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Review

Metabolic Efficacy of Phosphate Prodrugs and the Remdesivir Paradigm

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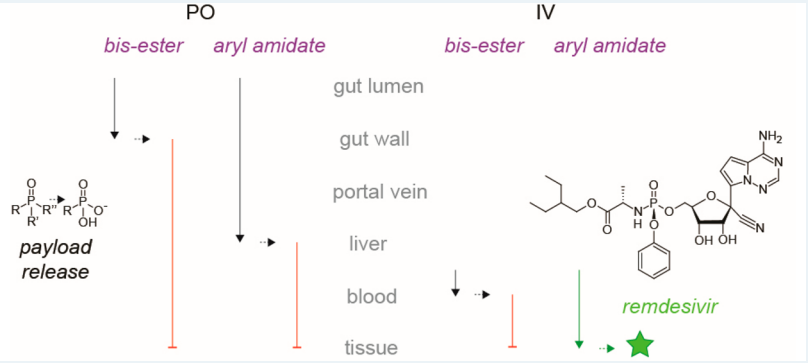

ABSTRACT: Drugs that contain phosphates (and phosphonates or phosphinates) have intrinsic absorption issues and are therefore often delivered in prodrug forms to promote their uptake. Effective prodrug forms distribute their payload to the site of the intended target and release it efficiently with minimal byproduct toxicity. The ability to balance unwanted payload release during transit with desired release at the site of action is critical to prodrug efficacy. Despite decades of research on prodrug forms, choosing the ideal prodrug form remains a challenge which is often solved empirically. The recent emergency use authorization of the antiviral remdesivir for COVID-19 exemplifies a new approach for delivery of phosphate prodrugs by parenteral dosing, which minimizes payload release during transit and maximizes tissue payload distribution. This review focuses on the role of metabolic activation in efficacy during oral and parenteral dosing of phosphate, phosphonate, and phosphinate prodrugs. Through examining prior structure−activity studies on prodrug forms and the choices that led to development of remdesivir and other clinical drugs and drug candidates, a better understanding of their ability to distribute to the planned site of action, such as the liver, plasma, PBMCs, or peripheral tissues, can be gained. The structure−activity relationships described here will facilitate the rational design of future prodrugs.

KEYWORDS: *SARS-CoV-2, COVID-19, nucleotide, phosphorus, hepatitis, antiviral*

INTRODUCTION

Phosphates are involved in a variety of biochemical processes which are critical to life and underlie human health and disease.[1] These include nucleic acid biosynthesis and polymerization, glycolysis, and lipid biosynthesis, among others. To maximize ligand−target interactions, drug candidates that pursue these processes often must include a phosphate, or a more metabolically stable phosphonate or phosphinate,[2] as few suitable isosteres exist that can capture the unique size and geometry of phosphates.[3]

Incorporation of phosphates into drugs is not without its shortcomings. First, these functional groups are charged at physiological pH (Figure 1A), which restricts their membrane permeability and cellular entry.[4,5] This restricted diffusion is useful for cells to compartmentalize their biochemical pathways. At the same time, low diffusion is problematic for drug absorption, making it difficult for drugs to reach their desired site of action. Second, phosphate esters themselves are metabolically labile. Sometimes, a phosphonate[6] or phosphinate[7] can be substituted, whereas at other times these groups inhibit binding to the molecular target or are less efficiently phosphorylated to an active form.

Therefore, prodrug approaches have been employed to enable use of phosphates, phosphonates, and phosphinates in drug molecules.[8−13] While the use of prodrug strategies in drug development is increasing (up to a third of small molecule drugs

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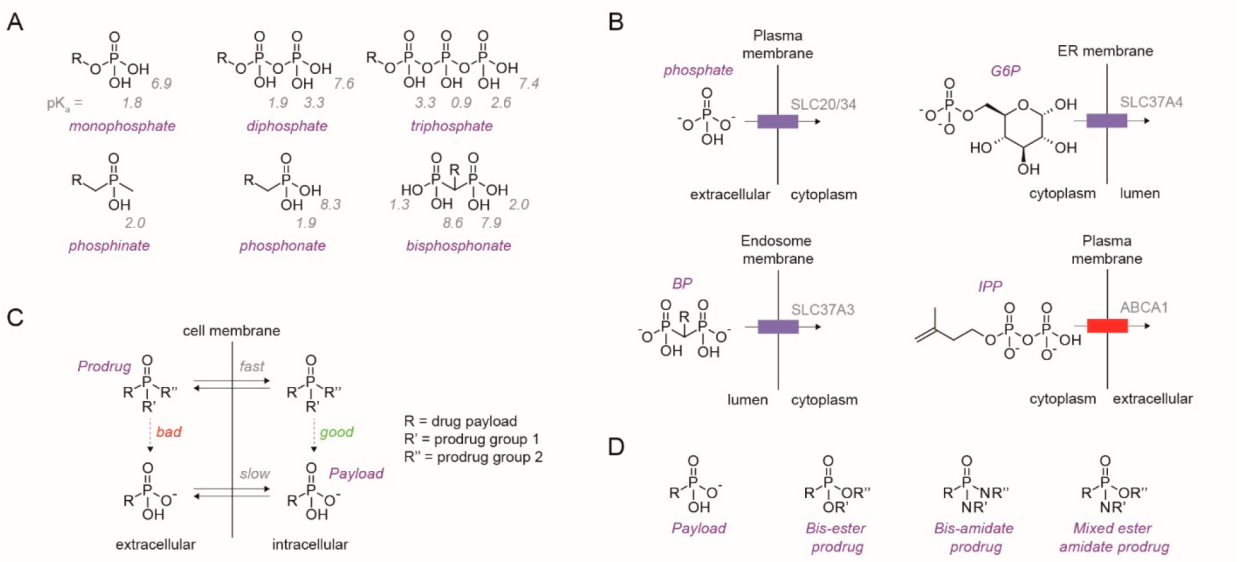

Figure 1. Phosphates can cross the cellular membrane barrier with transporters or prodrug modifications. (A) pK_a values of common organophosphorus functional groups. The acidity of phosphates, phosphinates, phosphonates, and bisphosphonates (BPs) makes them negatively charged at physiological pH values. (B) Membrane transport of phosphates. Organic phosphate can be internalized by SLC20 and SLC34. Glucose 6-phosphate (G6P) can be imported to the ER by SLC37A4. BPs enter cells from endosomes with the help of SLC37A3. ABCA1 can export endogenous isoprenoid diphosphates such as isopentenyl diphosphate (IPP). (C) Prodrug approach. Charge-neutral prodrugs can more rapidly diffuse across cell membranes relative to their charged counterparts. Because prodrug diffusion is rapid and reversible, the rate of prodrug internalization is dependent upon irreversible bioconversion to the less diffusible free acid form. (D) Some common forms of phosphonate prodrugs. Phosphoesters and phosphoamidates are typical prodrug components. These protecting groups may be symmetrical, asymmetrical or mixed ester/amide. Some monoprotected and cyclic forms have been evaluated (not shown).

can now be categorized as prodrugs),[8,14] the application of prodrugs to phosphorus-containing compounds is particularly important due to their low membrane permeability and esterase susceptibility. Planned application of prodrug technology to these drugs at an early stage in their discovery can avoid these associated pharmacokinetic problems and provide an opportunity to boost tissue specific payload release.

The usual strategy for phosphate prodrugs is to modify the acidic oxygen atoms with metabolically labile protecting groups to produce a charge-neutral compound with increased lipophilicity and decreased phosphoesterase susceptibility at the ester bond that bridges the phosphate and payload.[10,14] This strategy improves diffusion across cell membranes and enhances absorption and cellular potency. It can also be applied to the acidic oxygen atoms of phosphonates and phosphinates. For example, this effect is quite nicely illustrated on a bisphosphonate drug template in which masking of increasing numbers of the four acidic oxygen atoms yields sequentially stronger cellular potency.[15] The prodrug approach replaces intrinsic absorption and metabolism problems that arise from these phosphorus-containing compounds with planned metabolic activation that can be manipulated for therapeutic advantage.

Various prodrug forms have been evaluated in this context. These prodrug forms of the same payload are expected to achieve similar rates of membrane diffusion, yet the efficacy of the different forms is quite variable. The critical factor contributing to the varied efficacy of different prodrug forms is their ability to strike a desirable balance between unfavorable payload release during transit and favorable release at the intended site of action (i.e., their metabolism).

