

# Therapeutic Potential of Nitazoxanide: An Appropriate Choice for Repurposing versus SARS-CoV-2?

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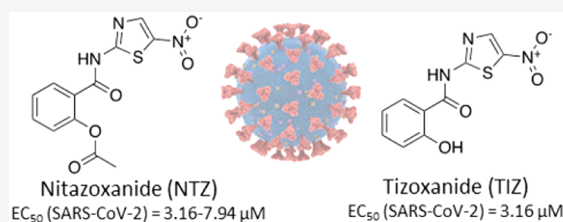


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**ABSTRACT:** The rapidly growing COVID-19 pandemic is the most serious global health crisis since the “Spanish flu” of 1918. There is currently no proven effective drug treatment or prophylaxis for this coronavirus infection. While developing safe and effective vaccines is one of the key focuses, a number of existing antiviral drugs are being evaluated for their potency and efficiency against SARS-CoV-2 *in vitro* and in the clinic. Here, we review the significant potential of nitazoxanide (NTZ) as an antiviral agent that can be repurposed as a treatment for COVID-19. Originally, NTZ was developed as an antiparasitic agent especially against *Cryptosporidium* spp.; it was later shown to possess potent activity against a broad range of both RNA and DNA viruses, including influenza A, hepatitis B and C, and coronaviruses. Recent *in vitro* assessment of NTZ has confirmed its promising activity against SARS-CoV-2 with an EC<sub>50</sub> of 2.12 μM. Here we examine its drug properties, antiviral activity against different viruses, clinical trials outcomes, and mechanisms of antiviral action from the literature in order to highlight the therapeutic potential for the treatment of COVID-19. Furthermore, in preliminary PK/PD analyses using clinical data reported in the literature, comparison of simulated TIZ (active metabolite of NTZ) exposures at two doses with the *in vitro* potency of NTZ against SARS-CoV-2 gives further support for drug repurposing with potential in combination chemotherapy approaches. The review concludes with details of second generation thiazolidines under development that could lead to improved antiviral therapies for future indications.



**KEYWORDS:** COVID-19, SARS-CoV-2, coronavirus, antiviral, pharmacokinetics, nitazoxanide, tizoxanide

## INTRODUCTION

When reports of a few cases of a new strain of pneumonia caused by an unknown pathogen were reported in Wuhan, a city of central China, in December 2019, there was little international concern.<sup>1</sup> A few weeks later almost 3000 cases, leading to 81 deaths, had already been recorded in China, and the disease had spread not merely to other areas of Asia but also to Europe, North America, and Australia.<sup>2</sup> By then the pathogen responsible for cases of pneumonia had been identified as a novel strain of coronavirus, which was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), that bears a close resemblance to the severe acute respiratory syndrome (SARS) virus responsible for outbreaks of disease in 2002–2003.<sup>3</sup> At the time of writing (late November 2020), over 53 million cases of the COVID-19 infection have been reported worldwide with over 1.35 million deaths.<sup>4</sup>

This rapidly growing pandemic is widely regarded as the most serious global health crisis since the “Spanish flu” of 1918. Currently, there is no proven effective small molecule treatment or prophylaxis for the disease although a number of them have been reviewed.<sup>5</sup> Many groups are actively searching for an effective vaccine and a number of them have entered late

stage (phase 3) clinical trials with some very positive results as of the end of November 2020.<sup>6,7</sup> Early small molecule screening reported by Wang<sup>8</sup> revealed several hit molecules *in vitro* with a focus on the known antiviral remdesivir (1)<sup>9,10</sup> and the 4-aminoquinoline antimalarial chloroquine (2),<sup>11,12</sup> which were the main focus of this seminal publication. Subsequently, multiple clinical trials have commenced with remdesivir, chloroquine, and the related hydroxychloroquine (3),<sup>13</sup> a drug used for autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (Figure 1).

