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ORIGINAL ARTICLE

Chemical stability of 4′-azidocytidine and its prodrug balapiravir

Fujun Li[1], Xiaoyang Wu[2], Xu Hadig[2], Sujuan Huang[2], Lei Hong[2], Tony Tran[2], Michael Brandl[2] and Tom Alfredson[2]

[1]*XenoPort, Inc., Santa Clara, CA, USA and* [2]*Roche Palo Alto LLC, Palo Alto, CA, USA*

Abstract

Background: R1479, a 4′-azidocytidine nucleoside analog, was developed for the treatment of Hepatitis C virus infection. Balapiravir (R1626) is the tri-isobutyrate ester prodrug of R1479 under clinical development to improve exposure of R1479 upon oral administration. *Objective*: The chemical stability and the rate of azide release of R1479 and balapiravir were studied. *Methods*: R1479 and balapiravir solutions were prepared at different pH values and stored at various temperatures. An ion pair high-performance liquid chromatography (HPLC) method with gradient elution was employed to analyze the prodrug, parent, and degradation products. Azide was measured using a reversed phase HPLC method with UV detection after formation of the 3,5-dinitrobenzoyl azide derivative with 3,5-dinitrobenzoyl chloride. The data were analyzed using initial rate and conventional first-order kinetic methods. *Results*: R1479 degrades to cytosine and azide in aqueous solutions, whereas balapiravir mainly degrades to R1479 and mono- and diesters of R1479. The rates of azide release from R1479 and balapiravir were generally comparable with the corresponding amount formed of cytosine. *Conclusion:* Azide release is pH dependent and is faster in acidic solutions than in neutral solutions. The amount of azide released is significantly less from balapiravir than that from R1479, suggesting a potential advantage of the prodrug over the parent drug.

Key words: *Azide; azido moiety; initial rate method; nucleoside analog; prodrug*

Introduction

R1479, a 4′-azido-substituted cytidine, is a potent and selective inhibitor of hepatitis C virus (HCV) RNA polymerase[1–3]. Preclinical and early clinical studies of R1479 showed suboptimal oral bioavailability because of limited absorption. Like other nucleoside analogs, R1479 is a polar molecule with poor intestinal permeability[4,5]. Prodrugs of R1479 were developed to increase transcellular permeability. Balapiravir (R1626, the tri-isobutyrate ester of R1479) was identified as the lead prodrug for further development[6,7]. The prodrug significantly improved oral bioavailability and dose proportionality[8]. In addition, balapiravir has shown a potent antiviral activity in chronic HCV patients[9–11].

The chemical reactivity of balapiravir has been reported previously[4]. Balapiravir mainly degrades to mono- and diester analogs as well as the parent drug R1479 following first-order kinetics. Since both R1479 and balapiravir contain an azido moiety, the release of azide was of interest[12,13]. Other azido-containing drugs have been approved for oral use, including azidocillin and zidovudine (AZT). However, the information on potential azide formation from these drugs is limited. AZT is a 3′-azido-3′-deoxythymidine, where the azido group has been reported to be fairly stable[14,15]. A 4′-substituted-2′-deoxynucleoside, 4′-azidothymidine (ADRT), has been synthesized and studied extensively for its chemical stability[14,16]. ADRT was found to be 340 and 130 times more reactive than AZT at acidic and neutral conditions, respectively. Because of the structural similarity of R1479 and ADRT, R1479 was hypothesized to be less stable than AZT with respect to azide release in aqueous solutions.

In this article, the rate of azide release and chemical stability of R1479 and balapiravir were studied using an initial rate method and conventional first-order kinetic

Address for correspondence: Dr. Fujun Li, XenoPort, Inc., 3410 Central Expressway, Santa Clara, CA 95051, USA. E-mail: fujun_li@yahoo.com

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method for monitoring the loss of drug or the formation of degradation products. The results were compared with other azido-substituted nucleosides. Analytical methods were developed to determine R1479, balapiravir, azide, and other degradation products.

Materials and methods

Materials

R1479 and balapiravir were prepared by chemical synthesis, Roche Palo Alto LLC (Palo Alto, CA, USA). Sodium azide and cytosine were obtained from Aldrich (Milwaukee, WI, USA). 3,5-Dinitrobenzoyl chloride was purchased from Fluka (Buchs, Switzerland). A 35 mg/mL 3,5-dinitrobenzoyl chloride solution was prepared in acetonitrile (150 mM). All other reagents were of analytical grade.

pH determination

pH measurements were made at room temperature using an Orion Triode^{TM} pH meter (model 611, Thermo Scientific, Waltham, MA, USA) electrode calibrated with aqueous standard buffer solutions. pH values were not corrected for temperature.