Like many drugs, the in vivo metabolism of phosphate, phosphonate, and phosphinate prodrugs is dependent upon the route of administration. Orally dosed prodrugs can be metabolized in the gastrointestinal lumen, the GI wall, portal vein, liver, blood, and target tissues, whereas the latter two compartments are most critical to parenteral dosing. Thus, the timing of payload release must consider the different features of these compartments. Metabolism of orally dosed prodrugs will differ substantially from parenteral prodrugs despite their similar chemical composition.

This review focuses on the historical development of phosphate, phosphonate, and phosphinate prodrugs in the context of the exciting recent clinical use of the antiviral remdesivir, a parenteral phosphate prodrug. Most of the examples are derived from the clinical development of nucleotide analogs, though relevant examples of other molecules such as angiotensin-converting enzyme (ACE) inhibitors,[16] fructose-1,6-bisphosphatase inhibitors,[17] squalene synthase inhibitors,[18] bisphosphonate drugs,[19] Rab geranylgeranyl transferase inhibitors,[20] and phosphoantigens[21] will be considered. Taken together, these studies illustrate how routes of administration and the rates of payload release in different compartments determine their efficacy and tissue specificity.

CELLULAR UPTAKE OF PHOSPHATES AND THE MEMBRANE BARRIER

The cellular uptake of phosphates is relatively slow because these charged compounds have low rates of transmembrane diffusion. Inorganic phosphate cannot cross cell membranes by passive diffusion under physiological conditions.[4] Cellular internalization of inorganic phosphate requires transporter proteins such as the phosphate: Na+ symporter family members (SLC20 and SLC34; Figure 1B).[22] Similarly, studies on nucleotides[5] or sugar phosphates[23] concluded that it was unlikely these phosphorylated molecules could diffuse across

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membranes at physiologically relevant rates. In the case of nucleotides, the nonphosphorylated nucleoside forms can cross membranes using equilibrative or concentrative transporters, though subsequent cellular phosphorylation to the nucleotide forms is inefficient.[24] In the case of sugar phosphates, their transport into the endoplasmic reticulum is controlled by proteins such as the sugar phosphate transporter (SLC37A4), which exchanges inorganic phosphate for sugar phosphates.[25] This shows a variety of phosphate transporters are used to control internalization of phosphate esters and their analogs.

In addition to transporters, vesicle acidification during endocytosis reduces the charge of these compounds which can increase their rate of diffusion into the cytosol from endocytic vesicles. This is evident in the cellular uptake of bisphosphonates[26] and phosphoantigens[27] which require energy-dependent fluid-phase endocytosis. However, even during endocytosis, transporters contribute to cellular uptake. For example, the entry of bisphosphonates is enhanced by the transporter SLC37A3 working in endosomes.[28]

Larger molecules with more hydrophobic character such as phospholipids can insert into the membrane and spontaneously flip, but this process is relatively slow. It can be improved by phospholipid flippases such as ATP8A1.[29,30] Organophosphorus compounds can also be secreted from cells. For example, isopentenyl diphosphate is effluxed by the ABCA1 transporter.[31] Taken together, naturally occurring organic phosphates generally have low membrane permeability and usually require dephosphorylation, acidification of endocytic vesicles, or use of transporters to cross biological membranes. Charge-neutral prodrug forms have the potential to bypass these normal routes of cell entry and exit and promote entry via transmembrane diffusion.

IMPORTANCE OF METABOLISM IN THE EFFICACY OF PHOSPHATE, PHOSPHONATE, AND PHOSPHINATE PRODRUGS

In contrast to a typical xenobiotic in which the liver and kidney are often the primary sites of metabolism, prodrugs are susceptible to (and sometimes targeted toward) metabolism in peripheral tissues as a feature of their design. Because prodrugs differ substantially from their payloads, when prodrug metabolic activation occurs prematurely it impacts both the absorption and distribution of the payload. Therefore, it could be argued that metabolism is the most critical pharmacokinetic parameter as it relates to phosphate, phosphonate, and phosphinate prodrug pharmacodynamics and efficacy.

Unlike their payloads, prodrug forms are usually charge-neutral and capable of transmembrane diffusion (Figure 1C). Once internalized by cells, the prodrug is metabolized to release the fully deprotected payload. Because payloads carry a formal charge of negative one or two, they cannot readily diffuse back out of cells. Although diffusion is the mode of entry, the diffusion of prodrugs is impacted by their enzymatic activation. Because diffusion usually happens faster than payload release and reaches equilibrium, the enzymatic payload release functions as the rate limiting step of cell entry. This is an example of Le Chatelier's principle: As the payload is irreversibly released by the cell, the diffusion equilibrium changes, allowing cells to internalize the payload at high levels. In fact, with sufficient exposure time the internal payload concentration could be expected to exceed the extracellular concentration of the prodrug form. If the payload is too difficult to liberate within the cell, then the prodrug form could diffuse out of the cell. If the payload is liberated too easily prior to cell entry, then diffusion into the cell is reduced. In some cases, slow cellular metabolism is beneficial. For example, slow payload release in the intestinal epithelium would be desirable for an orally dosed prodrug to enter the circulation. Eventually, that activation must be fast at the intended site of action.

For prodrugs that are orally administered, key questions include the following: How long they can last in the prodrug form? How far they can distribute systemically before releasing their payload? On one end of the spectrum, prodrug forms that lack acid stability are essentially useless for oral dosing, as they would release the payload in the low pH of stomach environment. In contrast, for a prodrug to reach peripheral tissue in the prodrug form, it must distribute to these tissues at a rate that is faster than its chemical instability as well as its enzymatic payload release in the GI wall, the liver, and the plasma. Parenteral dosing, while inconvenient for patients, is less complicated. Here, it is essential for the rate of tissue internalization to exceed the rate of plasma metabolism and hepatic and/or renal clearance. Collectively, prodrug efficiency depends on diffusion but requires a balance between rate of payload release outside and inside of target tissues and is influenced by route of administration.

EVALUATION OF PRODRUG EFFICACY

Prodrug efficacy can be defined as the ability to distribute the payload to the site of action. At the site of action, the payload is released to engage the drug target. Ideally, prodrug efficacy would be determined directly by its ability to produce either the intended pharmacodynamic effect at the site of action or alternatively the payload quantified at the relevant site of action. It is not always possible to measure that in animal models and not often possible to measure that in human patients. Plasma or urine payload levels are relatively easy to obtain, though they may not be representative of payload release into tissue. Leukocytes may provide an acceptable proxy for tissue uptake at times, though this assumes their rate of prodrug activation is similar to that of the target tissue.

In vivo assays are low-throughput and require complex analytical methods. To speed structure−activity relationship (SAR) analysis, in vitro and cellular approaches have been employed. Cell-free metabolism assays are often used as predictors of prodrug efficacy. Prodrug candidates can be assessed for metabolic stability in homogenate from each compartment the prodrug is expected to pass during transit to the target tissue. This includes chemical stability at a range of neutral to acidic conditions which is required for transit through the stomach and GI lumen and metabolic stability in the body. For orally administered prodrugs, stability assessment in intestinal and liver homogenates can estimate the likelihood that the prodrug form will survive those compartments.[32] Plasma stability assays can predict stability in the overall circulatory system including the portal vein. The rate of payload release in tissue homogenate obtained from the intended site of action would ideally exceed that of release in the preceding tissues.

Metabolism studies using isolated cells also can at times predict activation in target tissues in vivo. These cellular assays tend to be most useful when both dose and time are varied to enable rate of cellular activation to be determined.[33] Washout experiments work especially well for phosphate, phosphonate, and phosphinate prodrugs due to the charge differential between prodrug and payload, which restricts payload diffusion back out of cells. For prodrugs targeting the liver, transformation in

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human hepatocytes or hepatocyte cells lines can be assessed.[34] Cellular assays of prodrug potency in some ways already model parenteral dosing, as most known orally dosed phosphate, phosphonate, and phosphinate prodrugs are quickly metabolized in the liver, making it unlikely the payload would actually reach the target tissue in the prodrug form.

Some traditional approaches should be interpreted with caution when used to evaluate phosphate, phosphonate, and phosphinate prodrug efficacy. Oral bioavailability assays that measure prodrug or payload in the blood may fail to account for the amount of active form of the payload in the target tissue, where it can accumulate beyond the level of either the prodrug or payload in the plasma. The plasma or urine concentrations may not necessarily correlate with tissue concentration of the payload as it might with a typical drug. In cellular assays, endpoint studies could miss the window of maximum differences among prodrug forms which happens within minutes of cellular exposure. Transcellular permeability assays are difficult to interpret as they are impacted by cellular payload release in addition to permeability. Taken together, this collection of in vivo, cellular, and biochemical metabolism studies has revealed key insights into the localization of payload and how it varies dependent upon prodrug form and route of administration.

