via incubation with thymidine. We expect that EdC combined with thymidine would have potential applications for the long-term detection of proliferating cells and for tracking of cells in vivo.

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