RSC Medicinal Chemistry

ROYAL SOCIETY OF CHEMISTRY

RESEARCH ARTICLE

View Article Online
View Journal | View Issue

Cite this: *RSC Med. Chem.*, 2024, **15**, 1973

Cytotoxicity of phosphoramidate, bis-amidate and cycloSal prodrug metabolites against tumour and normal cells†

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Received 15th February 2024,
Accepted 16th April 2024

DOI: 10.1039/d4md00115j

rsc.li/medchem

Phosphonate and phosphate prodrugs are integral to enhancing drug permeability, but the potential toxicity of their metabolites requires careful consideration. This study evaluates the impact of widely used phosphoramidate, bis-amidate, and cycloSal phosph(on)ate prodrug metabolites on BxPC3 pancreatic cancer cells, GL261-Luc glioblastoma cells, and primary cultured mouse astrocytes. 1-Naphthol and 2-naphthol demonstrated the greatest toxicity. Notably, 2-naphthol exhibited an ED_{50} of 21 μM on BxPC3 cells, surpassing 1-naphthol with an ED_{50} of 82 μM. Real-time xCELLigence experiments revealed notable activity for both metabolites at a low concentration of 16 μM. On primary cultured mouse astrocyte cells, all prodrugs exhibited reduced viability at 128 to 256 μM after only 4 hours of exposure. A cell-type-dependent sensitivity to phosph(on)ate prodrug metabolites was evident, with normal cells showing greater susceptibility than corresponding tumour cells. The results suggest it is essential to consider the potential cytotoxicity of phosph(on)ate prodrugs in the drug design and evaluation process.

Introduction

Phosphates and phosphonates are important, biologically active functional groups in drug design and development. However, their negative charge (either mono- or di-) at physiological pH gives these groups high polarity, hindering their cell membrane permeability. A range of phosph(on)ate prodrugs have been developed, where the negative charge is masked by prodrug moieties that are cleaved chemically or enzymatically to release the charged biologically active drug intracellularly (Fig. 1).[1] These prodrugs have led to significant improvements in the activity of nucleoside analogue drugs such as tenofovir, remdesivir and azidothymidine (AZT), allowing for the effective permeability of the mono-phosph(on)ate derivatives to bypass the rate-limiting first phosphorylation step.[2] Following the development of nucleotide analogue prodrugs, these protecting groups were applied to other non-nucleotide scaffolds including pyrophosphates and bis-phosphonates for anti-cancer and anti-viral applications.[3,4]

Early phosph(on)ate prodrugs include the pivaloyloxymethyl (POM) and isopropyloxymethyl carbonate (POC) group (Fig. 2A). The development of these prodrugs led to the clinical use of adefovir dipivoxil and tenofovir disoproxil fumarate. Concerns related to toxicity, stemming from the release of pivalic acid and formaldehyde upon liberation of the prodrug, alongside stability issues, drove the subsequent innovation of new phosph(on)ate prodrug types including phosphoramidate, bis-amidate, and cycloSal prodrugs (Fig. 2B).[5]

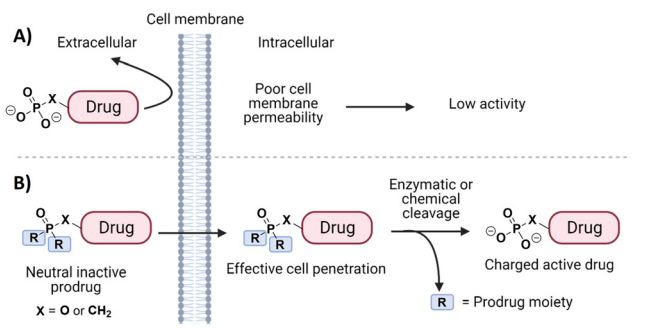

Fig. 1 Representation of the prodrug concept. A) Phosph(on)ate is deprotonated at physiological pH resulting in poor membrane permeability. B) Neutral phosph(on)ate prodrug allows for efficient cell membrane permeability before being cleaved intracellularly into the charged active drug. Created with **https://Biorender.com**

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† Electronic supplementary information (ESI) available: Evaluation of BxPC3 viability with phosphoramidate metabolites in combination (Fig. S1) and confirming purity of primary mouse astrocytes (Fig. S2). See DOI: **https://doi.org/10.1039/d4md00115j**