PRODRUG FORMS AND THEIR PROPOSED METABOLIC ACTIVATION

To function as an effective prodrug, the masking group must maximize payload release in target cells and minimize payload release in other tissue. Typically, phosphate, phosphonate, and phosphinate prodrugs take advantage of naturally occurring enzymes that metabolize phosphoesters and phosphoamides. Alternatively, enzymes may initiate spontaneous hydrolysis of a phosphoester or phosphoamide. Several different protecting groups have been used in phosphate, phosphonate, and phosphinate prodrugs (Figure 1D).[9] The most clinically relevant forms consist of monoester, symmetrical bis-esters or asymmetrical mixed aryl ester amidates (aryl amidates). Bis-amidates, mixed esters, monoacids, and cyclic forms are also under preclinical investigation for a variety of payloads. The linked protecting group cannot be a simple small phosphoester, such as a methyl or ethyl ester, as these are too metabolically stable to release the payload. Rather, the protecting group is normally a larger biologically relevant molecule, such as an amino acid, lipid, sterol, and so on, that can provide an enzymatic handle to metabolize and reduce potential byproduct toxicity.[14] Importantly, these protecting groups can be chemically manipulated to increase or decrease rate of payload release. There are only a handful of marketed phosphate, phosphonate, and phosphinate prodrugs, including the phosphinate fosinopril (an ACE inhibitor from Bristol-Myers Squibb; Figure 2).[35] With the recent emergency use authorization of remdesivir for SARS-CoV-2,[36] at least five different nucleotide prodrugs of four different payloads are now in clinical use (all currently antivirals from Gilead Sciences). These include bis-ester and aryl amidate forms and include phosphonate and phosphate payloads.

While the phosphinate monoester prodrug fosinopril (Monopril) reached clinical application in 1991, the first nucleotide prodrugs to obtain FDA approval include the phosphonates tenofovir disoproxil (Viread) in 2001 and adefovir dipivoxil (Hepsera) in 2002. Tenofovir disoproxil utilizes a bis-POC (isopropyloxycarbonyloxymethyl) prodrug form, while adefovir dipivoxil uses the bis-POM (pivaloyloxymethyl) prodrug form. These ester prodrugs liberate the

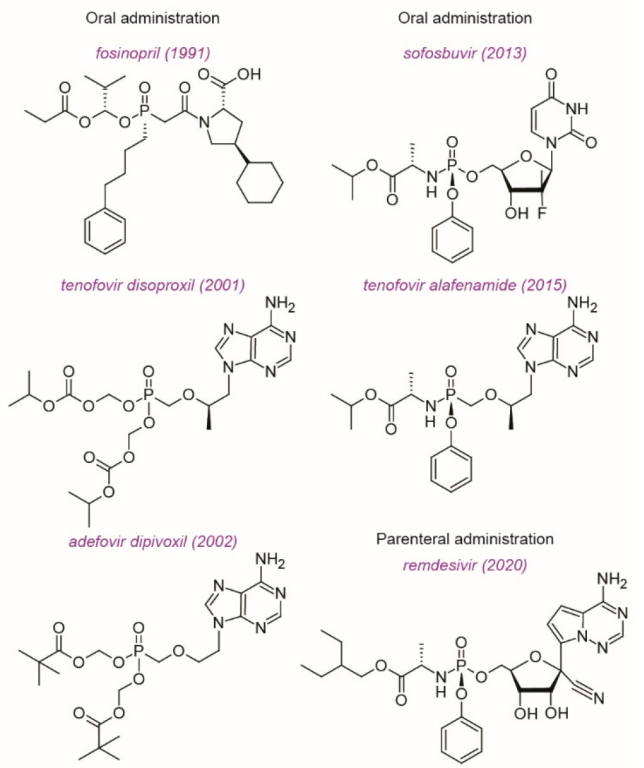

Figure 2. Clinical prodrugs that contain a phosphate, phosphonate, or phosphinate. Early prodrugs were developed for oral administration, including: fosinopril, a phosphinate monoester; tenofovir disoproxil, a phosphonate symmetrical bis-ester; adefovir dipivoxil, a phosphonate symmetrical bis-ester; sofosbuvir, a phosphate aryl amidate; and tenofovir alafenamide, a phosphonate aryl amidate. Remdesivir, a phosphate aryl amidate, has been developed for parenteral administration by the IV or inhalation routes. This strategy boosts tissue uptake from the extracellular fluid, in contrast to prior forms which do not systemically distribute in the prodrug form. The date of the first clinical approval is listed in parentheses.

payload first by enzymatic hydrolysis of the carboxylic acid ester followed by spontaneous release of a formaldehyde molecule. In the case of the bis-esters, this same process repeats to remove the second acyloxyalkyl protecting group and liberate the payload (Figure 3A).[37] The esterase activity is thought to be nonspecific, with multiple plasma and cellular esterases capable of this hydrolysis.

Aryl amidate prodrugs such as the phosphate ester sofosbuvir (Sovaldi, in 2013), the phosphonate tenofovir alafenamide (Vemlidy, in 2015), and now the phosphate ester remdesivir (Veklury, in 2020) have similarly reached clinical use. These prodrugs are activated first by carboxypeptidases that remove the amino acid ester. The remaining anionic amino acid can spontaneously cyclize to release the aryl ester, though this bond may additionally be susceptible to enzymatic hydrolysis. Subsequently, amidase activity can hydrolyze the amide bond to liberate the payload (Figure 3B).[38] The enzyme cathepsin A efficiently catalyzes the first step in the activation of some aryl amidate prodrugs including sofosbuvir.[39] In general, multiple proteases are capable of this hydrolysis, and the specific enzymes involved vary by cell type, prodrug form, and payload.[40,41] For sofosbuvir, cathepsin A is the key contributor, because its activity correlates most closely with cellular potency, and depletion of cathepsin A reduces activation.[42] The prodrug efficacy is dependent on phosphorus stereochemistry. Likewise,

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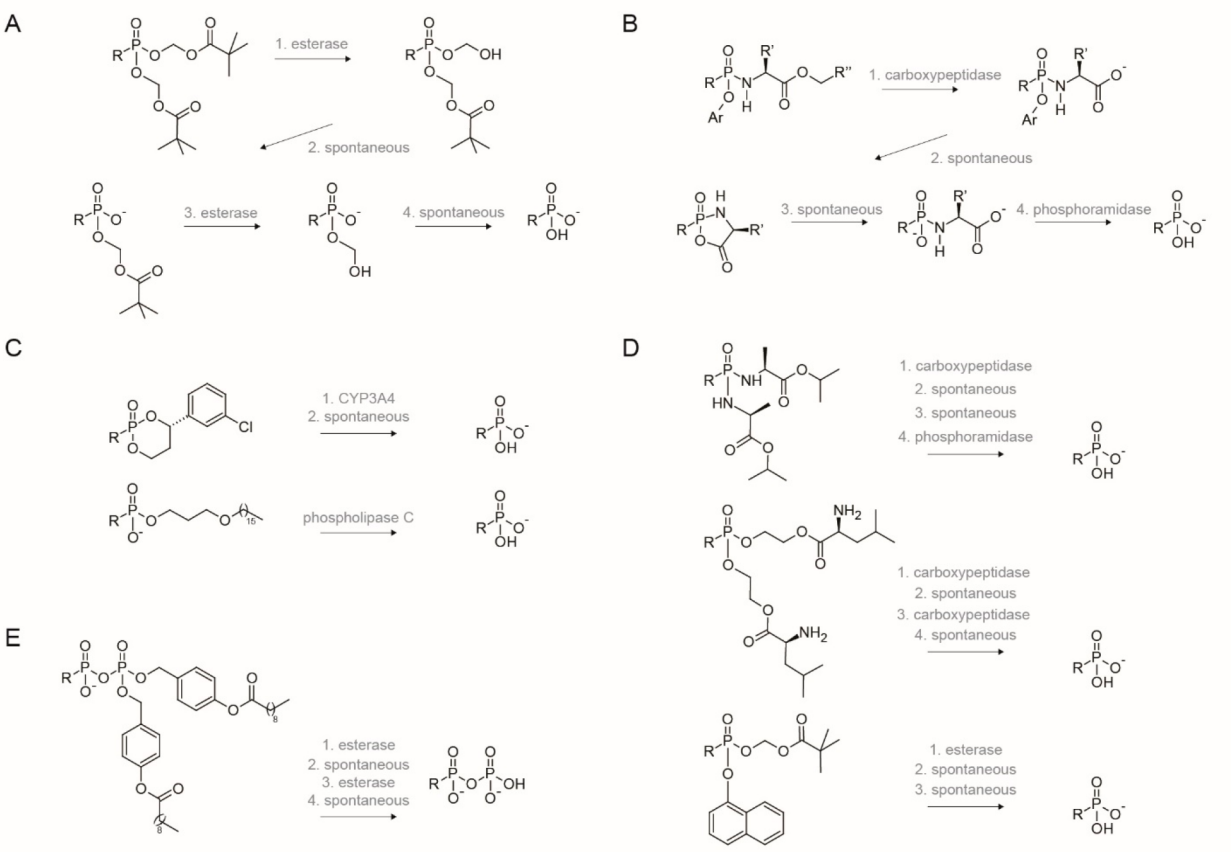

