International Journal of Pharmaceutics 498 (2016) 205–216

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

L-Type amino acid transporter 1 (lat1)-mediated targeted delivery of perforin inhibitors

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ARTICLE INFO

Article history:
Received 3 November 2015
Received in revised form 9 December 2015
Accepted 12 December 2015
Available online 15 December 2015

Keywords:
Prodrug
Perforin inhibitor
L-type amino acid Transporter 1 (LAT1)
Targeted drug delivery
Transporter-mediated drug delivery

ABSTRACT

Perforin is a cytolytic pore-forming glycoprotein secreted by cytotoxic effector cells. It is a key component of the immune response against virus-infected and transformed cells and has been implicated in a number of human diseases. Perforin activity can be inhibited by small-molecular-weight compounds, although less is known about their delivery to the site of action. Therefore, in the present study, it was explored if perforin inhibitors could be efficiently and site-selectively delivered firstly into the cytotoxic effector cells and secondly into lytic granules, in which perforin is stored. This was accomplished by designing and synthesizing four prodrugs of perforin inhibitors that could utilize L-type amino acid transporter (LAT1), since activated immune cells are known to over-express LAT1. The results demonstrate that cellular uptake of perforin inhibitors can be increased by LAT1-utilizing prodrugs (into human breast adenocarcinoma cells (MCF-7)). Furthermore, these prodrugs were also able to deliver perforin inhibitors into the cell organelles having lower pH (rat liver lysosomes). Therefore, by using these prodrugs, intracellular mechanisms of perforin inhibitory activity can be studied more thoroughly in future. Moreover, this prodrug approach can be applied for other drugs that would benefit from targeted delivery into cells expressing LAT1, such as cancer.

1. Introduction

Perforin is a cytolytic pore-forming glycoprotein secreted by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (Lichtenheld et al., 1988; Podack, 1992; Trapani and Smyth, 2002). It is a key component of the immune response against virus-infected and transformed cells. Perforin has also been implicated in a number of human immunopathologies and therapy-induced conditions, such as cerebral malaria, autoimmune neuroinflammation, insulin-dependent diabetes, allograft rejection and graft-versus-host disease (Kagi et al., 1997; Liesz et al., 2011; Potter et al., 2006; Veale et al., 2006). The mechanism of perforin function is complex. CTLs and NK cells contain secretory vesicles (granules) that store perforin along with other cytotoxic proteins, including granzymes (pro-apoptic serine proteases) (Brennan et al., 2011; Thiery et al., 2011). After killer cell conjugation to the target cell, exocytic delivery of the granule contents into the calcium-rich immune synapse results in calcium binding by perforin, dramatically changing its conformation, followed by the formation of trans-membrane pores composed of highly ordered perforin oligomers (Baran et al., 2009; Law et al., 2010). Formation of perforin pores facilitates entry of granzymes into the target cell, which in turn activates various apoptotic mechanisms. Perforin-mediated cytotoxicity can been inhibited by small-molecular-weight compounds both in vitro and in vivo (Lena et al., 2008; Spicer et al., 2013), but its is not yet clear at what point in the process these inhibitors interact with perforin; whether extracellularly or if they also have intracellular function(s).

To study if perforin inhibitors could be efficiently and selectively delivered into the cytotoxic effector cells, we utilized a L-type amino acid transporter (LAT1) as a (pro) drug carrier. LAT1 is a sodium-independent heterodimeric trans-membrane protein that is mainly expressed in the blood–brain barrier, but also in placenta, tumors (Kanai et al., 1998) and activated immune cells (Hayashi et al., 2013; Nii et al., 2001; Sinclair et al., 2013). LAT1 transports not only large and neutral amino acids, but also

Abbreviations: BBB, blood–brain barrier; CTLs, cytotoxic T lymphocytes; HPLC, high-performance liquid chromatography; LAT1, L-type amino acid transporter; MCF-7, human breast adenocarcinoma cell line; MCTs, monocarboxylate transporters; NK cells, natural killer cells; OATPs, organic anion transporting polypeptides; PSA, polar surface area.

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http://dx.doi.org/10.1016/j.ijpharm.2015.12.034
0378-5173/

several clinically used drugs and prodrugs, such as levodopa, gabapentin and melphalan (Boado et al., 1999; Uchino et al., 2002). The relatively high and selective expression of LAT1 at the blood–brain barrier (BBB), in activated immune cells and several types of cancer cells (Fuchs and Bode, 2005; Yanagida et al., 2001), makes it an important means of targeted drug delivery, especially in the case of infectious and inflammatory brain diseases as well as some forms of cancer.

In the present study, we prepared four LAT1-utilizing prodrugs of selected perforin inhibitors and studied their ability to bind to LAT1 and to be delivered into LAT1-expressing cells (human breast adenocarcinoma cells; MCF-7). Moreover, the ability of these prodrugs and their parent compounds to be accumulated into cell organelles that have lower pH (rat liver lysosomes), was also evaluated, since such lysosomes closely resemble the secretory vesicles of NK cells and CTLs (Burkhardt et al., 1990; Dell'Angelica et al., 2000).