In this review, we concentrate on the significant potential of nitazoxanide, 2-(acetyloxy)-N-(5-nitro-2-thiazolyl) benzamide (NTZ, 4), as a broad spectrum antiviral agent against COVID-19. NTZ, initially developed as an antiparasitic agent especially

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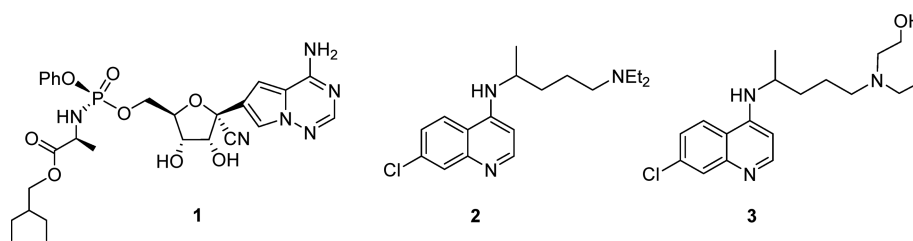


Figure 1. Structures of remdesivir (1), chloroquine (2), and hydroxychloroquine (3).

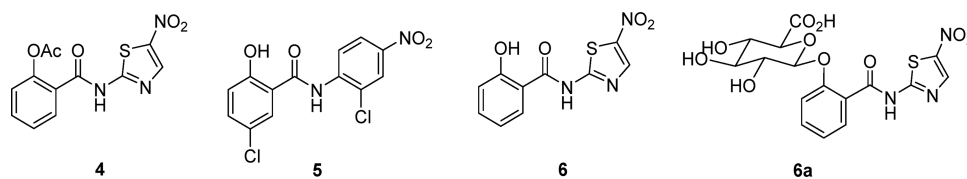


Figure 2. Structures of nitazoxanide (NTZ) (4), niclosamide (5), tizoxanide (TIZ) (6) and tizoxanide glucuronide (TG) (6a).

Table 1. Physicochemical Properties of NTZ and TIZ<sup>a</sup>

	HBD	HBA	pK <sub>a</sub> <sup>21,25</sup>	pK <sub>a</sub> (strongest acidic)	pK <sub>a</sub> (strongest basic)	LogP	LogD	LogS (pH 7.4)	human protein binding (% bound) <sup>15,30</sup>
NTZ	1	8	6.18	8.3	-4.2	2.322	2.322	2.601	
TIZ	2	7		7.81	-4.2	2.157	2.157	2.85	>99.9

<sup>a</sup>Values for HBD, HBA, LogP, LogD, and LogS were calculated or predicted by StarDrop (version 6.5). Values for pK<sub>a</sub> (strongest acidic) and pK<sub>a</sub> (strongest basic) were predicted by Chemaxon via DrugBank.<sup>20</sup>

against *Cryptosporidium* spp.,<sup>14</sup> was later shown to have broad-spectrum antiviral activity<sup>15,16</sup> including activity against influenza A (Figure 2). In a recent SARS-CoV-2 screening publication by Wang and co-workers, NTZ was somewhat overlooked despite expressing a similar antiviral EC<sub>50</sub> to chloroquine. Here, we summarize and analyze the current literature on NTZ, on which there is a large body of clinical data, and related thiazolides with a recommendation that trials of this agent either alone or in combination as a curative agent for COVID-19 are warranted.

## ■ AN APPROVED CRYPTOSPORIDIUM DRUG WITH BROAD ANTI-INFECTIVE SPECTRA

NTZ (4) was first synthesized in the early 1970s and is a prototype of a class of compounds known as the thiazolides.<sup>15,16</sup> Its structure is based on the scaffold of the anthelmintic niclosamide (5), which also displays broad spectrum antiviral activity including against coronaviruses (IC<sub>50</sub> of 0.28 μM versus SARS-CoV-2) (Figure 2).<sup>15,17,18</sup> *In vivo*, the active form of NTZ is known as tizoxanide (TIZ, 6) (*in vitro* against SARS CoV-2 NTZ and TIZ have similar potencies, *vide infra*) and together with niclosamide represent the potential of salicylamide derivatives to be developed as a class of compounds against SARS-CoV-2 (an overview of niclosamide is outside the scope of this review).<sup>19</sup>

