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Phosphorylated 5-ethynyl-2′-deoxyuridine for advanced DNA labeling†

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The representative DNA-labeling agent 5-ethynyl-2′-deoxyuridine (EdU) was chemically modified to improve its function. Chemical monophosphorylation was expected to enhance the efficiency of the substrate in DNA polymerization by circumventing the enzymatic monophosphorylation step that consumes energy. In addition, to enhance cell permeability, the phosphates were protected with bis-pivaloyloxymethyl that is stable in buffer and plasma, and degradable inside various cell types. The phosphorylated EdU (PEdU) was less toxic than EdU, and had the same or a slightly higher DNA-labeling ability *in vitro*. PEdU was also successfully applied to DNA labeling *in vivo*. In conclusion, PEdU can be used as a less toxic DNA-labeling agent for studies that require long-term cell survival or very sensitive cell lines.

Introduction

Detection of DNA synthesis in growing organisms is important for biological science including cell proliferation and differentiation, cell cycle dynamics, and carcinogenesis.[1–3] DNA labeling is conducted for detection using pyrimidine nucleosides such as [3*H*]thymidine, bromodeoxyuridine (BrdU), and 5-ethynyl-2′-deoxyuridine (EdU).

DNA labeling with [3*H*]thymidine is a powerful technique but it has been replaced by BrdU in many studies because autoradiography requires special equipment and is labor intensive and very time consuming.[4] As an alternative, BrdU labeling is faster and provides better results in microscopic analyses. However, after incorporation of BrdU into replicated DNA during S phase, the technique requires an anti-BrdU antibody that cannot bind to BrdU in double stranded DNA without harsh DNA denaturation for conversion to single stranded DNA.[5]

Recently, DNA labeling techniques using EdU have been described as an alternative to both [3*H*]thymidine and BrdU.[9–12] It contains an alkyne group that reacts with fluorescent azide in a copper-catalyzed azide–alkyne cycloaddition (CuAAC) by click chemistry.[6–8] The main advantage of using EdU for DNA detection is that the labeled DNA can be detected without denaturation because of the much smaller size of the fluorescent azide allows access to the alkyne group of the incorporated EdU. Despite these advantages, EdU is a highly toxic antimetabolite that induces cell death.[13,14] Its cytotoxicity is an impediment to DNA labeling studies that require subsequent cell survival. Therefore, EdU has been modified to reduce its cytotoxicity by substitution of fluoro, hydroxyl and methyl groups with 2′ hydrogen.[15] Not only pyrimidine but also purine nucleoside analogues containing an alkyne group have been established.[16–18] These compounds were designed to bypass the antimetabolic pathway of EdU and maintain the alkyne group for click chemistry with fluorescent azide.

Here, we applied a different approach by phosphorylation of EdU. Based on the structure of EdU, chemical phosphorylation was carried out on the 5′ hydroxyl group. The chemical monophosphorylation was expected to increase the efficiency of the substrate in DNA polymerization by circumventing the enzymatic monophosphorylation step that consumes energy. However, a phosphorylated compound is generally ineffective at penetrating the cell membrane because of its negative charges. Therefore, cell permeability was enhanced through protection of the phosphates by bis-pivaloyloxymethyl [bis-(POM)] that is quite stable in buffer and plasma, and reversible inside various cell types.[19–22] After penetrating the cell membrane, progressive degradation of bis(POM)-protecting groups regenerates EdU monophosphate gradually and can prevent excessive concentrations of thymidine-like antimetabolites, resulting in a reduction of cytotoxicity.

In this study, bis(POM)-phosphorylated EdU (PEdU) was synthesized by conjugation of chlorobis(POM) phosphate to

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EdU.[23] The synthesized PEdU was compared with commercial EdU in terms of cytotoxicity and sensitivity *in vitro*, and its labeling capability *in vivo* was evaluated using mice.