Solution reactivity study

Sample preparation for determination of chemical reactivity of R1479

R1479 solutions at a concentration of approximately 0.2 mM in 0.001–0.1 N HCl, 0.01 M potassium acetate, 0.01 M potassium phosphate dibasic, and 0.01 M potassium carbonate anhydrous at various pH values were prepared. The ionic strength of the aqueous solutions was adjusted to 0.15 M with potassium chloride.

All sample solutions were flame-sealed into 1 mL clear glass ampoules and placed in ovens at 40°C, 50°C, and 60°C. Several samples at each pH were stored at −20°C to serve as control samples 'zero time' for the calculation of percent remaining at the time of assay. At selected time intervals, individual samples were removed from the elevated temperature ovens and stored at −20°C. Samples were allowed to warm to room temperature before analysis by high-performance liquid chromatography (HPLC).

Sample preparation for determination of azide release from R1479 and balapiravir

R1479 or balapiravir samples were prepared in 0.1 N HCl (pH 1), 0.01 N HCl (pH 2), 0.001 N HCl (pH 3), and 0.01 M potassium phosphate dibasic (pH 7) solutions. The ionic strength was adjusted to 0.15 M with potassium chloride.

Approximately 4 mM of R1479 was dissolved in pH 1, 2, 3, and 7 solutions. Approximately 4 mM of balapiravir was dissolved in pH 1 and 2 solutions and only 2 mM of balapiravir was dissolved in pH 3 solution because of limited solubility. The study of balapiravir at higher pH was not conducted as the solubility was inadequate to obtain acceptable sensitivity by HPLC analysis. pH values were measured.

All sample solutions were prepared in glass vials and incubated in a 37°C water bath agitated at 50 rpm. Aliquot samples were withdrawn at different time points and divided into two portions for different HPLC analyses.

High-performance liquid chromatography methodology

Samples were analyzed by HPLC using either a Waters 2690 or Agilent 1100 systems.

Method for determination of cytosine, R1479, and balapiravir

The pH 1 sample solutions of balapiravir were diluted immediately with 0.03 M KOH to raise the pH value to near pH 3 to prevent further degradation of balapiravir. Other sample solutions were diluted with 3% acetonitrile in water to achieve a final concentration of approximately 1 mM. All R1479 samples at 0.2 mM concentration were directly injected onto the HPLC.

The HPLC analysis was performed with a Zorbax SB-phenyl column (4.6 × 150 mm, 5 μm) using UV detection at 276 nm. The mobile phase A consists of 5 mM 1-heptane sulfonic acid, sodium salt with 0.05% phosphoric acid in water, and mobile phase B contains 5 mM 1-heptane sulfonic acid, sodium salt with 0.05% phosphoric acid in 90% acetonitrile and 10% water. Gradient elution was applied from 2% B to 90% B in 25 minutes, followed by an isocratic condition for 5 minutes with a flow rate of 1 mL/min.

The amount of cytosine, R1479, or balapiravir was calculated by comparing to standard solutions or control samples. Mono- and diesters of R1479 were determined by percent HPLC area normalization using a response factor of 1.0.

Method for azide determination

All samples were diluted with 50 mM potassium phosphate, monobasic buffer (1:2 dilution). The pH was adjusted with 1 N KOH or 1 N HCl to between pH values 4.0 and 5.5. The samples were then centrifuged to remove insoluble material.

Five milliliters of the above supernatant was transferred into a 10-mL volumetric flask. Acetonitrile (4.8 mL) was added to the flask and then 200 μL of 3,5-dinitrobenzoyl chloride solution was added to form the 3,5-dinitrobenzoyl azide derivative. The samples were mixed and

allowed to react for at least 10 minutes to let the reaction to go to completion. 3,5-Dinitrobenzoyl azide standard solutions were prepared similarly from sodium azide.

The amount of 3,5-dinitrobenzoyl azide was determined using a reversed phase HPLC method with UV detection at 230 nm. A Zorbax SB-CN column (4.6 × 150 mm, 3.5 μm) was used, and the separation was performed using water and acetonitrile as mobile phases with a gradient elution from 15% acetonitrile to 65% in 30 minutes at 1 mL/min. The amount of azide was calculated by comparing to azide standard solutions.