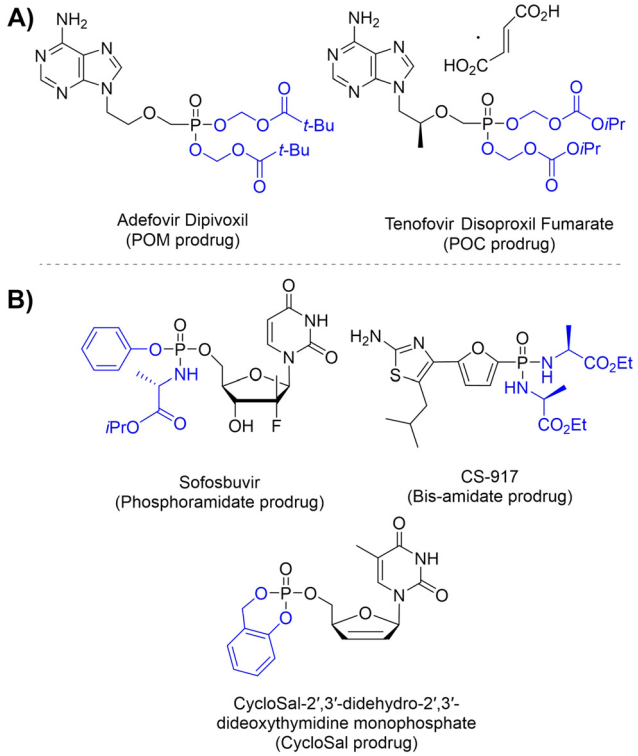

Fig. 2 A) Chemical structure of clinically used anti-virals with pivaloyloxymethyl (POM) and isopropyloxymethyl carbonate (POC) prodrugs. B) Example applications of the phosphoramidate, bis-amidate and cycloSal prodrugs.

The phosphoramidate prodrug, first developed by McGuigan *et al.*, consists of an aryloxy group and an amino acid ester group.[6] It is one of the most successful phosph(on)ate prodrugs to date, leading to the clinical use of the anti-viral agents tenofovir alafenamide, remdesivir and sofosbuvir (Fig. 2B).[7] Additionally, several anti-cancer agents using the phosphoramidate strategy are in clinical trials including NUC-1031, a prodrug of gemcitabine, and NUC-3373, a prodrug of 5-fluoro-2′-deoxyuridine.[8,9] Drugs with phosphoramidate prodrugs have shown superior stability and lower toxicity than their POM and POC analogues.[10] Several aryloxy moieties have been used in phosphoramidate prodrugs, the most common being a phenyl group, followed by 1-naphthyl and 2-naphthyl derivatives. Numerous amino acid derivatives have also been studied. It was found the amino acid moiety has a drastic effect on the activation of the phosphoramidate prodrug, with L-alanine becoming the most widely used derivative in the clinic.[11]

Further prodrug developments led to using two amino acid ester groups to form a bis-amidate prodrug that removes the formation of a chiral centre at the phosphorus and reduces the toxicity observed with corresponding POM prodrugs.[12] As with phosphoramidate prodrugs, L-alanine esters are the most commonly used derivative. One bis-amidate prodrug to reach clinical trials was CS-917 for treatment of type 2 diabetes, which showed 10-fold greater bioavailability than the free phosphonic acid (Fig. 2B).[13] Furthermore, cycloSal prodrugs that were originally introduced by Meier *et al.* consist of a salicyl alcohol-derived prodrug that can undergo pH-dependent chemical hydrolysis rather than enzymatic release.[14] This prodrug has significantly improved the activity of the mono-phosphate derivatives of several anti-viral nucleotides including AZT, acyclovir and 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T, stavudine) (Fig. 2B).[15,16]

The use of phosph(on)ate prodrugs is expanding, especially to non-nucleotide scaffolds, with compounds being evaluated at micro-molar concentrations during *in vitro* studies.[17–20] The metabolites liberated upon activation of the prodrug are rarely taken into account during the evaluation process and are often considered inert. Herein, the current generation of prodrugs, namely the phosphoramidate, bis-amidate and cycloSal prodrugs are evaluated for their potential toxicity. The most common phosphoramidate prodrug metabolites phenol, 1-naphthol and 2-naphthol were examined. We also look at the widely used L-alanine benzyl ester (L-Ala Bn ester) and L-alanine isopropyl ester (L-Ala *i*-Pr ester) moieties, which are cleaved by esterases to release L-alanine and the corresponding alcohol. Lastly, the cytotoxicity of salicyl alcohol, the metabolite of cycloSal phosph(on)ate prodrugs, is explored (Fig. 3). We investigated the potential cytotoxicity of these moieties on BxPC3 human pancreatic cancer cells, GL261-Luc mouse glioblastoma cells and primary mouse astrocytes using novel CyQUANT direct cell proliferation assays, luciferin-luciferase assay, xCELLigence real time impedance measurements, and caspase 3/7 activation measurements.