Materials and methods

Materials

5-Iodo-2′-deoxyuridine (IdU) was purchased from Tokyo Chemical Industry Co., Ltd. All other reagents were purchased from Sigma-Aldrich, Tokyo Chemical Industry Co., Ltd, or Wako Pure Chemical Industries, Ltd, and used without further purification. All non-aqueous reactions were conducted using super dehydrated solvent in an argon atmosphere. Flash column chromatography was performed using silica 60 (Spherical, 40–50 nm; Kanto Chemical). Electrospray ionization mass spectra (ESI-MS) were obtained on an Agilent 1100 series LC/MSD. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL AL-400 (400 MHz). HeLa and 3T3 cells were maintained in Dulbecco's modified Eagle's medium (high glucose with L-glutamine and phenol red; Wako Pure Chemical Industries, Ltd) supplemented with 10% fetal bovine serum. Cells were cultured at 37 °C in an atmosphere with 5% CO_2.

Synthesis of PEdU

A scheme of the synthesis is shown in Fig. 1.

Synthesis of EdU (1). A round flask was flushed with argon, and then IdU (371 mg, 1.04 mmol), palladium tetrakis (triphenylphosphine) (113 mg, 98 mmol), and copper iodide (47 mg, 250 mmol) were added to the flask. Anhydrous DMF (12 mL) was added *via* a syringe, and the mixture was stirred briefly at room temperature. After dissolving, triethylamine (0.4 mL, 2.9 mmol) and trimethylsilylacetylene (0.71 mL, 5.0 mmol) were added to the flask. The crude mixture was stirred at room temperature for 3.5 h and then concentrated by a rotary evaporator. The resulting residue was purified by flash column chromatography (MeOH–DCM = 1 : 9), producing 444 mg of yellow foam. The foam, 5-(trimethylsilyl)ethynyl-2′-deoxyuridine, was dissolved in THF (4 mL), and TBAF in THF (1.2 mL, 1 M, 1.2 mmol) was added to mixture, followed by stirring at room temperature for 3 h. Then, the mixture was filtered to remove the white precipitate that was washed with a small quantity of THF and eluted (MeOH–DCM = 1 : 9). The resulting filtrates were concentrated and purified by flash column chromatography (MeOH–DCM = 1 : 9), producing 181 mg (69%) of white solid. ^{1}H NMR (400 MHz, DMSO-d_6) 2.12 (2H, m, J = 6.3 Hz), 3.59 (2H, q, J = 4.5 Hz), 3.80 (1H, d, J = 3.0 Hz), 4.1(1H, s), 4.23(1H, m), 5.13(1H, t, J = 4.8 Hz), 5.24 (1H, d, J = 4.5 Hz), 6.1 (1H, t, J = 6.3 Hz), 8.29(1H, s), and 11.62(1H, s). ESI-MS: $[M + Na]^+$ calculated for $C_{11}H_{12}N_2O_5Na^+$ 275.06, found m/z 275.10.

Synthesis of chlorobis(POM) phosphate (2). Trimethyl phosphate (1.50 g, 10.7 mmol) was dissolved in CH_3CN (9 mL), followed by NaI (4.82 g, 32.1 mmol) and chloromethyl pivalate (6.28 g, 41.7 mmol). The crude mixture was heated to reflux and stirred for 1 day. Et_2O (100 mL) was added to quench the reaction, and the organic phase was washed with water three times and then dried over Na_2SO_4. After filtration of Na_2SO_4, the filtrate was concentrated by the rotary evaporator and purified by flash column chromatography (EtOAc–Hexane = 1 : 5) to produce 2.24 g (46%) of viscous oil. The oil was dissolved in piperidine (20 mL) and stirred at room temperature overnight. The mixture was concentrated by the rotary evaporator and dried in a vacuum to a constant weight. The resulting residue was dissolved in water (30 mL) and then passed through a column filled with ion exchange resin (Bio-rad AG 50W-X8). The column was washed with sufficient water, and the eluent was collected and lyophilized to produce 2.05 g (93%) of white solid. After ion exchange, the white solid was dissolved in a solution of oxalyl chloride (145 μL, 1.53 mmol) in DCM (3 mL), and then DMF (3 μL) in DCM (3 mL) was added slowly at 4 °C. After 10 min of stirring, the mixture was removed from the ice bath and stirred for 2 h at room temperature. The