Figure 3. Routes of prodrug metabolic activation. (A) Metabolism of acyloxyalkyl prodrugs. Carboxylesterases hydrolyze the carboxylic acid ester, followed by spontaneous release of formaldehyde. The process repeats if a second protecting group exists to release the free drug. (B) Metabolism of aryl amidate prodrugs. Carboxypeptidases hydrolyze the carboxylic acid ester, followed by spontaneous cyclization and hydrolysis of the aryl ester. Cellular phosphoramidases hydrolyze the amide to release the free drug. (C) Metabolism of preclinical compounds. HepDirect prodrugs undergo hydroxylation by CYP3A4 in the liver to enable spontaneous hydrolysis. Long-chain fatty acid prodrugs can be activated by phospholipase C. (D) Bis-amidites are activated similarly to aryl phosphoramidates by carboxypeptidases and phosphoramidases. Carboxypeptidases can also work on ester linked amino acids. Mixed acyloxyalkyl prodrugs are metabolized by cellular esterases. (E) DiPPro prodrugs enable di- or triphosphate delivery.

depletion of histidine triad nucleotide binding protein 1 (Hint1) increases concentration of intermediates in the cellular activation of sofosbuvir, suggesting that Hint1 plays a role in the phosphoramide hydrolysis during sofosbuvir activation.[42] However, phosphoamidates that contain amino acids other than alanine may be hydrolyzed by alternative phosphoramidases.[43]

A variety of additional prodrug forms are undergoing preclinical development. Cyclic prodrugs employing HepDirect technology such as pradefovir[44] and VK2809[45] have recently reached clinical trials. These compounds impart liver specificity because they require hydroxylation by the liver enzyme CYP3A4 to release the payload (Figure 3C). However, long-chain monoester conjugates that require activation by the enzyme phospholipase C may allow the payload to bypass the liver. For example, conjugation of hexadecyloxypropyl ester to the phosphonate cidofovir (forming brincidofovir) results in compounds that are quite stable to liver metabolism[46] but release the payload in target tissues, allowing for oral dosing.[47,48]

Symmetrical bis-amidates are activated in a manner similar to that for the aryl amidates, with initial formation of a carboxylate followed by cyclization and amide hydrolysis to liberate the drug (Figure 3D).[49,50] Like with the aryl amidates, Hint1 likely metabolizes some of these bis-amidates, but it is not solely responsible for metabolism of this step. For example, sphingomyelin phosphodiesterase, acid-like 3A (SMPDL3A) metabolizes a non-nucleotide bis-amidate with greater activity than Hint1.[51] Symmetrical amino acid bis-esters have been evaluated. Some of these compounds show increased potency, better selectivity, and more plasma stability relative to adefovir dipivoxil.[52,53] Likewise, some mixed aryl POM prodrugs have good plasma stability without loss in potency relative to bis-POM compounds.[54,55] Exciting recent studies have examined DiPPro and TriPPro approaches to generate prodrugs of di- and triphosphates that are plasma stable but are activated by cellular esterases (Figure 3E).[56−59]

OPTIMIZING PRODRUG METABOLISM FOR TISSUE TARGETING BY ORAL ADMINISTRATION

The expanding HIV epidemic in the late 1980s and early 1990s triggered a wave of research into nucleotide analogs as antiviral agents. The earliest nucleotide analog prodrugs were developed to increase the oral bioavailability of these drugs. At that time, nucleotide analogs such as adefovir had demonstrated strong HIV activity in cellular assays but displayed poor oral bioavailability due to the phosphonate, yet the phosphonate form was preferred because it could be more readily converted to the active diphosphate form (triphosphate analog) relative to the parent nucleoside.[24] Acyloxyalkyl moieties such as the POM group had been identified as mild-base-labile protecting groups during organic synthesis[60] and applied to enable cellular uptake

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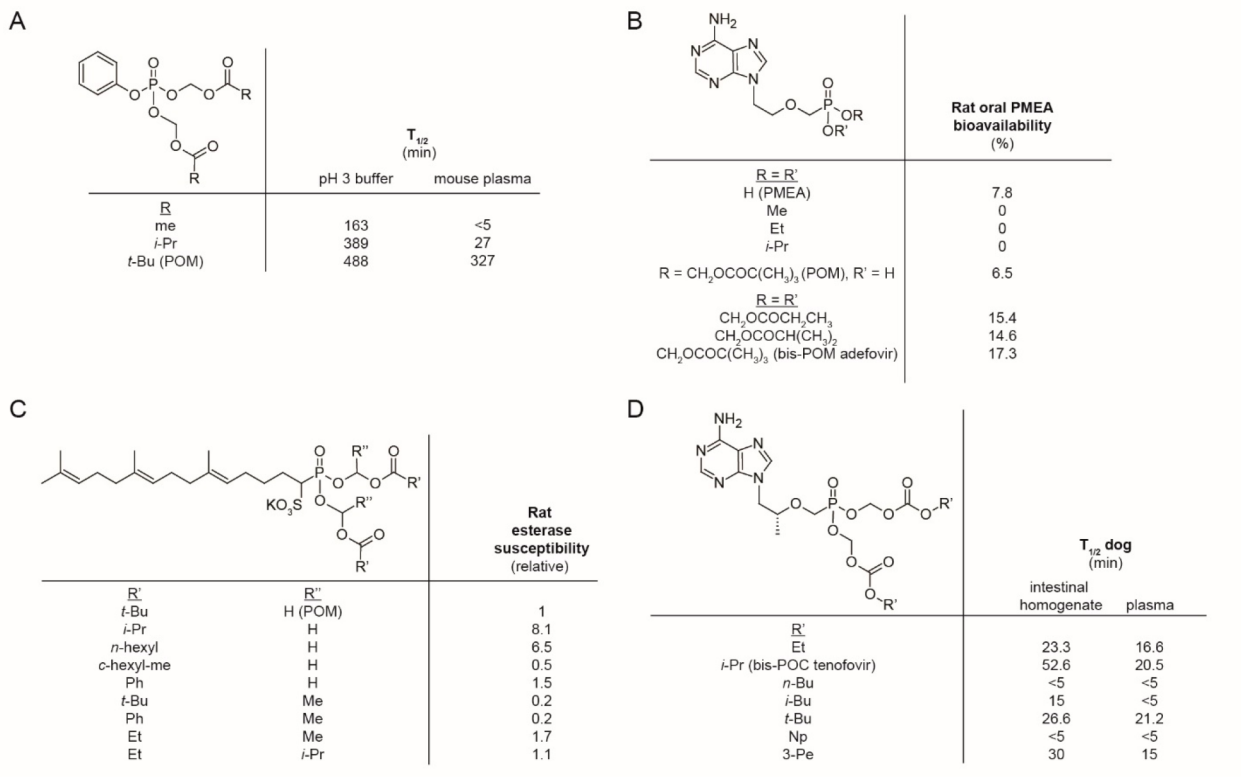