The main indication of NTZ is as an oral antiparasitic agent, and it is registered in Latin America, Egypt, India, and Bangladesh for the treatment of intestinal protozoa and helminths. In addition to this, NTZ is FDA-approved in the US for the treatment of diarrhea caused by *Cryptosporidium parvum* and *Giardia intestinalis*.<sup>15</sup> Today it remains the only FDA-approved treatment for *Cryptosporidium* infections.<sup>15,16</sup> Currently, NTZ is available orally both as a tablet (500 mg) and in the form of a suspension (100 mg/5 mL) [Alinia-Romark] for the treatment of adults and pediatric patients, respectively.<sup>20,21</sup> In addition to NTZ's key role as an

antiparasitic, it has shown promising activity as a broad spectrum antibacterial and antiviral agent (*vide infra*).<sup>22</sup> Over the years, NTZ has been involved in more than 40 clinical trials across a broad range of drug targets, highlighting its multifunctional chemotherapeutic significance.<sup>20</sup>

Reports of NTZ's remarkable broad-spectrum antibacterial activity first began to appear in the 1990s.<sup>23</sup> Over the past two decades, numerous studies and clinical trials have confirmed its activity across a wide variety of both Gram-negative and Gram-positive anaerobic bacteria.<sup>15</sup> More recently, studies have revealed its activity against Gram-positive aerobic bacteria such as *Mycobacterium tuberculosis*, in addition to a number of Gram-negative aerobes.<sup>15,24</sup> There is great speculation surrounding the mechanisms of action of NTZ. Both its antiprotozoal and anaerobic antibacterial activity are believed to be as a result of its ability to inhibit pyruvate:ferredoxin oxidoreductase (PFOR), an enzyme essential for anaerobic energy metabolism.<sup>15,25</sup> In the case of aerobic bacteria there is strong evidence to suggest that NTZ acts as an uncoupler, disrupting membrane potential and intraorganism pH homeostasis.<sup>15,26</sup>

It was also in the late 1990s that the antiviral activity of NTZ was discovered by serendipity during its use in the treatment of AIDS patients who had developed cryptosporidiosis.<sup>27</sup> Since then reports have emerged confirming its activity against a broad range of both RNA and DNA viruses, including influenza A, influenza B, respiratory syncytial virus, parainfluenza, coronavirus, rotavirus, norovirus, hepatitis B, hepatitis C, dengue, yellow fever, Japanese encephalitis virus, and human immunodeficiency virus.<sup>15,28,29</sup> (see later section).

Overall, the oral bioavailability of the thiazolide class of compounds is generally quite poor due to low aqueous solubility.<sup>19</sup> NTZ has an aqueous solubility of 0.0075 mg/mL and an absolute oral bioavailability of just 3% in the rat.<sup>19–21</sup> As a prodrug, NTZ is partially absorbed from the gastrointestinal tract and rapidly hydrolyzed into the active form of

Table 2. Summary of EC<sub>50</sub>, IC<sub>50</sub>, and IC<sub>90</sub> Values for Key Indications of NTZ and TIZ