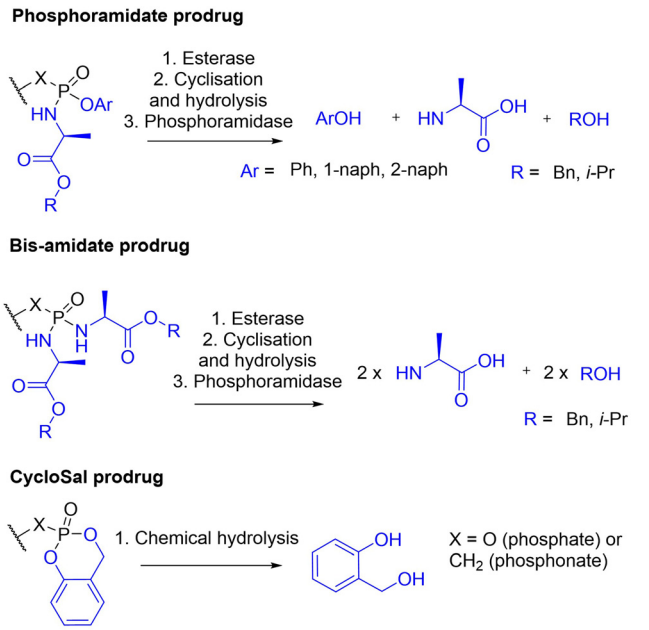

Fig. 3 Chemical structure and activation pathways of phosph(on)ate prodrugs and their metabolites explored in this study.

Results and discussion

For this study, a rapid and accurate method of determining cell viability was needed to evaluate the cytotoxicity of the prodrug moieties. To achieve this, we have developed a new highly sensitive and high throughput method of performing a CyQUANT direct cell proliferation assay. The CyQUANT assay uses a cell-permeable fluorescent DNA-binding dye to measure cell viability.[21] In this study, a fluorescence image of the plate after incubation with the CyQUANT reagents was read using the Amersham™ Typhoon 5™ laser scanning imaging platform (Fig. 4A). The Amersham™ Typhoon 5™ allowed each well to be scanned entirely at a resolution up to 100 μm. The image was then processed using ImageQuant™ TL to quantify the intensity of fluorescence of each well of the 96-well plate, which was normalised to the control (100% viability). This method was also applied to caspase 3/7 assays providing results that are more robust than measured with a plate reader as the entire well is imaged. Furthermore, the Typhoon 5™ platform was used to quantify the total number of cells stained with CellTag 700 to eliminate differences due to variation of cell viability during caspase 3/7 assays, demonstrating the wide applications of this system including In-Cell Western assays.

To investigate the effects of the phosph(on)ate metabolites on cell viability, human pancreatic BxPC3 cancer cells were treated with 1-naphthol, 2-naphthol, phenol, L-alanine benzyl ester, L-alanine isopropyl ester or salicyl alcohol for 72 hours. To initially identify which prodrugs showed significant toxicity all prodrug groups were tested at 128 μM, except for the L-alanine esters, with their concentration doubled to 256 μM to simulate their use as bis-amidate prodrugs with two metabolite groups liberated per phosph(on)ate. Cell viability was determined after 72 hours using the CyQUANT assay (Fig. 4A). 2-Naphthol showed the greatest cytotoxic effect at 128 μM on BxPC3 cells, reducing viability by 90%, followed by 1-naphthol which reduced viability by 53%. Phenol, L-alanine benzyl/isopropyl ester and salicyl alcohol had no significant effect on the viability of BxPC3 cells after 72 hours compared to the control (Fig. 4B). The cytotoxicity of 1-naphthol and 2-naphthol on BxPC3 cells was confirmed by dose–response curves produced using the xCELLigence Real Time Cell Analyser system. The xCELLigence platform uses impedance measurements to give a cell index value, which provides a non-invasive, real-time and sensitive readout of cell number and size.[22] Using cell index measurements, the ED_{50} values were 21 μM and 82 μM for a 72-hour exposure time for 2-naphthol and 1-naphthol, respectively (Fig. 5).

Additionally, BxPC3 cells were incubated with a combination of the aryl alcohols (1-naphthol, 2-naphthol and