Data analysis

The observed first-order rate constant for the disappearance of drug was determined by fitting the percent of drug remaining versus time to an exponential equation, whereas zero-order rate constant was determined from the slope of the linear plot of drug concentration versus time. The rate constants for the formation of degradation products were obtained from the slope of the curves plotted with the degradation product concentrations as a function of time.

Results and discussion

The structures of R1479, balapiravir, ADRT, and AZT are shown in Figure 1.

Chemical reactivity of R1479 and balapiravir

The chemical reactivity of R1479 was studied in aqueous solutions at pH range of 1–11. The observed first-order rate constants (k_{obs}) were determined by monitoring the loss of R1479 with time. The degradation of R1479 is catalyzed by both acid and base. Figure 2a shows the pH-rate profiles for the degradation of R1479 in aqueous solutions at 40°C, 50°C, and 60°C. R1479 is most stable between pH values 5 and 6. Cytosine, **2**, and azide are the major degradation products (Figure 3). In comparison with ADRT, where thymine and azide have been reported to be the major degradation products, the rate constants for the degradation of R1479 are approximately 20–40 times faster than that for ADRT from pH values 1 to 7 at 60°C (Table 1)[14]. However, the rate constants are similar for both compounds at pH 11, suggesting that the reaction mechanisms of the two compounds are likely different at acidic and neutral conditions compared to basic conditions. It is not known whether the higher reactivity of R1479 compared with ADRT at acidic and neutral pH is because of the difference of nucleoside base or the presence of hydroxyl group at the 2′ position of the ribose. Furthermore, AZT (3′-azido-3′-deoxythymidine) has been reported to have much slower reactivity than ADRT as azide is not in a labile position in AZT[14]. The instability of R1479 and ADRT is most likely a result of the 4′-azido substitution at the anomeric position of the furanose moiety[17,18].

We have previously reported that balapiravir, the prodrug of R1479, degrades under both acidic and basic

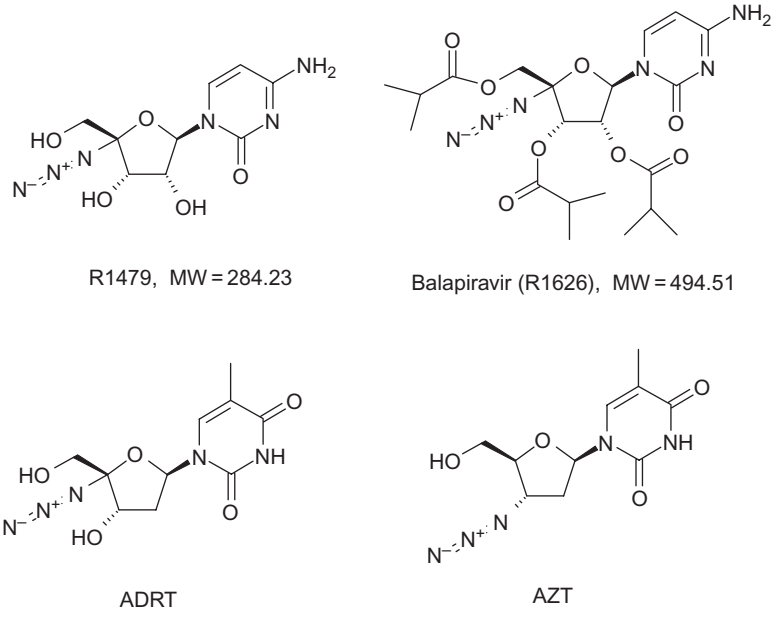

Figure 1. Structures of R1479, balapiravir, ADRT, and AZT.