virus	strain	NTZ EC <sub>50</sub> or IC <sub>50</sub> (μM)	TIZ EC <sub>50</sub> or IC <sub>50</sub> (μM)	ref
rotavirus	SA-11	3.3	1.9	Rossignol et al. <sup>44</sup> La Frazia et al. <sup>46</sup>
	WA-G1P	6.5	3.8	La Frazia et al. <sup>46</sup>
hepatitis B	wild-type	0.12, <sup>a</sup> 0.59 <sup>b</sup>	0.15, <sup>a</sup> 0.46 <sup>b</sup>	Korba et al. <sup>48</sup>
hepatitis C	genotype 1a	0.33	0.25	Korba et al. <sup>48</sup>
	genotype 1b	0.21	0.15	Korba et al. <sup>48</sup>
	1a, 1b, 2a, 4a	2.5 to >10.1	2.7 to >8.8	Khan et al. <sup>49</sup>
influenza A	H1N1 A/PR/8/34	3.3	3.8	Rossignol et al. <sup>61</sup>
	H1N1 A/WSN/33	1.6	1.9	Rossignol et al. <sup>61</sup>
	H5N9 A/Ck/It/9097/97	3.3	1.9	Rossignol et al. <sup>61</sup>
	H3N2v (4 variants)	0.88–18.3 <sup>c</sup>		Sleeman et al. <sup>62</sup>
	H1N1 (54 variants)		0.13 <sup>d</sup>	Tilmanis et al. <sup>63</sup>
	H3N2 (53 variants)		0.16 <sup>d</sup>	Tilmanis et al. <sup>63</sup>
			0.18 <sup>d</sup>	Tilmanis et al. <sup>63</sup>
influenza B	Victoria lineage (47 variants)		0.16 <sup>d</sup>	Tilmanis et al. <sup>63</sup>
	Yamagata lineage (56 variants)		0.16 <sup>d</sup>	Tilmanis et al. <sup>63</sup>
coronavirus	CCov S-378	3.3		Rossignol <sup>15</sup>
	murine coronavirus	3.3		Cao et al. <sup>70</sup>
	MERS-CoV	3.0	3.1	Rossignol <sup>28</sup>
	SARS-CoV-2	2.12		Wang et al. <sup>8</sup>
	SARS-CoV-2	3.16–7.94	3.16	NIH <sup>71</sup>

<sup>a</sup>Extracellular virion DNA. <sup>b</sup>Intracellular HBV replication intermediates. <sup>c</sup>IC<sub>90</sub> value. <sup>d</sup>Median value across multiple variants.

the drug, tizoxanide (TIZ, 6) (Figure 2),<sup>30</sup> which is heavily protein bound (Table 1).<sup>15</sup> As a result, coadministration with other highly protein bound drugs, such as warfarin, may be a concern. A phase I study into the effect of NTZ on the pharmacodynamics and pharmacokinetics of a single dose of warfarin concluded it to be safe and well tolerated.<sup>31</sup> It is not fully understood as to how or why TIZ is so highly plasma protein bound, but it is believed to primarily bind to albumins due to being slightly acidic (Table 1).<sup>32</sup>

Over the past two decades NTZ has been subjected to extensive pharmacological testing for its safety in both animals and humans.<sup>15</sup> Its use has been evaluated in a number of clinical trials with only minor adverse effects such as diarrhea, abdominal pain, headache, and nausea being reported at low rates similar to placebo groups.<sup>33,34</sup> Additionally, *in vitro* metabolic studies have demonstrated that there is no significant inhibitory effect on cytochrome P450 (CYP450) enzymes; therefore no drug–drug interactions are expected.<sup>35</sup> Overall, NTZ has an established safety profile, with one-time oral doses of up to a remarkable 4 g shown to be safely tolerated in healthy adult volunteers.<sup>36</sup>

## ■ THE ANTIVIRAL SPECTRA AND ANTI-CORONAVIRUS ACTIVITIES OF NTZ: *IN VITRO*, *IN VIVO*, AND CLINICAL

As noted, NTZ and its active metabolite, TIZ, along with newer thiazolide analogues, have been shown to exhibit broad-spectrum activity against a wide range of viruses.<sup>15,37,38</sup> The most significant indications have been those against intestinal viruses, hepatitis, and influenza. Key *in vitro* and clinical data for NTZ against these indications is summarized in Table 2 and Table 3 respectively. Other reports indicate significant *in vitro* activity against a host of viral species as described later.<sup>39–43</sup>

The antiviral activity of NTZ was first reported against rotavirus by Rossignol et al. in 2006.<sup>44</sup> This study included *in vitro* assays of TIZ in cells infected with simian rotavirus SA-11. It was found that TIZ inhibited rotavirus replication with an EC<sub>50</sub> of 1.9 μM and that this effect was sustained with