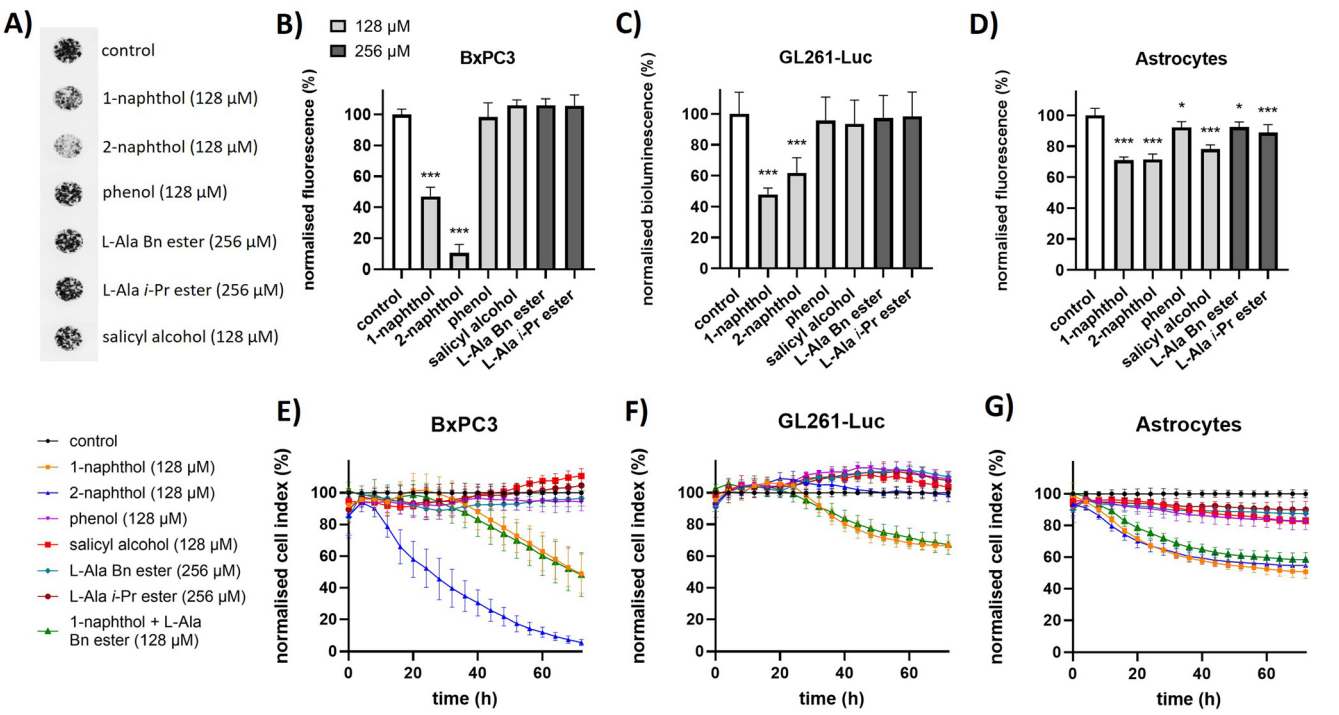

Fig. 4 Evaluation of the viability of BxPC3, GL261-Luc and primary mouse astrocytes treated with prodrug moieties. A) Fluorescence image of BxPC3 cells treated with prodrug moieties for 72 hours and then measured with CyQUANT. The image was produced using the Amersham™ Typhoon 5™. B) and D) cell viability measured 72 hours after treatment using CyQUANT assay. Fluorescence readings were quantified using ImageQuant™ TL and normalised to the control. C) Viability measured by luciferase assay 72 hours after treatment where luminescence was measured by plate reader and normalised to the control. E–G) Time-course of cell index reflecting viability over 72 hours using the xCELLigence system. The cell index measurements were taken every hour and normalised to the control. Statistical significance is shown relative to the control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Data is shown as mean ± S.D of three independent experiments.

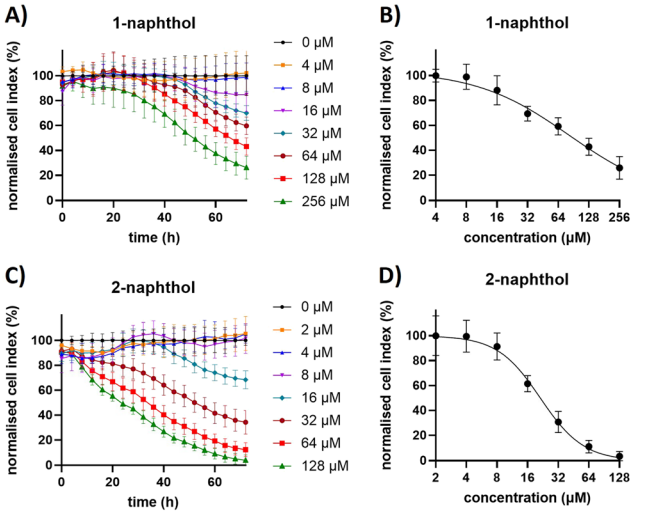

Fig. 5 Dose–response curves of 1-naphthol and 2-naphthol on BxPC3 cells over 72-hours. Cell index measurements using the xCELLigence system were taken every hour and normalised to the control. A) Cell index of BxPC3 cells decreased with increasing concentrations of 1-naphthol. B) Dose–response curve of 1-naphthol (ED_{50} = 82 μM). C) Cell index of BxPC3 cells decreased greatly with increasing concentrations of 2-naphthol. D) Dose–response curve of 2-naphthol (ED_{50} = 21 μM). Data is shown as mean ± S.D. of three independent experiments.

phenol) and L-alanine benzyl or L-alanine isopropyl ester at 128 μM as they would be used in combination in phosphoramidate prodrugs. The CyQUANT assay showed no significant difference between the toxicity of 1-naphthol, 2-naphthol or phenol alone compared to their toxicity in combination with either L-alanine benzyl or L-alanine isopropyl ester at 128 μM on BxPC3 cells (Fig. S1†). Furthermore, no significant difference was found between the use of either L-alanine benzyl ester or L-alanine isopropyl ester in these combinations.