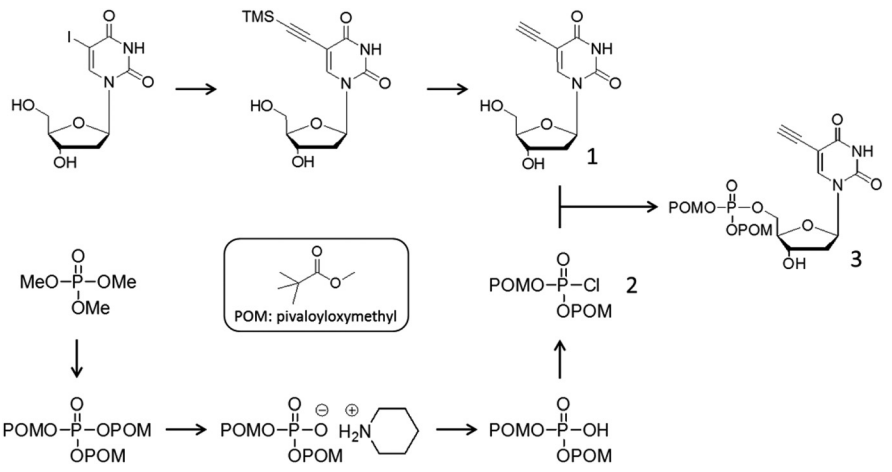

Fig. 1 Scheme of PEdU synthesis.

solvent was removed by the rotary evaporator and dried to a constant weight, producing 80 mg (75%) of yellowish oil. Because of its instability, the chlorobis(POM) phosphate was used immediately for the next step. ^{1}H NMR (400 MHz, $CDCl_3$) 1.24 (18H, s), 5.85 (4H, m), ESI-HRMS: $[M - 2CH_2O + H]^+$ calculated for $C_{10}H_{19}O_5PCl$ 285.0659, found *m*/*z* 285.0630.

Synthesis of PEdU (3). EdU (50 mg, 0.2 mmol) was dissolved in pyridine (2 mL) and then cooled to −40 °C. DMAP (13 mg) was added to the EdU solution followed by stirring for 20 min. A solution of chlorobis(POM) phosphate (100 mg, 0.29 mmol) in THF (2 mL) was added to the EdU solution dropwise *via* a syringe over 20 min. The mixture was stirred at −40 °C for 4 h and then at room temperature for 1 day. EtOAc (20 mL) was added to quench the reaction, and the organic phase was washed with water (10 mL) twice. The aqueous layer was back-extracted with EtOAc. The collected organic phase was washed with brine (10 mL), dried over Na_2SO_4, and concentrated by the rotary evaporator. The compound was purified by flash column chromatography with a gradient (MeOH–DCM = 1 : 45 to 1 : 10), producing 21 mg (18%) of yellowish powder. ^{1}H NMR (400 MHz, CD_3OD) 1.23 (18H, s), 2.13(2H, t, J = 3.0 Hz), 3.54 (1H, s), 3.76 (2H, t, J = 24.1 Hz), 3.92 (1H, d, J = 3.2 Hz), 4.11 (1H, d, J = 4 Hz), 4.40 (1H, d, J = 7.2 Hz), 5.70 (4H, d, J = 9.9 Hz), 6.21 (1H, t, J = 2.85 Hz), 7.98 (1H, s), 8.38(1H, s), ^{13}C NMR (100 MHz, CD_3OD) 27.2, 39.8, 41.0, 62.5, 71.7, 76.0, 82.8, 82.8, 84.4, 87.5, 89.2, 100.0, 145.7, 151.3, 164.5, and 178.0. ESI-HRMS: $[M + Na]^+$ calculated for $C_{23}H_{33}N_2O_{12}PNa^+$ 583.1663 found *m*/*z* 583.1625.

Cytotoxicity assay

Hela and 3T3 cells were applied to a cytotoxicity assay using cell counting kit-8 (CCK-8). The cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated overnight. The medium was removed and various concentrations (10^{-1}, 10^0, 10^1 and 10^2 μM) of EdU or PEdU in DMSO were added to the fresh medium. The final concentration of DMSO in each well was 1%, and the control was treated with 1% DMSO only. The cells were incubated for 24, 48 and 72 h. After incubation, a CCK-8 solution was added to each well followed by incubation for 3 h. Absorbance at 450 nm was recorded using a microplate reader (Model 680, BIO-RAD).