2. Materials and methods

2.1. General synthetic procedures

All reactions were performed with reagents obtained from Sigma–Aldrich (St. Louis, MO, USA), Acros Organics (Waltham, MA, USA) or Merck (Darmstadt, Germany). Reactions were monitored by thin-layer chromatography using aluminum sheets coated with silica gel 60 F_{245} (0.24 mm) with suitable visualization. Purifications by flash chromatography were performed on silica gel 60 (0.063–0.200 mm mesh) eluting with CH_2Cl_2/MeOH solution. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin, Fällanden, Switzerland) operating at 500.13 MHz and 125.75, respectively, using tetramethylsilane as an internal standard. pH-Dependent NH-protons of the compounds were not observed. ESI–MS spectra were recorded by a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionization source. Over 95% purities were obtained for the compounds **1–18** by elemental analysis (C, H, N) with a PerkinElmer 2400 Series II CHNS/O organic elemental analyzer (PerkinElmer Inc., Waltham, MA, USA).

2.2. Synthesis of the prodrugs ***1–8***

The compounds **1–4** were prepared as previously described by using 9-BBN to protect the amino acid group (Peura et al., 2013), coupling the amino acid and the perforin inhibitor by the aid of EDC and deprotecting the final compounds by ethylenediamine. More detailed procedures can be found from the supporting data. The compounds **5–8** were prepared by refluxing compound **13** or **14** with either hydroxylamine hydrochloride (compounds **5** and **7**, respectively) or with hydrazine hydrate (compounds **6** and **8**, respectively) in the presence of pyridine in EtOH or EtOH/DMSO mixture overnight.

2.2.1. (R)-2-Amino-3-(3-(((4-(5-(1-oxo-1,3-dihydroisobenzofuran-5-yl) thiophen-2-yl)benzyl)oxy)carbonyl)phenyl)propanoic acid (***1***)

Yellow solid (41%). ^1H NMR ($(CD_3)_2SO$): δ ppm 8.00 (s, 1H), 7.93–7.84 (m, 4H), 7.79 (d, J = 3.8 Hz, 1H), 7.77 (d, J = 8.2 Hz, 2H), 7.65 (d, J = 3.8 Hz, 1H), 7.58–7.54 (m, 3H), 7.44 (t, J = 7.7 Hz, 1H), 5.45 (s, 2 H), 5.39 (s, 2H), 3.62–3.58 (m, 1H), 3.20-3.09 (m, 1H), 2.96–2.85 (m, 1H); ^{13}C NMR ($(CD_3)_2SO$): δ ppm 172.33, 169.90, 165.60, 148.40, 144.17, 140.96, 139.15, 138.76, 136.07, 134.30, 132.80, 130.08, 129.38, 128.64 (2C), 128.40, 127.22, 127.04, 125.86, 125.52, 125.41 (2C), 125.25, 123.65, 118.83, 69.52, 65.51, 55.60, 37.77. MS (ESI^-) for $C_{29}H_{22}NO_6S$ (M-H)$^-$: calcd 512.55, found 512.10.

2.2.2. (R)-2-Amino-3-(3-((4-(5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)phenoxy)carbonyl)phenyl)propanoic acid (***2***)

Orange–yellow solid (81%). ^1H NMR ($(CD_3)_2SO$): δ ppm 7.97 (s, 1H), 7.92–7.85 (m, 5H), 7.82 (d, J = 7.8 Hz, 1H), 7.76–7.70 (m, 3H), 7.57–7.44 (m, 3H) 5.45 (s, 2 H), 3.96–3.89 (m, 1H), 3.13–3.05 (m, 1H), 2.98–2.90 (m, 1H); ^{13}C NMR ($(CD_3)_2SO$): δ ppm 175.92, 169.97, 165.60, 148.43, 146.82, 144.68, 140.10, 138.93, 138.11, 134.70, 134.66, 128.40, 128.35, 128.19, 127.24, 126.87, 125.76, 125.64 (2C), 125.53, 124.51, 123.49, 120.59 (2C), 118.69, 69.54, 55.30, 37.19. MS (ESI^-) for $C_{28}H_{21}N_2O_5S$ (M-H)$^-$: calcd 497.54, found 497.14.

2.2.3. (R)-2-Amino-3-(3-((4-(5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)phenyl)carbamoyl)phenyl)propanoic acid (***3***)

Yellow solid (86%). ^1H NMR ($(CD_3)_2SO$): δ ppm 8.07 (s, 1H), 8.02–7.98 (m, 2H), 7.94 (d, J = 8.2 Hz, 1H), 7.89 (d, J = 8.2 Hz, 1H), 7.83 (d, J = 8.6 Hz, 2H), 7.79 (d, J = 3.9 Hz, 1H), 7.68–7.63 (m, 2H), 7.53 (t, J = 7.6 Hz, 1H), 7.37 (d, J = 8.5 Hz, 2H), 5.45 (s, 2H), 3.49–3.44 (m, 1H), 3.26–3.20 (m, 1H), 3.03–2.97 (m, 1H); ^{13}C NMR ($(CD_3)_2SO$): δ ppm 175.78, 170.25, 164.73, 150.53, 148.70, 143.88, 141.18, 138.96, 138.60, 135.34, 131.13, 131.03, 129.01, 128.84, 128.16, 127.56, 126.96, 126.71 (2C), 126.05, 125.78, 123.87, 122.81 (2C), 119.01, 69.81, 55.33, 36.63. MS (ESI^-) for $C_{28}H_{20}NO_6S$ (M-H)$^-$: calcd 498.53, found 498.19.