To determine the effects of the phosph(on)ate prodrugs on another tumour cell type, the viability of GL261-Luc mouse glioblastoma cells following a 72-hour incubation with the compounds was determined using a luciferin-luciferase assay to measure ATP release. 1-Naphthol showed the greatest toxicity at 128 μM reducing ATP release by 53% compared to the control, while 2-naphthol showed less toxicity, reducing ATP release by 39%. No other prodrugs affected the GL261-Luc cells' proliferation by measuring ATP release (Fig. 4C).

The effect of the prodrug metabolites on normal murine cells was then determined using a CyQUANT assay on primary cultured mouse astrocytes, which are derived from the same cell type as GL261-Luc mouse glioblastoma cells (Fig. 4D). Both 1-naphthol and 2-naphthol had an equally cytotoxic effect at 128 μM, reducing viability by 29%. Phenol showed slight cytotoxicity, reducing viability by 8%. L-Alanine benzyl ester and L-alanine isopropyl ester also had a significant effect on cell viability at 256 μM (reduced by 8% and 12% respectively); however, no statistically significant difference between the two ester groups was found. Finally, salicyl alcohol showed the second-greatest cytotoxic effect on normal astrocytes reducing viability by 22% at 128 μM.

The xCELLigence system was used for real-time analysis of the cellular response to the prodrug moieties over 72 hours. 2-Naphthol at a concentration of 128 μM reduced the cell index of BxPC3 cells by 94% compared to the control after 72 hours (Fig. 4E). A rapid response to the prodrug moiety was observed as the cell index declined after only 8 hours of treatment. 1-Naphthol reduced the cell index by 51% after 72 hours and took 30 hours to begin to have a negative effect on viability. In comparison, only 1-naphthol alone or in combination with L-alanine benzyl ester showed cytotoxicity on the GL261-Luc cells using the xCELLigence system, which began after 28 hours reducing the cell index by 33% (Fig. 4F). Phenol, L-alanine benzyl ester, L-alanine isopropyl ester and salicyl alcohol showed no significant effect on either BxPC3 or GL261-Luc cells over an exposure period of 72 hours.

An unexpected finding was that all prodrug metabolites showed some cytotoxic effect at 128 μM or 256 μM on primary cultured mouse astrocyte cells after 72 hours. 1-Naphthol and 2-naphthol showed similar toxicity reducing the cell index by 54% and 46% respectively (Fig. 4G). The cell index of the primary cultured astrocytes declined within 4 hours of treatment with 128 μM of 1-naphthol or 2-naphthol. Phenol, L-alanine benzyl/isopropyl ester and salicyl alcohol all showed a negative effect on cell viability and reduced the cell index by 11–17% after 72 hours (Fig. 4G). In all experiments with xCELLigence impedance measurements, the cells were also treated with a combination of 128 μM of 1-naphthol and L-alanine benzyl ester. No significant difference was found between the combination of these metabolites and treatment at 128 μM of 1-naphthol alone (Fig. 4E–G).

Finally, the effect of the prodrug moieties on BxPC3 and GL261-Luc caspase 3/7 activation was measured to determine if the prodrug groups induced apoptosis. The cells were incubated with the compounds for 72 hours and then apoptotic cells were detected with CellEvent Caspase-3/7 Detection Reagent and the Amersham™ Typhoon 5™ laser scanning imaging platform. Caspase-3/7 activation was then normalised to cell number using the CellTag 700 stain. BxPC3 cells showed an 8.1-fold increase in caspase 3/7 activation when treated with 128 μM of 2-naphthol and a 2.2-fold increase when treated with 128 μM of 1-naphthol. GL261-Luc cells showed a 2.0-fold increase when treated with 128 μM of 1-naphthol. No significant increase in caspase 3/7 activation was produced by the other prodrugs on either cell line (Fig. 6).

Overall, we have evaluated the potential toxicity of widely used phosphoramidate, bis-amidate and cycloSal prodrug metabolites towards tumour and healthy cells to determine if these moieties can contribute to the off-target toxicity of a drug. The results reveal that the choice of aryl moiety in the design of phosphoramidate prodrugs has a significant impact on the toxicity of the prodrug metabolites. For example,

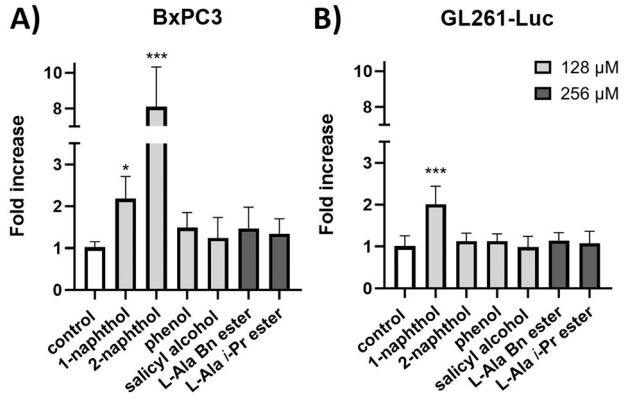

Fig. 6 Caspase 3/7 activation of BxPC3 and GL261-Luc cells treated with phosph(on)ate prodrug moieties for 72 hours. The fluorescence was normalised to cell number using CellTag 700. Statistical significance is shown relative to the control. * $p \leq 0.05$, *** $p \leq 0.001$. Data is shown as mean ± S.D. of three independent experiments.