Metabolic labeling of cellular DNA

To analyze metabolic labeling of cellular DNA, we used a Click-iT® EdU imaging kit (Invitrogen) according to the manufacturer's protocol. The culture supernatant was removed after incubation with the various concentrations of each nucleoside at 24, 48 and 72 h. The cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature. After washing the cells twice with 3% bovine serum albumin (BSA) in PBS, the cells were permeabilized with 0.5% Triton X-100 in PBS for 20 min. The cells were washed twice with 3% BSA in PBS again. Reaction cocktails containing Click-iT® reaction buffer, $CuSO_4$, Alexa Fluor® 488 azide, and reaction buffer additive were added to each well followed by incubation for 30 min at room temperature while protected from light. After removing the reaction cocktails, the cells were washed once with 3% BSA in PBS. The labeled cells were imaged under a microscope (Axio Observer. Z1, ZEISS).

In vivo analysis

Six mice (3 weeks old) were injected intraperitoneally (i.p.) with 0.2, 1, or 5 mg PEdU in 0.1 ml PBS. The negative control animal was injected i.p. with 0.1 ml PBS only. At 24 and 48 h after injection, one mouse from each experimental group was fixed by perfusion with 4% paraformaldehyde. After 24 h, the tissues were embedded in paraffin, sectioned at 2 μm, and mounted onto glass slides. After paraffin removal, the sections were rinsed with PBS and stained with 100 mM Tris (pH 8.5), 1 mM $CuSO_4$, and 10 μM Alexa 488 azide (from a 10 mM stock solution in DMSO), and 100 mM ascorbic acid (added last from a 0.5 M stock solution in water) for 30 min. After staining, the sections were washed twice with PBS and methanol, washed once with PBS, and then stained with DAPI.

Results and discussion

Design and synthesis of PEdU

EdU is a well-known compound for manageable DNA detection, but is also a cytotoxic antimetabolite. PEdU was designed to improve the EdU incorporation efficiency and decrease cytotoxicity (Fig. 2). By providing a monophosphate, we expected an improvement in the formation of triphosphate through circumventing the enzymatic monophosphorylation step that requires energy, resulting in efficient incorporation into DNA. However, a common problem of phosphorylated compounds is that the negative charges of the phosphates hinder cell membrane permeability. Therefore, a monophosphate was added with bis(POM) protecting groups that are widely used to neutralize negative charges in prodrug production. A POM protecting group can be cleaved by esterases inside of cells, resulting in gradual release of the active form, phosphorylated EdU in this study.[19,20] This gradual regeneration of the thymidine analogue was expected to prevent an excessive concentration of the antimetabolite.

Cytotoxicity assays

The cytotoxicities of EdU and PEdU were compared at various concentrations after 24, 48 and 72 h of incubation with mouse embryonic fibroblasts (3T3) and human cervical cancer cells (HeLa) as shown in Fig. 3. After 24 h, only the highest concentration of EdU, 100 μM, induced some cytotoxicity in both 3T3 and HeLa cells. After 48 h, 10 μM EdU induced cytotoxicity, and many cells were damaged in 100 μM EdU. In contrast, PEdU treatment did not lead to any decrease in the viability of both cell lines at all concentrations. After 72 h, the cell viability had decreased rapidly at 10 and 100 μM EdU in both cell lines. HeLa cells were relatively more affected than 3T3 cells. This cytotoxic phenomenon of EdU was also observed with PEdU. However, PEdU did not induce any cytotoxicity in 3T3 cells until 72 h at all concentrations or in HeLa cells until 48 h. Cytotoxicity was found only in HeLa cells after 72 h. It is

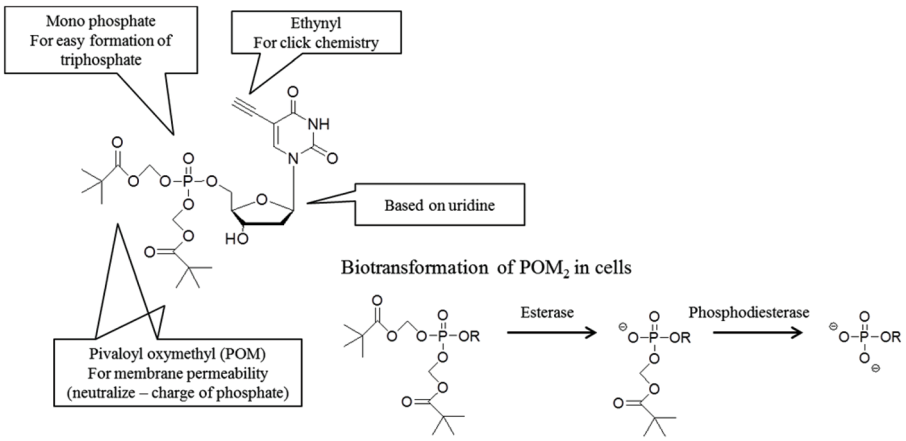

Fig. 2 Design of PEdU.