Figure 4. Discovery of bis-POM and bis-POC prodrug forms. (A) SAR of bis-acyloxyalkyl groups. Incorporation of a *t*-butyl group improves chemical and plasma stability relative to methyl or isopropyl groups. This is known as the POM protecting group. Adapted with permission from ref 66. Copyright 1984 Academic Press, Inc. (B) SAR of mono- and bis-acyloxyalkyl groups applied to adefovir. The nucleotide antiviral adefovir (PMEA) displays low oral bioavailability. The simple methyl, ethyl, or isopropyl esters are internalized but are unable to release the free payload as they cannot be metabolized to the acid form. The bis-acyloxyalkyl prodrugs improve oral availability, but the mono-POM does not. Adapted with permission from ref 68. Copyright 1994 American Chemical Society. (C) SAR of bis-acyloxyalkyl groups applied to a squalene synthase inhibitor. POM esters fall in the middle of a ~40 fold range of esterase susceptibility. Compounds metabolized much faster or slower decrease oral bioavailability. Other substituents with similar esterase susceptibility as the POM form may likewise provide good balance. Adapted with permission from ref 70. Copyright 1996 American Chemical Society. (D) SAR applied to tenofovir leading to the POC group. The POC isopropyl group provided the strongest stability in intestinal homogenate and plasma, although some additional promising candidates were identified. Adapted with permission from ref 32. Copyright 1997 Plenum Publishing Corporation.

of charged penicillin analogs[61,62] and carboxylic acids.[63] Some data suggested that this approach could be adaptable to phosphonates.[64,65] Fosinopril had shown that a monoacyloxyalkyl form could improve oral bioavailability of a phosphinate ACE inhibitor to allow payload distribution to this cell surface target.[35]

To further optimize the acyloxyalkyl moiety, the simple phosphate phenyl ester was used as a model system to evaluate protecting group stability. Chemical stability and plasma stability were increased when the acyloxyalkyl structure contained the *t*-butyl group as opposed to methyl or isopropyl groups (Figure 4A).[66] This approach was applied to discovery of oral prodrugs of adefovir (Figure 4B).[67,68] Here, prodrug forms were evaluated for bioavailability of the payload. The acyloxyalkyl forms gave the biggest payload concentration, which was a modest 2-fold increase over the free acid form. Further studies found better oral availability and suggested the prior experiments were saturated.[69] An SAR study on bis-acyloxyalkyl prodrugs of squalene synthase inhibitors provided additional information on esterase susceptibility. This study found that the *t*-butyl-containing form was effective because it had intermediate esterase susceptibility. Compounds that were more esterase susceptible were metabolized too quickly in the gut during absorption, while compounds that were metabolized slowly were not adequately released (Figure 4C).[70] Collectively, these studies concluded that the POM form provides an effective balance of stability relative to activation which promotes oral bioavailability. Because these esters are acid-stable, they survive the GI lumen. They promote gut wall penetration and improve oral availability of their payloads. However, because they are metabolized in the gut, liver, and plasma, they do not reach tissues beyond the liver in the prodrug form. This is quite suitable when the target is extracellular, such as fosinopril, but not ideal for intracellular targets such as those of the nucleotide analogs.

The synthetic nucleotide analog tenofovir was likewise initially developed for HIV and faced a similar bioavailability problem, as it differs from adefovir by addition of a lone methyl group. For tenofovir, an alkyloxycarbonyloxymethyl form was proposed, which was adapted to this phosphonate based on prior work on its use in amines[72] and phosphates.[73] A key study examined the payload release of a series of bis-alkyloxycarbonyloxymethyl esters of tenofovir (Figure 4D).[32] A set of seven prodrug forms revealed high variability in their chemical and enzymatic stability.[32] One compound was eliminated from consideration due to chemical instability and three were eliminated due to low stability in intestinal homogenate and plasma. Three prodrug forms had good stability profiles in a dog

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model in these tissues, and subsequently, the bis-POC form of tenofovir was chosen based on the highest stability in intestinal homogenate and plasma.[32] This stability compared favorably to bis-POM tenofovir. Both the bis-POM and bis-POC prodrug forms increased bioavailability of free tenofovir relative to the free acid form.

Aryl amidate prodrug forms were evaluated to increase the activity of azidothymidine (zidovudine) and related nucleotide analogs for HIV.[74] A systematic study on the d4T payload demonstrated that an alanine amidate provided optimal HIV activity in a cell model (Figure 5A).[71] Over the course of the next decade, these aryl amidates were understood to exhibit better gut wall and plasma stability relative to acyloxyalkyl prodrugs and were investigated for delivery of nucleotide analogs targeting HIV and other viruses including hepatitis B and C.[75] This line of research led to the discovery and clinical approval of sofosbuvir.[75] In the SAR study describing the selection of sofosbuvir as the clinical candidate, a variety of aryl groups and carboxylic acid esters were evaluated (Figure 6A).[76] Even compounds of structural similarity differed in biological activity. A group of seven compounds displayed excellent plasma stability and rapid liver metabolism, which was desirable for the hepatitis C target in the liver. Within this subset, two compounds strongly elevated the triphosphate form of the drug in the liver, including the phenyl Ala-*i*-Pr version which was chosen as the clinical candidate and became sofosbuvir.

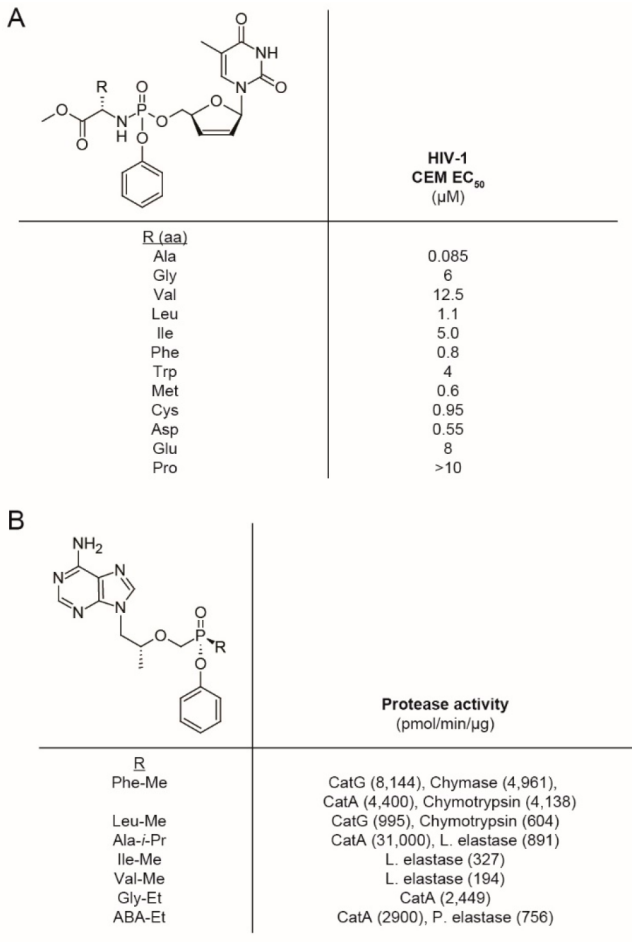

Figure 5. Optimization of aryl amidates for cathepsin A metabolism. (A) Optimization of the phosphoramidate amino acid. Different amino acids contribute to a range of cellular potencies of ∼150-fold. The alanine prodrug was most potent in this series, which is a common trend. The potency additionally varies by cell type, payload, and type of carboxylic acid ester. Adapted with permission from ref 71. Copyright 1997 Elsevier Ltd. (B) Enzymes of aryl amidate metabolism vary by amino acid and carboxylic acid ester. The prevalent isopropyl alanine combination is efficiently and specifically metabolized by cathepsin A. Other combinations are less efficient but can direct the compounds to different enzymes with different expression patterns. Adapted with permission from ref 40. Copyright 2008 The American Society for Pharmacology and Experimental Therapeutics.