phenol, the metabolite of the widely used phenyl prodrug moiety, exhibited no toxicity on either cancer cell line. In comparison, 1-naphthyl and 2-naphthyl groups are commonly used in phosphoramidate prodrugs to increase lipophilicity.[23–27] However their metabolites 1-naphthol and 2-naphthol displayed significant cytotoxicity on both BxPC3 and GL261-Luc cells. 2-Naphthol had an ED_{50} of 21 μM on BxPC3 cells, while 1-naphthol had an ED_{50} of 82 μM. Both ED_{50} values are within reported concentration ranges used for the *in vitro* biological evaluation of phosphoramidate prodrugs.[17,18,28]

Interestingly, 2-naphthol showed 4-fold greater cytotoxicity on BxPC3 cells than 1-naphthol as determined using real time xCELLigence monitoring (Fig. 5). The opposite was observed for GL261-Luc cells where 1-naphthol was more cytotoxic than 2-napthol (Fig. 6). These results reveal that the relative toxicity of 1-naphthol compared to 2-naphthol is dependent on cell type. For all phosphoramidate aryl metabolites, a greater and more rapid decline in cell viability was observed using real-time xCELLigence monitoring on primary cultured mouse astrocytes compared to GL261-Luc cells, suggesting that healthy cells are more sensitive to the prodrug metabolites. Using CyQUANT viability assays on BxPC3 cells (Fig. S1†) and real-time xCELLigence experiments on all three cell types (Fig. 4), no significant difference between the effects of the aryl alcohol metabolites of phosphoramidate prodrugs alone, or in combination with L-alanine benzyl ester and L-alanine isopropyl ester was found. This suggests that the primary factor driving cytotoxicity in phosphoramidate prodrugs is the aryl moiety.

From these results it is evident that the phenyl moiety is more favourable in the design of non-cytotoxic drugs, while the use of 1-naphthyl or 2-naphthyl could increase off-target toxicity. Future work should investigate whether these aryl moieties, or other aryl groups that have not yet been used in phosphoramidate prodrugs can produce selective cytotoxicity against a targeted tumour cell type to enhance the anti-cancer activity of current chemotherapeutic agents. For example, phosphoramidate prodrugs using 2-naphthol could be explored as a co-drug on chemotherapeutics using tumour-targeted delivery systems for pancreatic cancer.[29] These results also highlight the importance of screening the aryl alcohol metabolites when determining structure–activity relationships, as the toxicity of the 1-naphthol or 2-naphthol varies between cell types and could, in some cases, be a false positive for increased cytotoxic activity of the parent drug.

The use of two amino acid ester groups to form bis-amidate phosph(on)ate prodrugs is also common and carries the advantage of removing the stereocentre at the phosphorus atom. Furthermore, bis-amidate prodrugs have been widely explored as it is believed their metabolites are non-toxic, although this has not been reported until now. Here we treated the cells with L-alanine benzyl ester or L-alanine isopropyl ester, which are cleaved by esterases to release the corresponding alcohol and L-alanine. These prodrug moieties were tested at 256 μM, which is within reported concentration ranges for the biological evaluation of these prodrugs.[30,31] These prodrug moieties showed no significant increase in apoptosis or reduction in cell viability on BxPC3 and GL261-Luc cells at 256 μM as determined by CyQUANT assay and real-time xCELLigence monitoring (Fig. 4 and 6). In comparison, on primary cultured astrocyte cells a slight cytotoxic effect was observed, with no significant difference between either the L-alanine benzyl ester or the L-alanine isopropyl ester (Fig. 4D and G). While this toxicity is unlikely to be an issue at lower concentrations, it is an important consideration in the design of bis-phosphonate or pyrophosphate prodrugs where up to 4 equivalences of the prodrug metabolites are released.[19]

CycloSal phosph(on)ate prodrugs are favourable in their ability to mask two negative charges with a cyclic moiety, limiting the molecular weight of the drug and releasing salicyl alcohol upon release. Salicyl alcohol showed no effect at 128 μM on BxPC3 or GL261-Luc cell proliferation or apoptosis (Fig. 4 and 6). However, on normal mouse astrocyte cells slight toxicity was observed. Again, this demonstrates that normal cells may have greater sensitivity to phosph(on)ate prodrug metabolites than tumour cell lines and should be considered when testing cycloSal prodrugs at higher concentrations.[20]