2.2.4. (R,(E,Z))-2-Amino-3-(4-((4-(5-((2-oxo-5-thioxoimidazolidin-4-ylidene) methyl) thiophen-2-yl)phenoxy)carbonyl)phenyl) propanoic acid (***4***)

Dark red solid (52%). ^1H NMR ($(CD_3)_2SO$): δ ppm 7.78 (s, 1H), 7.66 (d, J = 7.4 Hz, 1H), 7.48 (d, J = 8.7 Hz, 2H), 7.38 (d, J = 7.5 Hz, 1H), 7.34 (d, J = 7.4 Hz, 1H), 7.19 (d, J = 3.8 Hz, 1H), 7.17 (d, J = 3.8 Hz, 1H), 7.78 (d, J = 8.6 Hz, 2H), 6.18 (s, 1 H), 3.65–3.62 (m, 1H), 3.21–3.15 (m, 1H), 2.82–2.75 (m, 1H); ^{13}C NMR ($(CD_3)_2SO$): δ ppm 191.27, 179.88, 172.38, 166.54, 157.03, 149.34, 138.67, 138.36, 134.64, 132.04, 129.00, 128.62, 128.08 (2C), 126.55, 126.33, 125.65, 125.24, 121.57, 115.85 (2C), 55.81, 37.81. MS (ESI^-) for $C_{14}H_9N_2O_2S_2$ (M-[$C_{10}H_{10}NO_3$])$^-$: calcd 301.01, found 300.96.

2.2.5. (E,Z)-4-((5-(4-((E,Z)-1-(Hydroxyimino)ethyl)phenyl)thiophen-2-yl) methylene)-5-thioxoimidazolidin-2-one (***5***)

Dark red solid (74%). ^1H NMR ($(CD_3)_2SO$), *E*/*Z* isomers were observed together: δ ppm 12.40 (s, 1H), 11.98 (s,1H), 11.30 (s, 1H), 7.84 (d, J = 4.0 Hz, 1H), 7.74–7.71 (m, 4H), 7.68 (d, J = 4.0 Hz, 1H), 6.64 (s, 1H), 2.17 (s, 3H); ^{13}C NMR ($(CD_3)_2SO$): δ ppm 178.40, 165.40, 152.36, 146.43, 139.23, 136.69, 135.60, 135.22, 133.05, 132.44, 126.29, 125.91, 125.89, 125.36, 104.18, 11.32. MS (ESI^-) for $C_{16}H_{12}N_3O_2S_2$ (M − H)$^-$: calcd 342.41, found 342.04.

2.2.6. (E,Z)-4-((5-(4-((E,Z)-1-Hydrazonoethyl) phenyl) thiophen-2-yl) methylene)-5-thioxoimidazolidin-2-one (***6***)

Red-brown solid (77%). ^1H NMR ($(CD_3)_2SO$), *E*/*Z* isomers were observed separately: δ ppm 10.98 (s, 1H), 9.08 (s,1H), 8.00 (d, J = 8.5 Hz, 1H), 7.77 (d, J = 8.2 Hz, 1H), 7.66 (d, J = 8.5 Hz, 1H), 7.63 (d, J = 3.7 Hz, 1H), 7.58 (d, J = 8.2 Hz, 1H), 7.47–7.36 (m, 1H), 6.57 (s, 0.5H), 6.55 (s, 0.5H), 4.71 (s, 2H), 2.54 (s, 3H); ^{13}C NMR ($(CD_3)_2SO$): δ ppm 197.42, 170.42, 145.21, 141.74, 139.27, 138.84, 135.60, 132.99, 130.60, 129.70, 127.94, 125.79, 125.23, 125.15, 123.98, 11.61. MS (ESI^-) for $C_{16}H_{13}N_4OS_2$ (M − H)$^-$: calcd 341.43, found 341.49.

2.2.7. (E,Z)-5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl) thiophene-2-carbaldehyde oxime (***7***)

Dark red solid (74%). ^1H NMR ($(CD_3)_2SO$), *E*/*Z* isomers were observed separately: δ ppm 12.17 (s, 0.5H), 11.40 (s, 0.5H), 8.35 (s, 0.5H), 8.03 (s, 0.5H), 7.99–7.93 (m, 1H), 7.90 (s, 1H), 7.89–7.85 (m, 1H), 7.75 (d, J = 4.0 Hz, 0.5H), 7.71 (d, J = 3.8 Hz, 0.5H), 7.53 (d, J = 4.0 Hz, 0.5H), 7.36 (d, J = 3.8 Hz, 0.5H), 5.44 (s, 0.5H), 5.44