increasing viral load (Table 2). Also reported was a randomized double-blind placebo-controlled trial of 50 children with rotavirus diarrhea who were treated with either NTZ or placebo. NTZ treatment was shown to decrease the median time to resolution of symptoms to 31 h, with no adverse effects reported, compared to 75 h for the placebo group (Table 3). Further clinical trials in adults demonstrated that NTZ treatment significantly reduced the median time to resolution of symptoms of both rotavirus and norovirus related gastroenteritis to 1.5 days, compared to 2.5 days for placebo (Table 3).<sup>45</sup> The clinical findings were corroborated by further *in vitro* studies against rotavirus,<sup>46</sup> showing that NTZ and TIZ inhibited viral replication in both the simian SA11-G3P rotavirus (EC<sub>50</sub> of 3.3 μM and 1.9 μM respectively) and human Wa-G1P rotavirus (EC<sub>50</sub> of 6.5 μM and 3.8 μM respectively). Both compounds also demonstrated significant cytoprotective effects in cells infected with rotavirus. Recent *in vitro* studies have also confirmed the dose-dependent activity of NTZ and TIZ against norovirus with no major cytotoxicity observed.<sup>47</sup>

NTZ and TIZ are also active against hepatitis B virus (HBV), inhibiting the production of both extracellular HBV DNA (EC<sub>50</sub> of 0.12 μM and 0.15 μM, respectively) and intracellular HBV replicons (EC<sub>50</sub> of 0.59 μM and 0.46 μM, respectively) with good selectivity and low cytotoxicity.<sup>48</sup> Similar results were obtained against replicating hepatitis C virus (HCV), with EC<sub>50</sub> values of 0.33 μM for NTZ and 0.25 μM against HCV genotype 1a.<sup>48</sup> For both HBV and HCV, antiviral activity was maintained across multiple genotypes (Table 2). These results have been corroborated in later screens along with novel thiazolide analogues.<sup>27,29</sup> However, in the case of HCV, other studies using alternative detection methods (qRT-PCR) have not consistently replicated this *in vitro* activity.<sup>49</sup> Further studies also demonstrated that both NTZ and TIZ possessed a high barrier to resistance by HCV.<sup>50,51</sup> A number of clinical trials have also been conducted, investigating the efficacy of NTZ treatment of both hepatitis B and C. Trials against hepatitis B have been somewhat limited, comprising case reports<sup>52</sup> and a small