The prodrug metabolite concentrations used in this study are within the reported concentration ranges used for phosphoramidate, bis-amidate and cycloSal prodrug compounds during *in vitro* evaluations. Furthermore, these concentrations are within the reported maximum plasma concentrations of several FDA approved anti-cancer agents.[17–20,28,32] The concentration of prodrug metabolite reached is influenced by the rate of metabolism. For example, phosphoramidate derivatives of ManNAc-6phosphate were metabolised by 80–90% within 2 hours in human serum.[18] Similarly, a cycloSal prodrug of PMEA was rapidly hydrolysed in phosphate buffer at a pH of 7.3 with a

half-life of 0.09 hours.[33] Bis-amidate prodrugs have shown variable stability depending on the parent structure and ester moiety used. For example, bis-amidate prodrugs of butyrophilin ligands showed varying stability in K562 cell extracts, with an L-alanine ethyl ester derivative showing 70% activation within 4 hours while an L-alanine isopropyl ester derivate showed 20% activation.[34] Overall, the time-period over which metabolism occurs will impact the dose of prodrug metabolite reached and should be considered on a case-by-case basis during drug development programs. Furthermore, the toxicity concerns of the prodrug metabolites will depend on the therapeutic window between the drug's effective dose and the onset of off-target toxicity from the metabolites.

Conclusion

The importance of phosph(on)ates in drug design makes prodrugs a vital area of development. Here we have examined the potential cytotoxicity of the widely used phosphoramidate, bis-amidate and cycloSal phosph(on)ate prodrug moieties at concentrations relevant to reported biological evaluations of these compounds. The findings highlight the significance of the aryl moiety of phosphoramidate prodrugs in off-target toxicity from liberation of the prodrug. Phenol was well tolerated by BxPC3 and GL261-Luc tumour cell lines. In comparison, 1-naphthol and 2-naphthol showed significant cytotoxicity on BxPC3 cells with an ED_{50} of 82 and 21 μM, respectively. Moreover, the use of L-alanine ester groups and salicyl alcohol did not induce significant cytotoxicity on the tumour cells. However, all prodrugs evaluated in this study showed greater and more rapid (as short as four hours) toxicity on primary cultured mouse astrocytes than GL261-Luc tumour cell lines. These results suggest that it is vital to consider the off-target activity of phosph(on)ate prodrug metabolites in the drug design and biological evaluation process.

Experimental section

Reagents

L-Alanine benzyl ester HCl and L-alanine isopropyl ester HCl were purchased from AK Scientific. Phenol and salicyl alcohol were purchased from Sigma Aldrich (St. Louris, MO, United States). 1-Naphthol and 2-naphthol were purchased from Aaron chemicals. D-Luciferin was purchased from Sigma-Aldrich. Invitrogen™ CyQUANT™ Direct Cell Proliferation Assay and Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent were purchased from ThermoFisher Scientific (Waltham, MA, United States). CellTag™ 700 stain was purchased from LI-COR Biosciences (Lincoln, NE, U.S.A).

Cell culture

BxPC3 Human pancreatic cancer and GL261-Luc mouse glioblastoma were cultured in Dulbecco's modified eagle's medium (DMEM, Sigma Aldrich) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin–streptomycin–glutamine (Sigma-aldrich). The cells were passaged twice per week and passage numbers between 10 and 20 were used for subsequent experiments.

All euthanised mice used for culture of astrocytes were approved by the ANSTO Animal Care and Ethics Committee. All procedures were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013), and comply with the ARRIVE guidelines. Whole brains were dissected from six twelve-week-old male C57BL/6 mice. After meninges were removed, brains were cut into small pieces and then incubated in porcine trypsin (0.125%) and dissolved in Hanks' balanced salt solution for 10 min at 37 °C. Cells were then dissociated by mechanical trituration. DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin–glutamine were added prior to filtering with 100 μm and then 40 μm cell strainers. Cells were plated onto 75 cm^2 flasks at 37 °C for 4 h, media was removed, and flasks washed twice. Glial cells were maintained at 37 °C in 5% CO_2 in the supplemented DMEM, and media was changed every 3–4 days. Fourteen days after initial plating, cultures were purified by shaking the flask at 350 rpm at 37 °C for 2 h to remove microglia and oligodendrocytes. Adherent astrocytes were removed from the flask after incubation with 0.125% porcine trypsin for 5–10 min. The pellet of cells after centrifugation at 100 g for 5 min was resuspended in the supplemented DMEM, counted, and plated for subsequent experiments. The purity of astrocytes was determined with immunohistochemistry by incubation with rabbit-anti mouse GFAP (glial fibrillary acidic protein) antibody (Abcam, Cambridge, United Kingdom) overnight at 4 °C and then Alexa-fluor 488 conjugated goat anti-rabbit secondary antibody (Abcam). The cells on coverslips were mounted with pro-Long Diamond with DAPI (Thermo Fisher Scientific). The fluorescence was viewed and captured with Olympus BX61WI microscope equipped with CoolSNAP HQ2 camera and images were processed and deconvoluted with CellSense Dimension software. The purity of astrocytes with positive GFAP staining was greater than 90% (Fig. S2†).