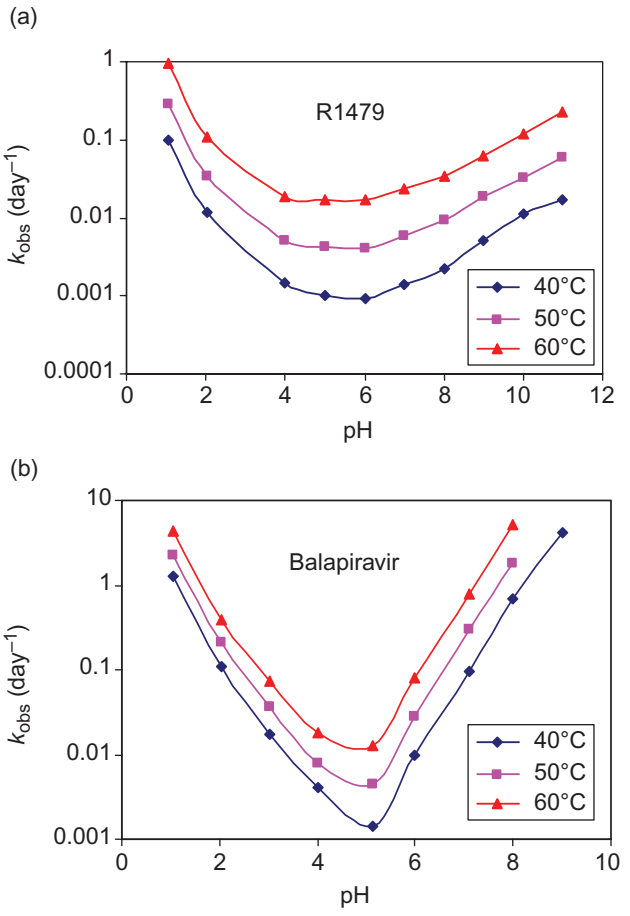

Figure 2. pH-rate profiles for the degradation of R1479 (a) and balapiravir (b) in aqueous solution.

conditions following first-order kinetics[4]. The most stable pH is near pH 5. The major degradation products are the parent drug R1479 and mono- and diester analogs. Cytosine and azide were not reported. In comparison to R1479, the rate of degradation of balapiravir is much faster under the same conditions except at pH 5 (Table 1 and Figure 2b). However, it was observed that the release of azide from balapiravir was significantly slower than that from R1479.

Release of azide from R1479 and balapiravir

More extensive studies were conducted to determine the degradation and azide release from R1479 and balapiravir in aqueous solutions at 37°C because of the pharmacological activity of azide[12]. The study was carried out at acidic and neutral pH and 37°C to simulate physiological conditions. An initial rate method was utilized to monitor the formation of the degradation products in a short time period. To detect low levels of azide and cytosine formed from the degradation of R1479 or balapiravir by HPLC/UV method, high concentrations of R1479 and balapiravir were used for this study. However, the study was not feasible for balapiravir at neutral pH because of its low solubility. Both R1479 and balapiravir have pH-dependent solubility with a pK_a of 3.9 and 3.6 and solubility of 10 and 0.19 mg/mL at neutral pH, respectively[4,5].

The disappearance of R1479 and the formation of cytosine and azide were monitored in the R1479 solutions, whereas, for balapiravir solutions, the disappearance of the prodrug and the formation of R1479, mono- and diesters of R1479, cytosine, and azide were determined.

Figure 4 shows the degradation of R1479 and the formation of cytosine as a function of time. Because the loss of R1479 is less than 5%, the concentration of the reactant can be considered constant so that the reaction can be treated as zero-order kinetics[19]. This initial rate method has been utilized to rapidly determine reaction order and rate constants[20,21]. The rate constants obtained from the degradation of R1479 and the formation of cytosine are comparable in pH 1 and 2 solutions, indicating that the formation of cytosine is the major degradation pathway (Table 2). These results are consistent with that obtained from conventional first-order kinetics studies. The rate constants could not be determined at pH 3 and 7 in this experiment because of very slow degradation of R1479.

As previously reported, balapiravir degrades to mono- and diester analogs as well as R1479. Even though balapiravir degraded to a large extent at pH 1, the data fit zero-order kinetics fairly well (Figure 5). Interestingly, the half-life of balapiravir (22 hours) is comparable to that reported for 5′-ester prodrug of AZT