Table 3. Summary of Clinical Trials Conducted with NTZ<sup>a</sup>

viral indication	phase	study population	dosing	main results	ref
rotavirus	II	38 <sup>b</sup>	7.5 mg/kg NTZ as an oral suspension	median time to resolution of symptoms: 31 h for NTZ treatment vs 75 h for placebo	Rossignol et al. <sup>44</sup> (NCT00302640)
rotavirus/norovirus	II	45	500 mg NTZ once daily	median time to resolution of symptoms: 1.5 days for NTZ treatment vs 2.5 days for placebo	Rossignol et al. <sup>45</sup>
hepatitis B	II	12	500 mg NTZ twice daily	negative serum HBV DNA levels in 9 of 12 patients; 3 of 4 patients initially HBsAg positive became HBsAg negative	Rossignol and Keefe <sup>53</sup>
hepatitis B	II	9	500 mg NTZ twice daily	negative serum HBV DNA levels in 8 of 9 patients, 2 patients initially HBsAg positive became HBsAg negative	Rossignol and Bréchet <sup>54</sup>
hepatitis B	II	48	600 mg NTZ once or twice daily, or 900 mg NTZ twice daily	NTZ vs placebo in patients undergoing treatment for chronic hepatitis B; ongoing	NCT03905655
hepatitis C (genotype 4)	II	50	500 mg NTZ twice daily or placebo	negative serum HCV RNA levels in 7 of 23 patients in NTZ group, 0 of 24 in placebo. Six of 7 responders achieved SVR <sup>c</sup>	Rossignol et al. <sup>55</sup> (NCT00418639)
hepatitis C (genotype 4)	II	96	500 mg NTZ twice daily + IFN + RBV or 500 mg NTZ twice daily + IFN	SVR <sup>c</sup> achieved in 79% of patients in NTZ + IFN + RBV group, 50% in IFN + RBV only	Rossignol et al. <sup>56</sup> (NCT00421434)
hepatitis C (multiple genotypes)	II	44	500 mg NTZ twice daily + IFN	SVR <sup>c</sup> achieved in 80% of patients treated with NTZ + IFN + RBV	Rossignol et al. <sup>57</sup> (NCT00763568)
hepatitis C (genotype 1)	II	64 <sup>d</sup>	500 mg NTZ twice daily + IFN + RBV	SVR <sup>c</sup> achieved in 7% of patients treated with NTZ + IFN + RBV, 0% for placebo + IFN + RBV	Shiffman et al. <sup>58</sup> (NCT00495391)
hepatitis C (genotype 1)	II	112	500 mg NTZ twice daily + IFN + RBV	SVR <sup>c</sup> achieved in 44% of patients treated with NTZ + IFN + RBV, 32% for placebo + IFN + RBV	Bacon et al. <sup>59</sup> (NCT00637923)
hepatitis C (genotype 4)	III	100	500 mg NTZ twice daily + IFN + RBV	SVR <sup>c</sup> achieved in 50% of patients treated with NTZ + IFN + RBV, 48% with IFN + RBV	Shehab et al. <sup>60</sup> (NCT01276756)
influenza (uncomplicated)	II/III	624	300 mg or 600 mg NTZ twice daily	median duration of symptoms: 95.5 h (600 mg NTZ), 109.1 h (300 mg NTZ), 116.7 h (placebo)	Hafizulla et al. <sup>68</sup> (NCT01227421)
multiple respiratory viruses	II	100 <sup>b</sup>	100–200 mg NTZ twice daily	median duration of symptoms: 4 days for NTZ treatment vs >7 days for placebo	Gamiño-Arroyo et al. <sup>69</sup>
multiple respiratory viruses	II	86	500 mg NTZ twice daily	median duration of symptoms: 4 days for NTZ treatment vs 7 days for placebo	Gamiño-Arroyo et al. <sup>69</sup>
multiple respiratory viruses	II	257 <sup>c</sup>	≥12 years: 600 mg NTZ twice daily, 4–11 years: 200 mg NTZ oral suspension twice daily, 1–3 years: 100 mg NTZ oral suspension twice daily	median duration of hospitalization: 6.5 days for NTZ + SOC <sup>e</sup> , 7.0 days for placebo + SOC <sup>e</sup>	Gamiño-Arroyo et al. <sup>69</sup> (NCT02057757)
influenza (uncomplicated)	III	1941	600 mg NTZ twice daily or 75 mg OST <sup>g</sup> twice daily or 600 mg NTZ and 75 mg OST twice daily	NTZ + OST vs NTZ and OST monotherapy; ongoing	NCT01610245
influenza (uncomplicated)	III	325	600 mg NTZ twice daily	NTZ vs placebo; ongoing	NCT02612922
influenza (uncomplicated)	III	1032	600 mg NTZ twice daily	NTZ vs placebo; ongoing	NCT03336619

D

<sup>a</sup>There are a number of newly registered clinical trials that included NTZ as a monotherapy or in combination with other agents against COVID-19; however, as most of these have not yet started or are only just starting enrolment, the details of these trials are not included in this table. Please see the later section for further details, such as the NCT identifiers of these trials. The usual adult dose of NTZ for diarrhea caused by *Cryptosporidium parvum* is 500 mg twice daily.<sup>72</sup> <sup>b</sup><12 years. <sup>c</sup>SVR defined as negative serum HCV RNA levels at 24 weeks after treatment end. <sup>d</sup>Patients previously nonresponsive to IFN+RBV therapy. <sup>e</sup>Hospitalized patients. <sup>f</sup>Standard of care (SOC) included fluid replacement therapy, supplemental oxygen, anti-influenza antivirals, and antibiotics, as determined by the treating physician. <sup>g</sup>OST = oseltamivir.



clinical trial<sup>53,54</sup> in adults with chronic hepatitis B (Table 3). Across the two clinical trials, reductions of serum HBV DNA to undetectable levels were observed in most patients (81% overall), as well as several patients who initially tested positive for hepatitis B e antigen (HBeAg) becoming HBeAg-negative (5 out of 6 patients total) over the treatment period. Furthermore, 29% of patients also tested negative for hepatitis B surface antigen (HBsAg) at the end of treatment (Table 3).