CyQUANT viability assays

CyQUANT viability assays were performed in 96-well, black-walled, clear bottom ½ area plates. Cells were seeded at 5 × 10^3 cell per well for BxPC3 cells, or 3 × 10^3 cell per well for primary mouse astrocytes. Twenty-four hours after plating, cells were incubated with compounds in supplemented DMEM containing a final concentration of 0.1% DMSO for 72 hours. For CyQUANT measurements, the media was removed and the cells were incubated with 25 μL of CyQUANT Direct nucleic acid stain (2 μL mL^{-1}) and CyQUANT Direct background suppressor (10 μL mL^{-1}) in Hank's buffered saline solution for 1 hour at 37 °C as specified by the manufacturer instructions. The fluorescence images after staining were captured using the Amersham™ Typhoon 5™

Biomolecular Imager using a Cy2 filter (Ex: 488 nm and Em: 520 nm) and a pixel resolution of 100 μm. The fluorescence intensity of the entire well in the 96-well plate for each treatment was quantified using ImageQuant™ TL 10.2 (Citiva) then normalised to the control (100% viability). At least three independent experiments were performed with at least four replicates per experiment.

Luciferin-luciferase assay

Luciferin-luciferase assay to measure ATP release from cells as an index of cell viability was performed in 96-well, white-walled, clear bottom ½ area plates. GL261-Luc cells with luciferase gene insertion and protein expression were seeded at 3×10^3 cell per well. Twenty-four hours after plating, cells were incubated with compounds in supplemented DMEM at a final concentration of 0.1% DMSO for 72 hours. The media was removed and the cells were incubated with 25 μL of D-luciferin in complete media at a concentration of 150 μg mL^{-1} for 15 minutes at 37 °C. Luminescence was then read using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA). At least three independent experiments were performed with at least four replicates per experiment.

Caspase 3/7 assay

Measurements of the early apoptosis marker caspase 3/7 were performed in 96-well, black-walled, clear bottom ½ area plates. Cells were seeded at 5×10^3 cell per well for BxPC3 cells, or 3×10^3 cell per well for GL261-Luc cells. Twenty-four hours after plating, cells were incubated with compounds in supplemented DMEM at a final concentration of 0.1% DMSO for 72 hours. Cell Event™ Caspase-3/7 Green Detection Reagent was added to the cells and incubated for 1 hour at 37 °C according to the manufacturer's protocols. The media was removed, and fluorescence of the whole well was then read using the Amersham™ Typhoon 5™ Biomolecular Imager using a Cy2 filter (Ex: 488 nm and Em: 520 nm) and a pixel resolution of 100 μm. To quantify the number of cells each well, the cells were then fixed with 3.7% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilised with 0.3% Triton X-100 in PBS for 2 min. The cells were then blocked with BSA in PBS for 30 minutes. CellTag 700 (25 μL per well) was added, and the plate was incubated for 1 hour according to the manufacturers protocol. The plate was then read again using the Amersham™ Typhoon 5™ Biomolecular Imager using an IRshort filter (Ex: 685 nm and Em: 720 nm) and a pixel resolution of 100 μm. Using ImageQuant™ TL 10.2 (Citiva) the intensity of the caspase 3/7 fluorescence was normalised to the number of cells as determined using CellTag 700. The caspase 3/7 activation was then normalised as a fold-increase to the control cells. At least three independent experiments were performed with at least four replicates per experiment.

xCELLigence real time monitoring of cell proliferation

To measure cell proliferation in real time, E16 (16-well) xCELLigence plates were seeded with 5×10^3 cell per well BxPC3 cells or 3×10^3 cell per well for GL261-Luc cells and primary mouse astrocytes. After 24 hours cells were treated with compounds in supplemented DMEM containing a final concentration of 0.1% DMSO. xCELLigence cell index measurements reflecting electrical impedance were performed every hour for 72 hours. The cell index at each time point was normalised to the control (100% viability). At least three independent experiments were performed with two replicates per experiment.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 9. Statistically significant differences were determined by one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test. ED_{50} values were determined using GraphPad Prism nonlinear regression curve fit. All data is shown as mean ± S.D.

Abbreviations

L-Ala Bn ester	L-Alanine benzyl ester
L-Ala *i*-Pr ester	L-Alanine isopropyl ester
AZT	Azidothymidine
d4T	2′,3′-Didehydro-2′,3′-dideoxythymidine
POC	Isopropyloxymethyl carbonate
POM	Pivaloyloxymethyl
S.D.	Standard deviation

Author contributions

Experiments were designed by R. F., D. S., and G.-J. L., and performed by R. F. and G.-J. L. Manuscript was written by R. F. with editing and review from all authors.

Conflicts of interest

There is no conflict of interest to declare.

Acknowledgements

R. Farrell and H. Steele would like to acknowledge the Australian Institute of Nuclear Science and Engineering (AINSE). This research was supported by an AINSE Ltd. Postgraduate Research Award (PGRA). R. Farrell and H. Steele would also like to acknowledge the support of the Australian Government Research Training Program (AGRTP).

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