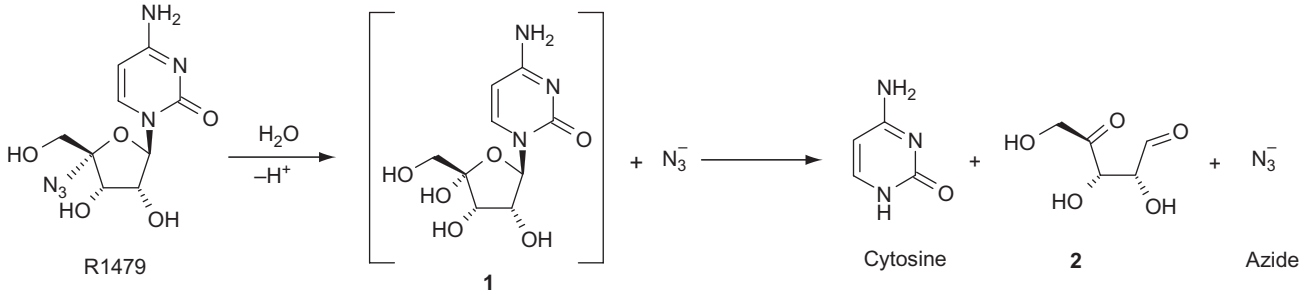

Figure 3. Degradation mechanism of R1479.

Table 1. Observed first-order rate constants for the degradation of R1479, balapiravir, and ADRT in aqueous solutions.

	k_{obs} (day^{-1}) at 60°C		
pH	R1479	ADRT	Balapiravir
1	0.96	0.030	4.29
2	0.11	0.0026	0.39
5	0.017	0.00037	0.013
7	0.024	0.0013	0.80
11	0.22	0.17	—

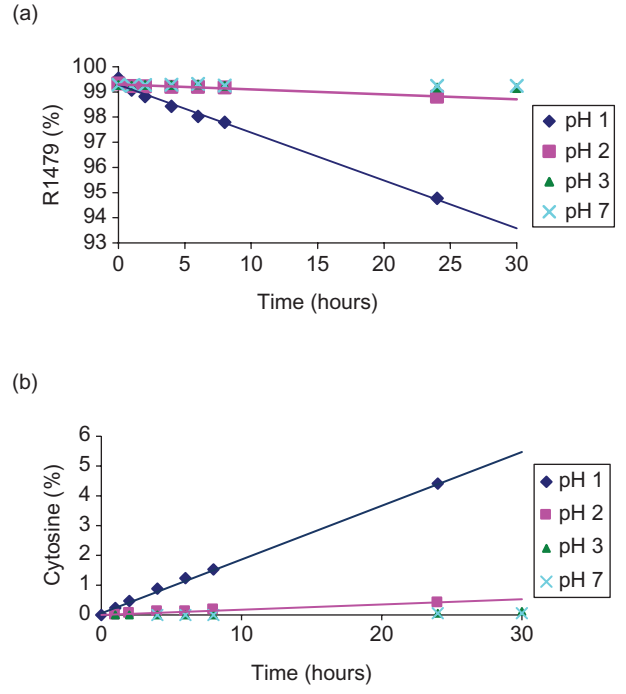

Figure 4. Percent change of R1479 (a) and cytosine (b) with time in various pH solutions of R1479 at 37°C.

in pH 1 solution at 37°C[22], indicating that the structures of the nucleosides apparently have little influence on the hydrolysis of the prodrugs. The formation of mono- and diesters of R1479 followed zero-order kinetics for the early part of the study as these esters had not degraded extensively during this study period. The initial rate constants are shown in Table 2. 2′,3′-Diester formed rapidly in the early stage of the degradation reaction followed by 2′,5′- and 3′,5′-diesters. In contrast, the initial rate for the formation of monoesters is rather slow. Figure 6 shows a plot of percent mono- and diesters versus time in pH 2 solution. When the degradation reaction is followed to a large extent, the reaction kinetics can be different because of consecutive decompositions of these esters[4]. The formation of R1479 and cytosine is markedly slower compared with the mono- and diesters (Table 2 and Figure 7). As shown in Figure 7, the data for R1479 and cytosine fit zero-order kinetics in the early part of the degradation reaction at pH 1. However, they increased exponentially with time when the reaction continued, suggesting that both R1479 and cytosine could be produced from multiple components. There was no significant amount of R1479 and cytosine formed at pH values 2 and 3.