The activation of aryl amidates varies as a result of the specific combination of amino acid, amino acid ester, and payload. Studies on aryl amidate analogs of tenofovir agreed that the Ala-*i*-Pr form used for sofosbuvir was most readily hydrolyzed by cathepsin A, which was 34-fold more active than the next most active enzyme, leukocyte elastase (Figure 5B).[40] While other combinations were also cathepsin A substrates, none were as efficient as this form. Some combinations were not hydrolyzed by cathepsin A at all, but rather by alternative serine or cysteine proteases. The second enzymatic step, hydrolysis of the phosphoramide bond in sofosbuvir, is dependent upon Hint1, but other amino acids are preferentially released by alternative enzymes.[78] Interestingly, this conversion may occur in the lysosomes, which would suggest additional membrane crossing is necessary to enter the cytoplasm.[78] There is a clear dependence upon the payload; for example, Hint1 has a preference for purines over pyrimidines. In other nucleotide phosphoramidates, Hint1 is not active, indicating that additional unknown phosphoramidases may play a role in release of payloads like d4T and azidothymidine (AZT).[43]

After the development of sofosbuvir, an aryl amidate form of tenofovir was developed. The aryl amidate form indeed improved liver uptake of tenofovir. This aryl amidate also has better distribution to leukocytes upon oral administration as compared to the bis-POC form. This was illustrated by examining in vivo PBMC uptake which was better with the aryl amidate form because the bis-POC form was unstable in plasma.[79,80] To further optimize payload distribution to PBMCs, an SAR study examined a large set of prodrugs of GS-9148 for ability to drive PBMC payload accumulation after oral dosing (Figure 6B).[41] The aryl Ala-*i*-Pr version of this compound was a surprisingly poor cathepsin A substrate. The bis-amidates that were evaluated had a good in vitro profile but were limited by hepatocyte metabolism or poor HIV potency. The bis-Ala-*n*-Bu was selected for in vivo studies where it had high liver clearance. The aryl alanine prodrugs also had good in vitro profiles, with better cathepsin A cleavage and hepatocyte stability. In vivo assays revealed excellent distribution to PBMCs following oral delivery by the Ala-cBu and Ala-Et forms. The Ala-Et form (GS-9131) was selected as the clinical candidate because it displayed slow liver metabolism and clearance.

THE REMDESIVIR PARADIGM

With the discovery of the potent antiviral activity of 4-aza-7,9-dideazaadenosine-containing nucleotides[81] and the Ebola outbreak of 2014, it became of interest to develop an antiviral for Ebola using the phosphoramidate approach.[82] Remdesivir is unique among these clinical prodrug examples in that it was developed for parenteral dosing. This strategy would enable greater exposure of the target tissue to the prodrug and therefore greater distribution of the payload to the target tissue. A large SAR study optimized remdesivir for IV administration for Ebola (Figure 6C).[77] A key innovation of the remdesivir prodrug form

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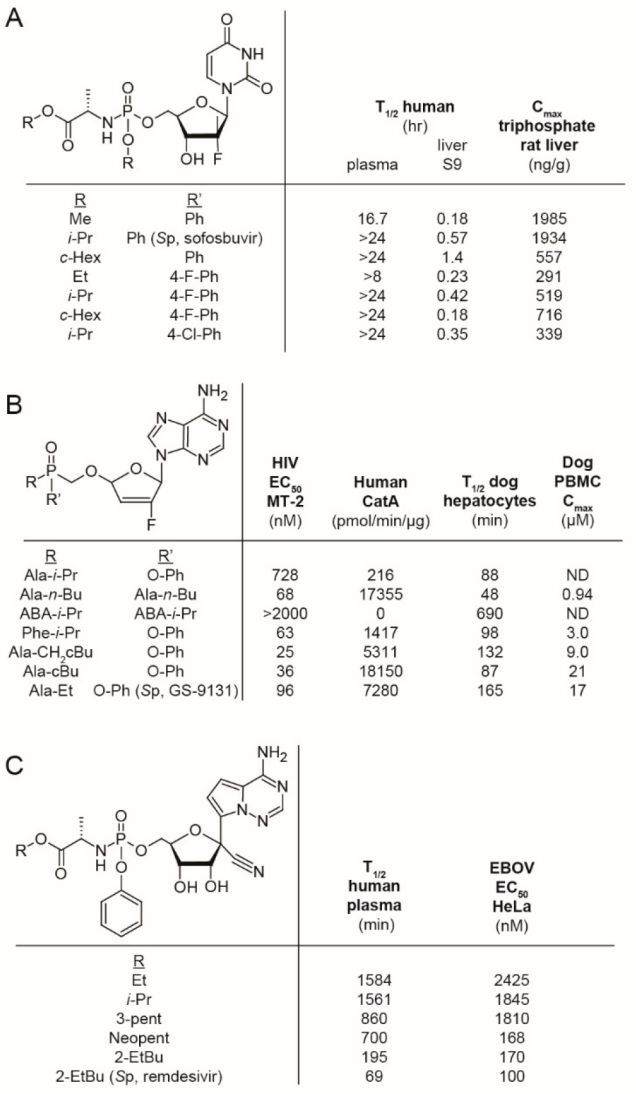
was the incorporation of the 2-EtBu group into the carboxylic acid ester. The study found that nonproximally branched alkyl



R	R'	$T_{1/2}$ human plasma (hr)	$T_{1/2}$ human liver S9 (hr)	C_{max} triphosphate rat liver (ng/g)
Me	Ph	16.7	0.18	1985
i-Pr	Ph (Sp, sofosbuvir)	>24	0.57	1934
c-Hex	Ph	>24	1.4	557
Et	4-F-Ph	>8	0.23	291
i-Pr	4-F-Ph	>24	0.42	519
c-Hex	4-F-Ph	>24	0.18	716
i-Pr	4-Cl-Ph	>24	0.35	339

R	R'	HIV EC_{50} MT-2 (nM)	Human CatA (pmol/min/μg)	$T_{1/2}$ dog hepatocytes (min)	Dog PBMC C_{max} (μM)
Ala-*i*-Pr	O-Ph	728	216	88	ND
Ala-*n*-Bu	Ala-*n*-Bu	68	17355	48	0.94
ABA-*i*-Pr	ABA-*i*-Pr	>2000	0	690	ND
Phe-*i*-Pr	O-Ph	63	1417	98	3.0
Ala-CH_2cBu	O-Ph	25	5311	132	9.0
Ala-cBu	O-Ph	36	18150	87	21
Ala-Et	O-Ph (Sp, GS-9131)	96	7280	165	17

R	$T_{1/2}$ human plasma (min)	EBOV EC_{50} HeLa (nM)
Et	1584	2425
i-Pr	1561	1845
3-pent	860	1810
Neopent	700	168
2-EtBu	195	170
2-EtBu (Sp, remdesivir)	69	100

Figure 6. Discovery of sofosbuvir, GS-9131, and remdesivir prodrug forms. (A) SAR leading to sofosbuvir for HCV. A variety of alanine-containing compounds were evaluated, but few had the right combination of plasma stability, speed of liver metabolism, and ability to produce the active triphosphate in rat liver. Adapted with permission from ref 76. Copyright 2010 American Chemical Society. (B) SAR leading to GS-9131 for HIV. A variety of aryl amidates and bis-amidates were evaluated for ability to drive PBMC levels after oral dosing. The Ala-cBu and -Et forms proved superior among the compounds tested. Adapted with permission from ref 41. Copyright 2010 Elsevier Ltd. (C) SAR leading to remdesivir for EBOV. A variety of alanine-containing compounds were evaluated. Those with nonproximal branched amino acid esters showed the greatest EBOV potency, which correlated with plasma stability. Adapted with permission from ref 77. Copyright 2017 American Chemical Society.

groups showed significant increases in cellular potency, though these correlated with decreases in plasma stability. Even though the plasma stability was relatively low, with $t_{1/2,plasma}$ = 69 min, this was not deemed critical due to the IV route of administration and the expectation that tissue diffusion would happen in a faster time frame. The choice of a clinical candidate was made largely based on in vitro potency toward Ebola and availability of chemical reactants to enable the difficult scaling of the chemical synthesis for prompt clinical trials against Ebola.[77]

As the Ebola outbreak dissipated remdesivir did not gain immediate clinical use. During the SARS-CoV-2 outbreak in 2019, remdesivir was found to display good in vitro potency toward coronaviruses, with EC_{50} values in Vero E6 cells ranging from 1.76[83] to 23.15 μM.[84] Although not optimized for SARS-CoV-2, the tissue distribution profile of remdesivir was deemed suitable for treatment of COVID-19, and the compound displayed activity in a monkey model of COVID-19.[85] The enzymes that metabolize the Ala-2-EtBu prodrug form are not clearly defined at this time, but they are likely expressed at sufficient levels in human lung tissue, which does contain high carboxyesterase expression.[86] Remdesivir treatment of humans with COVID-19 decreased time to recovery from 15 to 11 days,[87] and it was able to gain emergency use authorization for this disease. Remdesivir became the first drug to be used for COVID-19 that targeted the virus rather than the body's response to the virus. It was recently stated by Gilead that a 62% reduction in mortality of severely ill COVID-19 patients has been observed. Further clinical studies are ongoing, including for inhaled use of remdesivir to specifically target lung tissue.