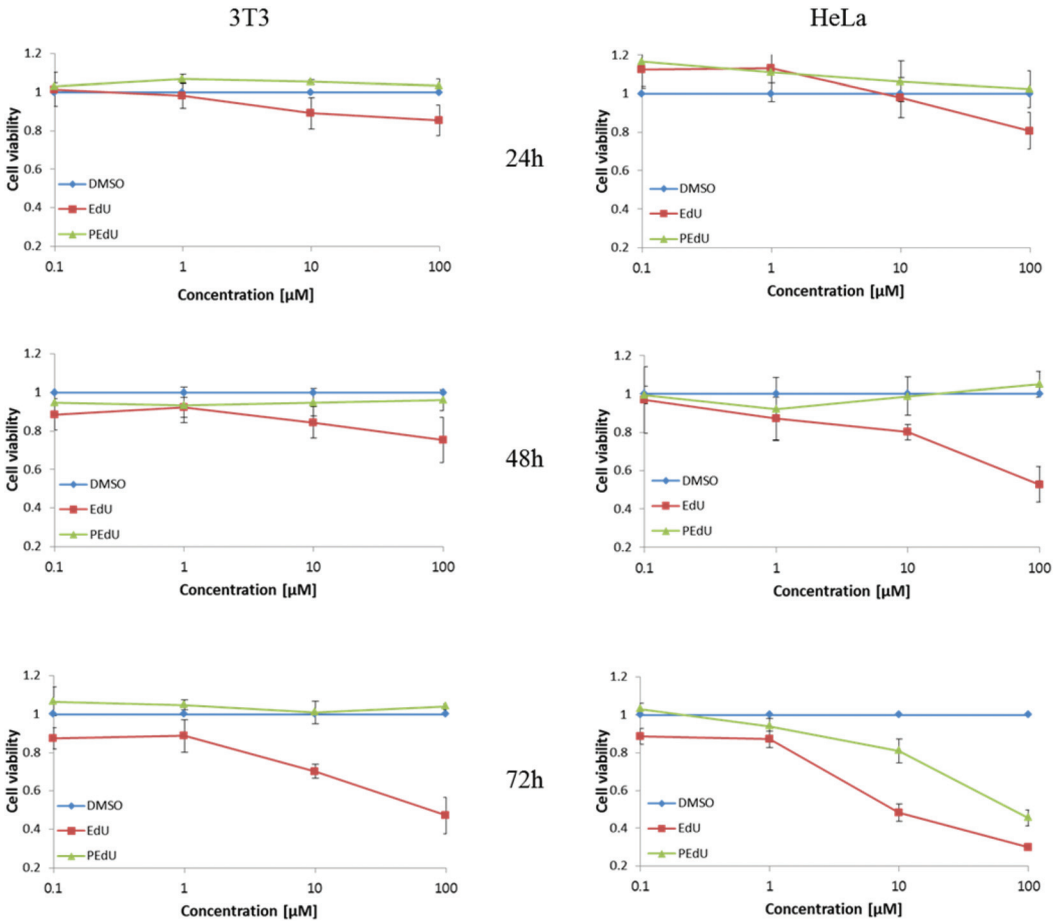

Fig. 3 3T3 and HeLa cells were treated with various concentrations of EdU or PEdU in DMSO. The control was treated with DMSO only. n = 4.

known that the cytotoxicity of EdU is mediated *via* two pathways (Fig. S1†). One is hydrolysis of glycosidic bonds, resulting in the generation of the 5-ethynyluracil antimetabolite[24–26] as well as EdUMP from EdU by phosphorylation, resulting in an antimetabolite that inhibits dTMP synthesis.[15] Although it is not known how the protective groups in PEdU affected the cytotoxicity *via* the second mechanism, the first mechanism must be considered to evade the cytotoxic pathways.