The initial rate of azide formation was also determined (Figure 8) and the results generally agree with the rate of cytosine formation for both R1479 and balapiravir after conversion of azide concentration to percent molar concentration. This is consistent with the proposed degradation mechanism for R1479. For balapiravir, the formation of cytosine and azide can result from balapiravir, mono- and diester analogs, and R1479. Consequently, it may not follow zero-order kinetics as shown in Figure 8b for pH 1 condition. Because of low levels of R1479, cytosine, and azide formed in balapiravir solutions, it could not be confirmed whether azide is

Table 2. Observed zero-order rate constants (percent change/hour) for the degradation of R1479 and balapiravir and the formation of degradation products in aqueous solutions at 37°C.

	pH 1	pH 2	pH 3	pH 7
R1479	−0.19	−0.020	<0.001	<0.001
Cytosine	0.18	0.018	<0.001	<0.001
Azide[a]	0.16 (68.7)	0.015 (6.2)	0.0020 (0.84)	0.0036 (1.5)
Balapiravir	−2.05	−0.32	−0.053	—
2′,3′-Diester	1.31	0.21	0.030	—
2′,5′-Diester	0.30	0.070	0.013	—
3′,5′-Diester	0.13	0.039	0.0058	—
2′-Monoester	0.28	<0.001	<0.001	—
3′-Monoester	0.12	0.0025	<0.001	—
5′-monoester	0.043	<0.001	0.0001	—
R1479	0.062	<0.001	<0.001	—
Cytosine	0.0034	<0.001	<0.001	—
Azide[a]	0.0067 (2.8)	0.00036 (0.15)	<0.0003	—

[a]Rate constant in parentheses is expressed in ng/mL/h for azide.

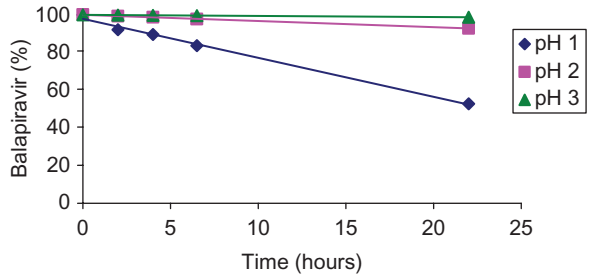

Figure 5. Percent change of balapiravir with time in pH 1, 2, and 3 solutions at 37°C.

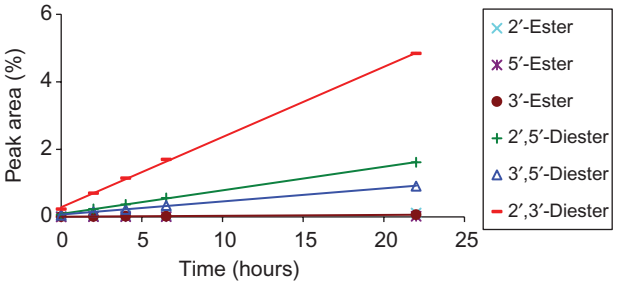

Figure 6. Percent change of mono- and diesters of R1479 with time in pH 2 solution of balapiravir at 37°C.

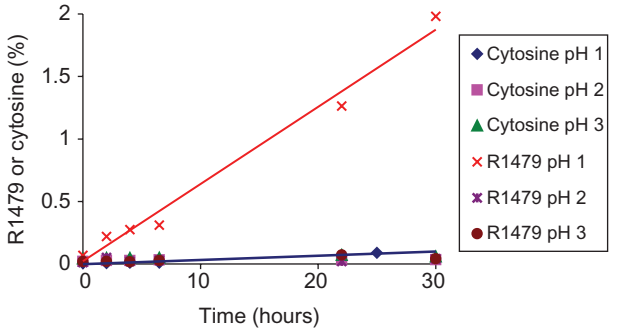

Figure 7. Percent change of R1479 or cytosine with time in balapiravir solutions at 37°C.

merely released from R1479. The overall rate of azide release from balapiravir is 20- to 40-fold slower than that from R1479 in pH 1 and 2 solutions. There was no significant amount of azide released from balapiravir at pH 3. Azide release from balapiravir is not experimentally available at pH 7 because of low solubility. However, the azide release from R1479 is pH dependent and is faster in acidic conditions than in neutral conditions. Thus, it can be estimated that the azide formed from balapiravir at pH 7 is similar or less than that from R1479.