The clinical use of remdesivir reveals a new paradigm for parenteral dosing of a phosphate prodrug by IV or potentially inhaled administration. In these routes, the factors that were important to development of sofosbuvir and tenofovir, such as high intestinal stability and high liver metabolism, are less critical. More crucial are the rates of cellular activation relative to plasma stability or extracellular stability. Complete plasma stability is not required; assuming as with other prodrugs the rate of prodrug diffusion is in a fast equilibrium, as long as the rate of cellular payload release exceeds the rate of plasma payload release, the dose will be sufficient to drive tissue accumulation of the payload (Figure 7A). The cellular balance of the rate of phosphorylation to the di- or triphosphate versus the rate of dephosphorylation to the free drug is also important. Dephosphorylation to the free alcohol would allow diffusion back out of the target cell.

Likely as a result of both intracellular and extracellular dephosphorylation, the parent nucleoside GS-441524 is the major remdesivir metabolite identified in plasma.[82] Some have argued that because of this finding, GS-441524 may be a better alternative for COVID-19 treatment.[88] However, it is unclear whether the two compounds have been directly compared in vivo against COVID-19 at this time, and again, plasma metabolite concentrations may not correlate with tissue concentrations of the active form. To surpass the in vivo activity of remdesivir, GS-441524 would need to be phosphorylated in tissues at a high rate, which is unlikely due to the inefficient activity of nucleoside kinases.[24] While both remdesivir and GS-441524 would both be diffusible into tissues, neither is trapped in tissue until it reaches the free phosphate form (due again to Le Chatelier's principle). Remdesivir should be expected to accumulate at higher tissue levels relative to GS-441524 if the rate of remdesivir payload release is higher than the rate of GS-441524 phosphorylation.

While not directly measured in the lung tissue, the cellular entry and conversion to the triphosphate does seem to occur at a faster rate than plasma degradation, at least in PBMCs, as remdesivir was able to cause accumulation of the triphosphate form in tissue at dose that exceeded the IC_{50} value and because the active metabolite is quite stable.[82] There is some question as to whether PBMCs are a suitable proxy for lung tissue because

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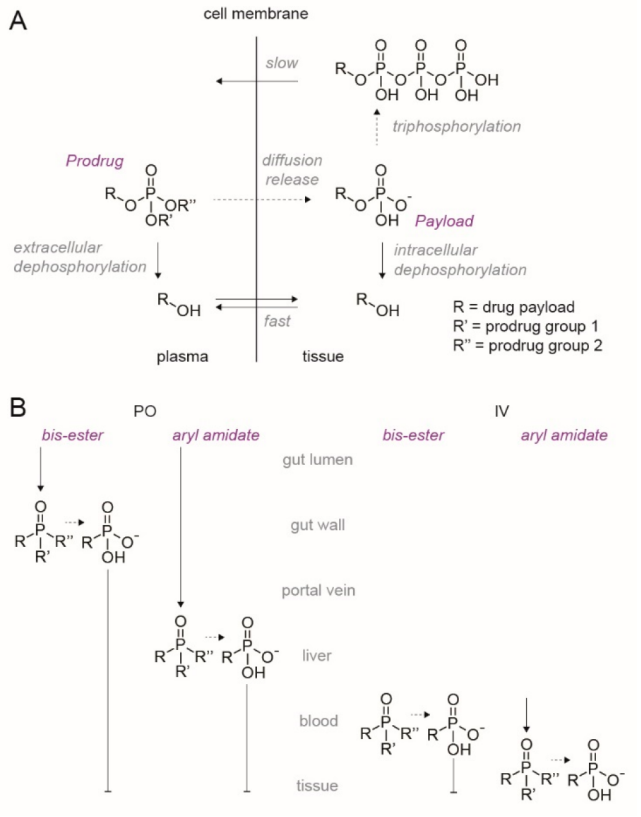

Figure 7. Prodrug activation and dependence on administration route. (A) Kinase bypass strategy of remdesivir-type prodrugs after IV administration. Diffusion and release of the monophosphorylated drug molecule promotes further cellular phosphorylation to the di- and triphosphate forms. Delivery of the monophosphate is preferred over the alcohol form which is not easily phosphorylated. Monophosphates are better substrates for phosphorylation and dephosphorylation relative to parallel monophosphonates. Remdesivir has only an intermediate plasma stability, but the intracellular payload release is likely faster. The intracellular triphosphate form is quite durable. (B) Prodrug metabolism varies by compartment and route of administration. Following oral administration, both esters and amides survive the digestive tract. Metabolism increases as compounds enter the gut wall and proceed to the liver. More metabolically stable compounds proceed further in the prodrug form, although few prodrug forms survive the liver. Some exceptions with good leukocyte activation may promote loading of blood cells prior to liver metabolism. Following parenteral administration, tissue payload levels from prodrugs like remdesivir depends primarily on the ratio of intracellular to extracellular payload release and intracellular stability of the triphosphate form; therefore, extended plasma stability is not a requirement. IV administration of plasma-stable amidate forms allows direct prodrug internalization in the tissues, whereas the other routes and forms result in premature payload release.

the expression of activating enzymes may differ. However, the ability of remdesivir to reduce COVID-19 progression in human trials after IV dosing[87] is important proof-of-concept and implies that suitable drug concentrations are obtained in the lung tissue following IV administration of this prodrug form.

REASSESSING PRODRUG METABOLISM FOR TISSUE TARGETING BY PARENTERAL ADMINISTRATION

There have now been several iterations of phosphate, phosphonate, and phosphinate prodrugs that differ in their route of administration and target tissues. Oral dosing of prodrugs such as fosinopril, bis-POM adefovir, and bis-POC tenofovir provide excellent absorption and release the payload into the gut wall, liver and plasma. Orally dosed sofosbuvir survives the intestinal cells and blood and rapidly releases the payload in the intended target of the liver. Oral dosing of tenofovir alafenamide not only results in liver release but also improves distribution to leukocytes. Remdesivir, dosed intravenously, promotes accumulation in leukocytes and peripheral tissues (Figure 7B). Together, these examples demonstrate that tissue specificity of prodrug release is a possibility, that the rules developed for orally dosed prodrugs may differ with parenteral administration, and that potential advantages of other prodrug forms should be reassessed in light of varied routes of administration.

During IV administration, tissue accumulation should depend less upon GI and liver metabolism and more upon the rate of tissue payload release relative to the rate of plasma release or clearance. Prodrugs that can maximize the rate of tissue payload release relative to plasma release would obtain a kinetic advantage. That kinetic advantage is similar in some ways to the kinetic advantage of drugs with slow K_{off} rates that allow for longer target engagement.[89] In the case of prodrugs, the kinetic advantage happens at the cellular level rather than the target protein level. This is a consequence of the charge differential between prodrug and payload forms and its resulting impact on cellular payload accumulation. Further development of prodrugs for IV dosing could attempt to maximize this kinetic advantage, either by speeding the uptake of amidate forms or slowing the plasma release of ester forms. While effective, it is unlikely that remdesivir has fully maximized the kinetics.

The various prodrug forms differ in their rates of cellular payload release. A study on bis-POC tenofovir found that within hours after exposure conversion to the di- and triphosphate analog forms had occurred.[90] This activation process involves multiple steps including two phosphorylation events. Hydrolysis of these prodrugs likely happens much faster, within minutes. For example, a fluorescent monoester showed how rapidly acyloxyalkyl prodrugs could be internalized.[91] Within hours, a large portion of the extracellular prodrug dose was internalized ($t_{1/2,\text{internalization}}$ = 55 min). This finding was mirrored in a study on bis-POM adefovir, which was quickly eliminated from extracellular media ($t_{1/2,\text{internalization}}$ = 100 min).[92] In the latter case, intracellular adefovir peaked at 2 h, while it took nearly 6 h for the triphosphate analog form to appear. These results suggest that experiments longer than a few hours are more sensitive to the total mass of prodrug exposed to the cells rather than the concentration, which has implications for evaluating potency under differing mass to volume ratios. They also suggest that exposure times of minutes to hours may be sufficient to yield a cellular drug concentration above that of the extracellular concentration. This was observed with remdesivir following IV administration, where after 2 h PBMCs obtained high levels of triphosphate in the midmicromolar range.[77]

The rate of cellular payload release varies substantially by cell type and depends upon the structure of the prodrug and expression of enzymes that metabolize it. This would cause tissues to differ in their rates of prodrug uptake. Studies on the tenofovir and adefovir payloads show that the bis-POM form is more quickly hydrolyzed than the bis-POC form,[93] in both cell extracts and intestinal homogenate.[32] In a direct comparison of the bis-POM and bis-POC forms of tenofovir, the bis-POC form was a stronger antiviral, increased the diphosphate concen-

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tration to a greater degree and was less toxic than the bis-POM form.[90] The authors attributed the cytotoxicity to the pivalate byproduct of the bis-POM form versus the isopropyl alcohol byproduct of the bis-POC form. It would be interesting to see whether bis-ester prodrugs optimized for the ratio of intracellular to extracellular payload release rather than oral bioavailability would be appropriate for IV administration and perhaps even work better than the aryl amidate technology.