Additional trials are ongoing to assess coadministration of NTZ with existing regimens for treatment of chronic HBV (NCT03905655) (Table 3). Clinical trials against hepatitis C have demonstrated that NTZ has modest efficacy as a monotherapy at the doses at which it has been tested, achieving undetectable serum HCV RNA in 7 out of 23 patients in the treatment group compared to 0 of 24 in the placebo group.<sup>55</sup> Subsequent trials examined the effect of coadministration of NTZ with Peginterferon alfa-2a (IFN) and ribavirin (RBV) in various regimens in 96 patients with chronic hepatitis C.<sup>56,57</sup> The results indicated that NTZ coadministered with RBV and IFN increased the rate of rapid viral response (64%) and sustained viral response (79%) compared to RBV and IFN only (38% and 50%, respectively). Further, two large trials (176 patients) demonstrated an increase in sustained viral response for the combined treatment compared to placebo plus RBV and IFN (7% and 44% for combined treatment compared to 0% and 32% for placebo group).<sup>58,59</sup> However, later clinical trials have failed to replicate these results, reporting no benefit for the addition of NTZ to IFN and RBV for treatment of hepatitis C.<sup>60</sup>

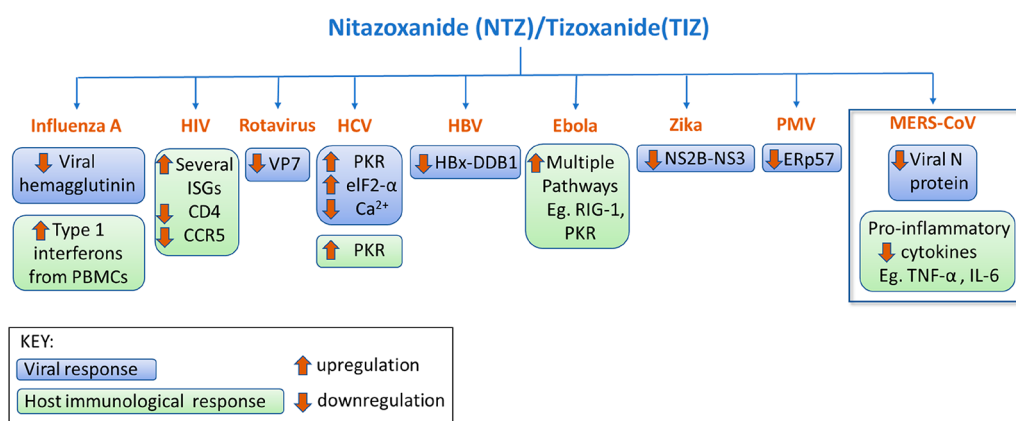
Influenza has become another important viral target for NTZ, with early studies reporting significant *in vitro* activity of both NTZ and TIZ against several strains of influenza A virus (IAV) including H1N1, H3N2, and H5N9 (Table 2).<sup>61</sup> This study demonstrated that NTZ and TIZ exhibited dose-dependent activity versus IAV in a variety of cell types, with EC<sub>50</sub> values of 1.7–5.7 μM (Table 2). NTZ was subsequently tested against a panel of H3N2 IAV variants circulating in the United States between 2011 and 2013,<sup>62</sup> reporting slightly higher activity with IC<sub>90</sub> values between 1.1 and 5.7 μM. These findings were expanded upon with a screen of 210 circulating IAV and influenza B virus (IBV) strains against TIZ.<sup>63</sup> The results were in line with previous studies, with median EC<sub>50</sub> values of 0.48 μM and 0.62 μM against H1N1 and H3N2 IAV strains, respectively, and 0.66 μM and 0.60 μM against Victoria lineage and Yamagata lineage IBV. Further *in vitro* studies showed this activity was also present against canine influenza virus with comparable potency (EC<sub>50</sub> = 1.63 μM and 1.89 μM for both NTZ and TIZ, respectively).<sup>64</sup> *In vitro* studies with oseltamivir and zanamivir have also demonstrated potential for use of NTZ as a combination therapy.<sup>65</sup> Once more, NTZ demonstrated significant activity against a number of strains of IAV (IC<sub>50</sub> values between 1.0 and 3.2 μM) as well as synergistic effects with both oseltamivir and zanamivir, compared to individual administration of each compound. NTZ has also emerged as one of 41 hits from a screen of 1280 compounds against IAV,<sup>66</sup> as well as being identified in a screen for potential protein disulfide isomerase inhibitors of IAV/IBV.<sup>67</sup> A number of clinical trials have been conducted for NTZ treatment of influenza based on its significant *in vitro* activity. A phase 2b/3 trial on 624 patients conducted in the United States found that individuals with uncomplicated influenza-like illness experienced a significant reduction in time to alleviation of symptoms at high doses (median reduction of