The addition of electron withdrawing groups to the α-carbon in α-azido ethers has been reported to reduce

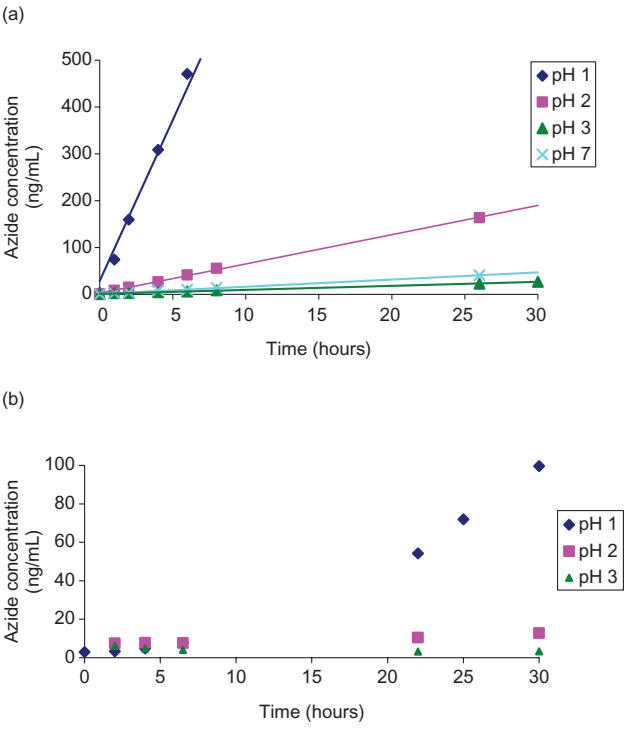

Figure 8. Change of azide concentration with time in solutions of R1479 (a) and balapiravir (b) at 37°C.

the solvolysis rate[23]. As the carbonyl group is a stronger electron withdrawing group than the hydroxyl group, the 4′-azido group in the ester prodrug, balapiravir, is assumed to be more stable than that in R1479. Furthermore, ester groups in balapiravir may provide steric hindrance and thereby reduce the rate of azide release.

HPLC method development

A HPLC method was developed to analyze balapiravir, R1479, mono- and diesters of R1479, and cytosine. Because of a wide range of polarity for these compounds from very polar for cytosine (log *P* –2.4) to fairly hydrophobic for balapiravir (log *P* 2.45), a gradient HPLC method with ion pair reagent, 1-heptane sulfonic acid, was developed to resolve all components. Figure 9 shows the HPLC chromatograms for stressed solutions of R1479 and balapiravir. All degradation products were well resolved except 3′,5′- and 2′,3′-diesters, which were partially resolved. The method is suitable for determining the degradation products as low as 0.02%.

Because a direct detection of azide by HPLC/UV is impractical due to its lack of chromophore and retention, 3,5-dinitrobenzoyl azide derivative was prepared by reaction of azide with 3,5-dinitrobenzoyl chloride (Figure 10) and then determined using a reversed phase HPLC method with UV detection at 230 nm[24]. The sample pH was controlled between pH values 4.0 and 5.5 to maintain

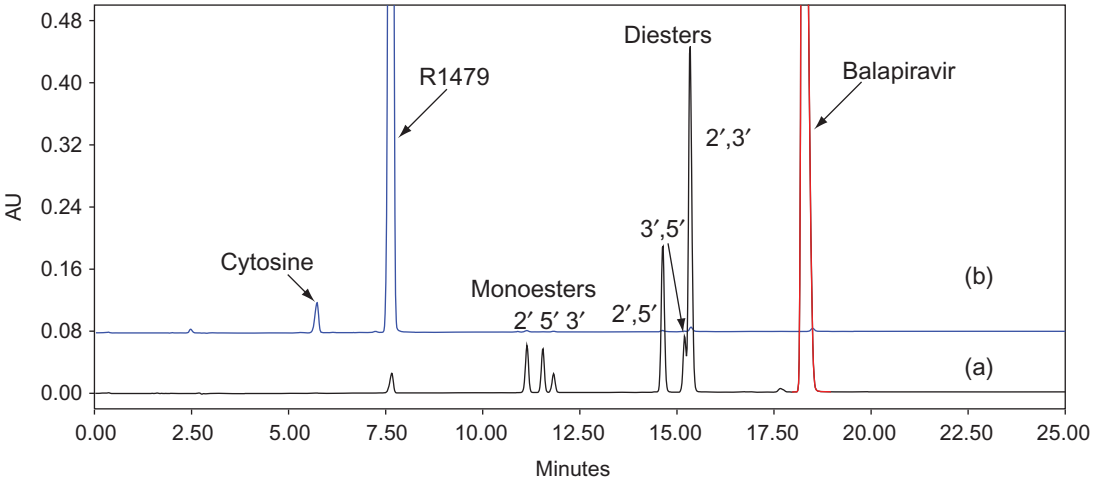

Figure 9. HPLC chromatograms of balapiravir (a) and R1479 (b) in pH 1 solution at 37°C after 8 hours.