In contrast to bis-esters, the payload release from aryl amidates is slower in the intestines, plasma, and tissue. The aryl Ala-*i*-Pr prodrug GS-465124 had stability in intestinal homogenate and plasma ($t_{1/2,\text{plasma}}$ = 261 min and $t_{1/2,\text{intestinal-homogenate}}$ = 360 min) higher than that in liver homogenate ($t_{1/2,\text{liver-homogenate}}$ = 5.3 min).[96] This suggests that aryl amidates have about 10-fold greater intestinal and plasma stability relative to the numbers typically reported for relevant bis-esters. Like the bis-ester forms, the aryl amidates were metabolized quickly in liver homogenate. Together, there is a greater probability of aryl amidates to reach the liver in the prodrug form relative to bis-esters after oral dosing, making them a preferred choice when liver is the intended site of action, such as hepatitis. This rate has been determined for the prodrug PSI-353661 which is converted to the triphosphate form in hepatocytes in about 4 h.[34] A similar result was seen with prodrug INX-08189 which caused cellular accumulation of the triphosphate in about 6 h.[97] The slower cellular payload release of amidates in the GI is attractive here to promote liver distribution, but a more rapid tissue payload release may be desirable for a prodrug administered by the IV route.

A few studies in the literature compare the kinetics of multiple prodrug classes, such as bis-esters versus aryl amidates. An interesting SAR study during the development of a fructose 1,6 bisphosphatase inhibitor targeting the liver compared the bis-POM, aryl amidate, and bis-amidate forms, among others. This study showed that the bis-POM form was at least 5-fold more susceptible to liver esterase relative to the aryl amidate form. The aryl amidate form was itself was 5-fold more susceptible than was the corresponding bis-amidate form. Bioavailability was inversely related, though bis-amidates had better bioavailability relative to the aryl amidate (Figure 8A).[94] The bis-Ala-Et form was selected as the in vivo candidate due to its high bioavailability. This finding is consistent with another study that compared nucleoside analog bis-amidates to aryl amidates and showed greater susceptibility of bis-amidates to hepatocyte metabolism.[41] The latter paper did examine IV administration of at least one bis-amidate and found it to undergo relatively high clearance relative to the aryl amidate forms and a worse PBMC loading efficiency. Together this suggests that some bis-amidates may not be ideal for IV administration due to their liver metabolism even by that route, though this may vary with respect to the amino acid ester employed, which has not yet been fully characterized. A larger SAR study on bis-amidates could likely improve their qualities in this regard.

An SAR study using a α-phosphonocarboxylate Rab geranylgeranyl transferase inhibitor directly compared POM, POC, and three bis-amidate prodrug forms (Gly-Me, Ala-Me, and Phe-Me) for their susceptibility to metabolism in intestinal homogenate.[95] Here, the authors observed that the bis-esters were metabolized more quickly than were the bis-amidates, with the bis-POM form activated slightly faster than the bis-POC form (Figure 8B). Interestingly, while the bis-Gly-me and Ala-Me forms were quite stable, the Phe-Me form was activated more quickly. This is reminiscent of the aryl Phe-Me form of

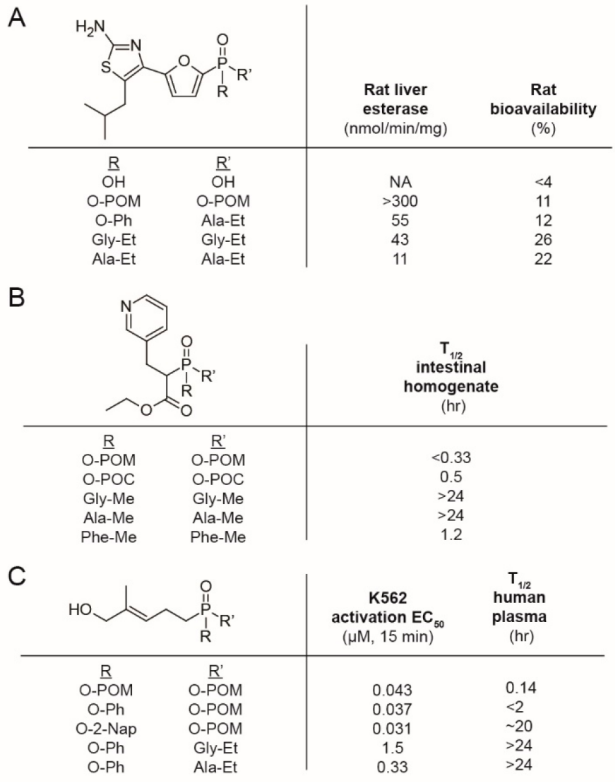

Figure 8. Metabolism of various prodrug forms impacts their suitability for parenteral administration. (A) SAR of fructose 1,6 bisphosphate inhibitors. A group of bis-acyloxyalkyl, bis-amidate, and aryl amidates were evaluated. The bis-amidates showed the fastest liver metabolism and greatest bioavailability and were selected for further study. Adapted with permission from ref 94. Copyright 2007 American Chemical Society. (B) SAR of Rab geranylgeranyl transferase inhibitors. Initial hydrolysis of the bis-esters in intestinal homogenate was more rapid compared to bis-amidates, though the Phe-Me combination was intermediate. Adapted with permission from ref 95. Copyright 2015 The Royal Society of Chemistry. (C) SAR of phosphoantigen prodrugs. Cellular potency at early time points is inversely related to plasma stability, although a mixed aryl POM form provided a good mix of stability and potency. Adapted with permission from ref 33. Copyright 2018 Elsevier Inc.

tenofovir, which was susceptible to other enzymes than cathepsin A including intestinal chymotrypsin. It would be interesting to see whether bis-amidates such as this would undergo faster tissue uptake following IV administration relative to the amidate forms contain that contain alanine.

SAR studies on the phosphoantigen payload have compared the bis-POM,[33] aryl amidate,[98,99] and mixed aryl POM[54,55] forms (Figure 8C).[33] In short exposure experiments, the mixed aryl POM forms were as efficiently activated as the bis-POM form. The aryl amidates (Gly-Et and Ala-Et) were less efficient, demonstrating slower uptake kinetics of these forms relative to the esters. It is quite striking that nanomolar potency was achieved in by the bis-esters after just a 15 min exposure, further demonstrating that active payload concentrations can be rapidly achieved with phosphonate prodrugs. The aryl POM forms demonstrated the best ratio of intracellular activation to plasma metabolism. As such, they may be viable candidates for phosphonate delivery by the IV route. These findings also suggest that aryl amidates, such as remdesivir, may not yet be

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fully optimized for their speed of tissue distribution after IV administration.

CONCLUSIONS

Research on phosphate, phosphonate, and phosphinate prodrugs has accelerated over recent years, culminating in clinical use of three prodrugs in the past decade (sofosbuvir, tenofovir alafenamide, and now remdesivir). A number of additional prodrugs are in active clinical trials (GS-9131, pradevofir, VK-2809, brincidofovir, etc.). The efficacy of these prodrugs is highly dependent on their metabolism, the details of which vary by route of administration. Most important for IV administration is the ratio of intracellular to extracellular payload release, which if done well can drive higher intracellular concentrations relative to the extracellular concentration. Remdesivir has demonstrated the clinical utility of a prodrug form by IV administration, though its specific prodrug moieties may not be fully optimized for this route and may not be fully applicable to other payloads. However, the clear efficacy of remdesivir by the IV route means it will set the standard for further development of parenterally dosed phosphate, phosphonate, and phosphinate prodrugs. Improving the rate of intracellular payload release of the amidates or decreasing the rate of plasma payload release of the esters may both prove beneficial to deliver future prodrugs by IV administration.

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Notes

The author declares the following competing financial interest(s): A.J.W. is a founder of Terpenoid Therapeutics. The current work did not involve the company.

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