In vitro metabolic DNA labeling

To evaluate the ability of PEdU to incorporate into DNA, we conducted CuAAC with a fluorescent azide (Alexa Fluor 488). Various concentrations (0.1–100 μM) of EdU or PEdU were incubated with 3T3 and HeLa cells for 24 h. After CuAAC with Alexa Fluor 488 azide, the incorporated DNA was detected by fluorescence microscopy (Fig. 4A). In 3T3 cells, fluorescence was observed at 1, 10 and 100 μM EdU and PEdU, but fluorescence was observed rarely at 0.1 μM. In HeLa cells, fluorescence was observed clearly at 10 and 100 μM EdU and PEdU, but hardly observed at 0.1 and 1 μM. There were no significant differences between EdU and PEdU. Fluorescence intensities were measured by image analysis software Image J (Fig. 4B) to compare the incorporation abilities. As a result, we found that the fluorescence intensity of PEdU incorporation was almost the same as that of EdU or slightly higher. Importantly, even though PEdU was less toxic than EdU, its DNA labeling ability was similar to EdU.

Salic and Mitchison[10] confirmed DNA labeling by EdU using hydroxyurea as an inhibitor. They reported that EdU labeling is greatly reduced in cells with DNA replication blocked by hydroxyurea.[10] Considering the similar structures of PEdU and EdU, we expected that PEdU would also label DNA. It has been reported that EdU is fully replaced with dT during PCR.[27] In the case of BrdU, CsCl density gradient ultracentrifugation has shown that the ratios of BrdU/dT are about 28.5% and 68% in cells treated for 2d and 3d, respectively, after subculture.[28] Because of the structural similarity of EdU released from PEdU and BrdU, we expected a similar incorporation ratio.