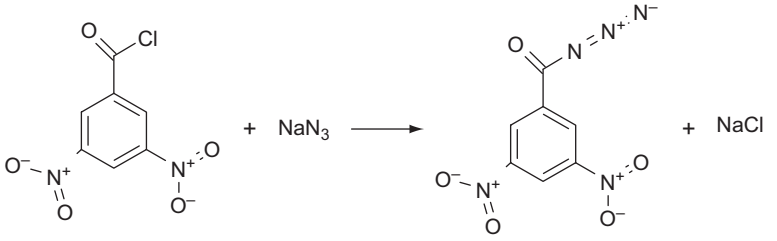

Figure 10. Reaction of azide with 3,5-dinitrobenzoyl chloride to form 3,5-dinitrobenzoyl azide.

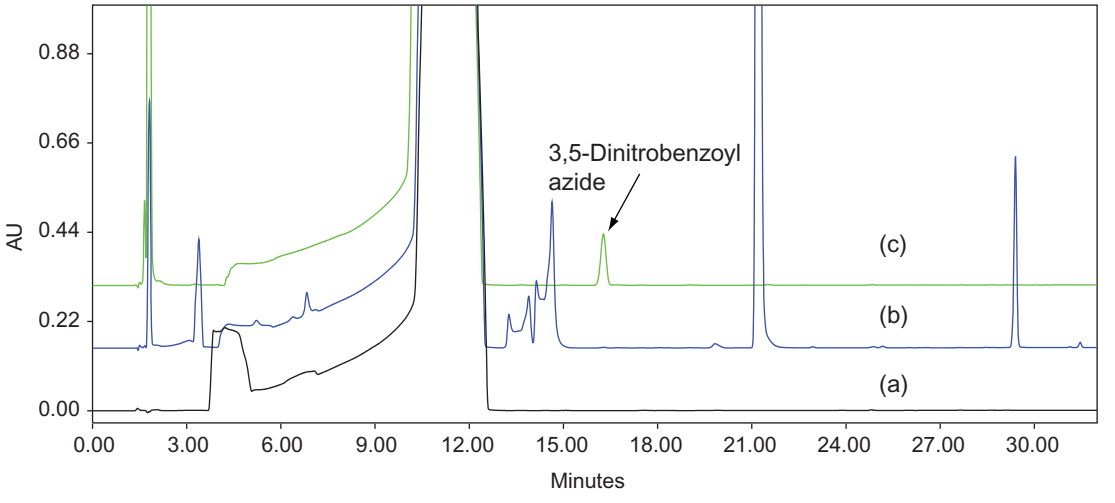

Figure 11. HPLC chromatograms for azide determination in reagent blank (a), balapiravir (b), and R1479 (c) in pH 1 solution at 37°C after 6 hours.

adequate stability of balapiravir as well as suitable derivatization conditions. Mobile phase conditions and HPLC column were optimized to separate 3,5-dinitrobenzoyl azide derivative from other components. Typical chromatograms are shown in Figure 11 for the azide determination in stressed R1479 and balapiravir solutions. The derivatization method has a limit of quantitation of 10 ng/mL[25].

Although the molar concentrations of cytosine and azide formed from R1479 and balapiravir are equivalent, the analytical method for azide determination is more

sensitive than that for cytosine. Therefore, direct measurement of azide is advantageous to study the release of azide from these 4′-azido-substituted nucleoside analogs.

Conclusion

Chemical stability of R1479 and its tri-isobutyrate ester prodrug, balapiravir, was studied in aqueous solutions. The degradation of both R1479 and balapiravir followed first-order kinetics and the formation of the degradation products followed zero-order kinetics when the initial rate method was used. Cytosine and azide are the major degradation products for R1479. Mono- and diester analogs and R1479 are the main degradation products for balapiravir, whereas cytosine and azide are minor degradation products. The azide formation from both R1479 and balapiravir is pH dependent. The release rate of azide is faster under acidic conditions than neutral conditions. The overall rate of azide release from balapiravir was significantly slower than that from R1479, possibly due to the presence of strong electron withdrawing carbonyl groups and steric hindrance of the ester groups in balapiravir. The results of this study suggest that the ester prodrugs of 4′-azido-substituted nucleoside analogs reduce the release of azide from anomeric centers and thereby enhance the stability in aqueous solutions.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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