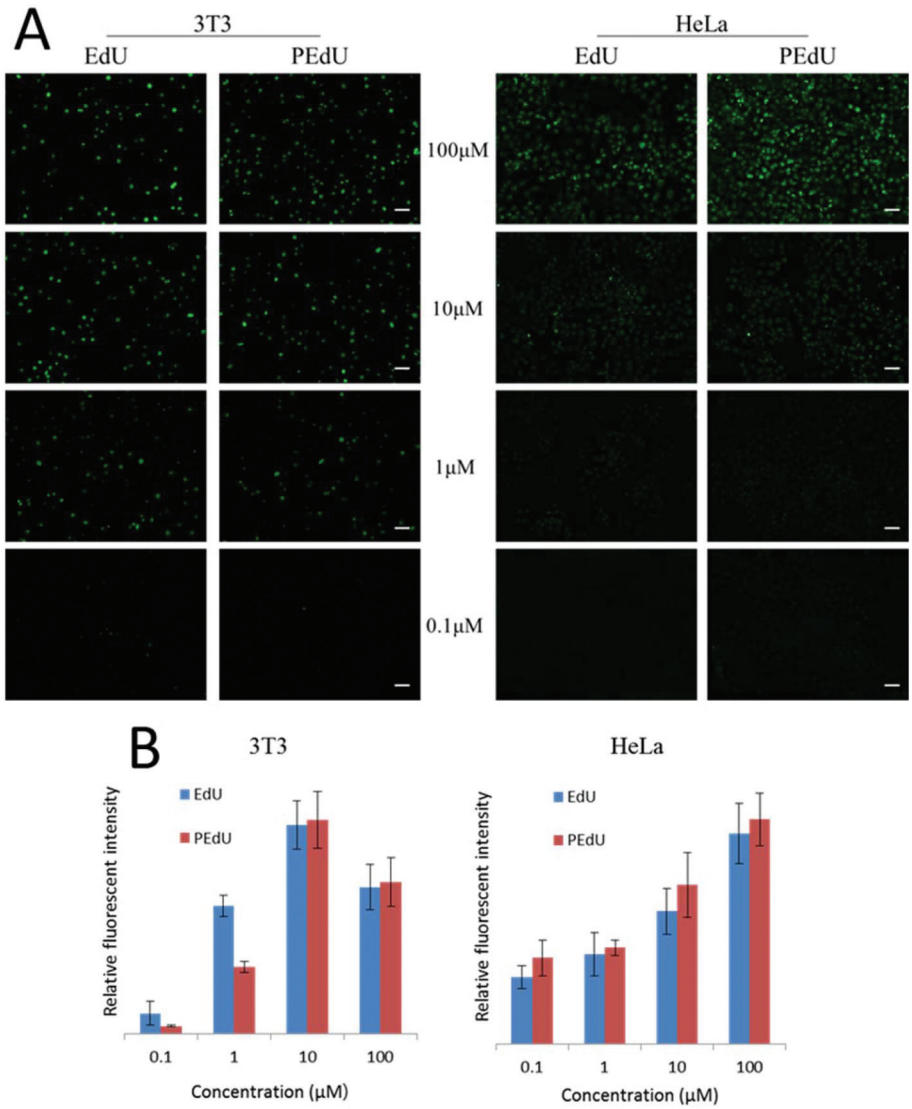

Fig. 4 Metabolic labeling of DNA in 3T3 and HeLa cells at various concentrations (0.1–100 μM) of EdU or PEdU. After 24 h, incorporated DNA was stained with Alexa Fluor 488 azide (A). Scale bar: 50 μm. The relative fluorescence intensity was measured using image analysis software (B). n = 4.

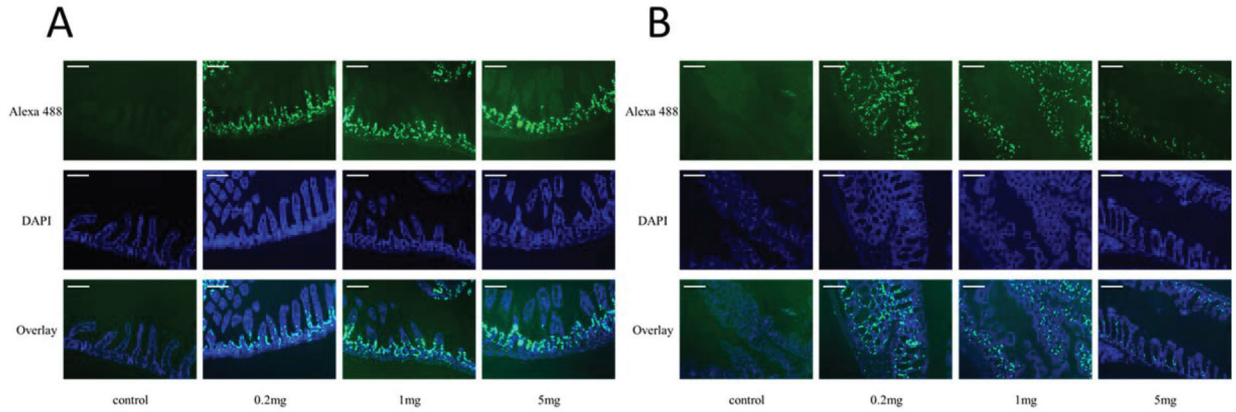

Fig. 5 Mice were injected i.p. with 0.2, 1 or 5 mg PEdU. After 24 h, ileums (A) and colons (B) were harvested. Sections from the organs were fixed and stained with Alexa Fluor 488 azide. Total DNA was stained with DAPI. Scale bar: 100 μm.

In vivo metabolic DNA labeling

To evaluate the ability of PEdU to label DNA in animals, 3-week-old mice were injected i.p. with various concentrations of PEdU in PBS. After 24 h, their tissues were harvested and stained with Alexa Fluor 488 and DAPI. Alexa Fluor 488 staining indicated newly synthesized DNA after PEdU injection, and DAPI staining showed total DNA. Even at the lowest amount of PEdU (0.2 mg), PEdU-incorporated DNA was detected clearly (Fig. 5A and B, ileum and colon, respectively). A total of 0.2 mg PEdU was also sufficient for DNA labeling of the liver, kidney, and thymus (Fig. S7†). These results showed that PEdU could be used to detect DNA synthesis in animals.

Conclusion

PEdU was designed as a low toxic and efficient DNA labeling agent. PEdU exhibited a remarkable reduction in cytotoxicity compared with EdU. Nevertheless, PEdU was incorporated as efficiently as EdU in metabolic DNA labeling at the same concentration and time. Accordingly, PEdU can be used as a less toxic DNA labeling agent in studies that require subsequent cell survival over long time periods or a very sensitive cell line or animal. This study indicates the possibility for modification of nucleoside analogues and prodrugs to enhance their biological activities.

References

1 J. A. M. Borghans and R. J. De Boer, *Immunol. Rev.*, 2007, **216**, 35–47.
2 D. W. Stacey and M. Hitomi, *Cytometry, Part A*, 2008, **73A**, 270–278.
3 A. Quiñones-Hinojosa, N. Sanai, J. S. Smith and M. W. McDermott, *J. Neurooncol.*, 2005, **74**, 19–30.
4 V. W. Hu, G. E. Black, A. Torres-Duarte and F. P. Abramson, *FASEB J.*, 2002, **16**, 1456–1457.
5 P. Taupin, *Brain Res. Rev.*, 2007, **53**, 198–214.
6 C. W. Tornøe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057–3064.
7 V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599.
8 F. Himo, T. Lovell, R. Hilgraf, V. V. Rostovtsev, L. Noodleman, K. B. Sharpless and V. V. Fokin, *J. Am. Chem. Soc.*, 2005, **127**, 210–216.
9 S. Limsirichaikul, A. Niimi, H. Fawcett, A. Lehmann, S. Yamashita and T. Ogi, *Nucleic Acids Res.*, 2009, **37**, e31.
10 A. Salic and T. J. Mitchison, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 2415–2420.
11 J. Gierlich, K. Gutsmiedl, P. M. E. Gramlich, A. Schmidt, G. A. Burley and T. Carell, *Chemistry*, 2007, **13**, 9486–9494.
12 H. Hua and S. E. Kearsey, *Nucleic Acids Res.*, 2011, **39**, e60.
13 S. Diermeier-Daucher, S. T. Clarke, D. Hill, A. Vollmann-Zwerenz, J. A. Bradford and G. Brockhoff, *Cytometry, Part A*, 2009, **75A**, 535–546.
14 H. H. Ross, M. Rahman, L. H. Levkoff, S. Millette, T. Martin-Carreras, E. M. Dunbar, B. A. Reynolds and E. D. Laywell, *J. Neurooncol.*, 2011, **105**, 485–498.
15 A. B. Neef and N. W. Luedtke, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 20404–20409.
16 L. Guan, G. W. van der Heijden, A. Bortvin and M. M. Greenberg, *ChemBioChem*, 2011, **12**, 2184–2190.
17 A. B. Neef, F. Samain and N. W. Luedtke, *ChemBioChem*, 2012, **13**, 1750–1753.
18 D. Qu, G. Wang, Z. Wang, L. Zhou, W. Chi, S. Cong, X. Ren, P. Liang and B. Zhang, *Anal. Biochem.*, 2011, **417**, 112–121.
19 D. Farquhar, R. Chen and S. Khan, *J. Med. Chem.*, 1995, **38**, 488–495.
20 S. R. Khan, B. Nowak, W. Plunkett and D. Farquhar, *Biochem. Pharmacol.*, 2005, **69**, 1307–1313.
21 S. Zhao and F. A. Etzkorn, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 6615–6618.

22 J. D. Rose, W. B. Parker, H. Someya, S. C. Shaddix, J. A. Montgomery and J. A. Secrist III, *J. Med. Chem.*, 2002, **45**, 4505–4512.
23 Y. Hwang and P. A. Cole, *Org. Lett.*, 2004, **6**, 1555–1556.
24 D. J. T. Porter, *J. Biol. Chem.*, 1994, **269**, 27932–27940.
25 L. D. Thornburg and J. Stubbe, *Biochemistry*, 1993, **32**, 14034–14042.
26 J. G. Niedzwicki, M. H. el Kouni, S. H. Chu and S. Cha, *Biochem. Pharmacol.*, 1983, **32**, 399–415.
27 J. Gierlich, K. Gutsmiedl, P. M. E. Gramlich, A. Schmidt, G. A. Burley and T. Carell, *Chem. – Eur. J.*, 2007, **13**, 9486–9494.
28 J. C. Thomas, C. Nessler and F. Katterman, *Plant Physiol.*, 1989, **90**, 921–927.