21.2 h at 600 mg/day, Table 3) compared to placebo, although the reduction at the lower dose (median reduction of 7.6 h at 300 mg/day) was not statistically significant.<sup>68</sup> Trials in children with influenza-like illness in Mexico (186 children across 2 studies) demonstrated that NTZ treatment reduced time to resolution of symptoms by 3 days.<sup>69</sup> This was followed by a trial in 257 patients who had been hospitalized with severe influenza-like illness (caused by a variety of viruses including influenza), which found no statistically significant reduction in duration of hospital stay associated with NTZ plus standard of care (including fluid replacement therapy, supplemental oxygen, antivirals, and antibiotics) compared to placebo plus standard of care.<sup>69</sup> A number of further large phase 3 trials are ongoing (NCT01610245, NCT02612922, NCT03336619) to further determine the role of NTZ in influenza therapy.

Early testing of the antiviral properties of NTZ and other analogues revealed high activity against canine coronavirus (CCov, IC<sub>50</sub> = 5.2–21.2 μM) among a wide range of other coronaviruses (Table 2).<sup>15,38</sup> Following the 2012 Middle East respiratory syndrome (MERS) outbreak, screening of the NIH clinical collection identified NTZ as a potent inhibitor of murine coronavirus (IC<sub>50</sub> = 3.3 μM, Table 2) and was recommended as a potential therapy for the disease.<sup>70</sup> Direct screening of TIZ and NTZ against MERS-CoV also showed high activity with IC<sub>50</sub> values of 3.1 μM and 3.0 μM, respectively.<sup>28</sup> Most recently, NTZ was also reported to be active against SAR-CoV-2 *in vitro* (see later section). In general, TIZ showed either equal or slightly higher potency than NTZ in most of the reported antiviral assays.

## ■ IN VITRO ACTIVITY OF NTZ AND ITS METABOLITES AGAINST SARS-COV-2

Only one published paper to date has been reported by Wang et al. for the *in vitro* activity of NTZ against SARS-CoV-2 with no data for the major metabolite TIZ.<sup>8</sup> The reported EC<sub>50</sub> was 2.12 μM (0.651 μg/mL) for NTZ against SARS-CoV-2 (BetaCoV/Wuhan/WIV04/2019) in Vero E6 cells. In another recent report,<sup>73</sup> after reanalysis of the data from the same study reported by Wang et al., the EC<sub>90</sub> of NTZ was estimated as 4.65 μM (1.43 μg/mL). It is well documented in the literature that NTZ is readily turned over to its active metabolite TIZ *in vivo* (NTZ *in vivo* t<sub>1/2</sub> ≤ 6 min) and NTZ is undetectable in plasma.<sup>30</sup> However, the turnover rates of NTZ to TIZ *in vitro* could vary significantly under different temperature and pH conditions, for example, the half-life of NTZ at 37 °C was 33 h at pH 5 and only 3 h at pH 8. Under the assay conditions, it is fair to say that the dynamic ratio of NTZ/TIZ within the reported assay could lead to a misinterpretation of the *in vitro* activity of either NTZ or TIZ against the virus *in vitro*. So, it is critically important that the active metabolite, TIZ, is assessed in any antiviral assay against SARS-CoV-2 to determine the potency unambiguously. Most recently, from a non-peer-reviewed source, the potency of TIZ against SARS-CoV-2 *in vitro* (EC<sub>50</sub> = 3.16 μM) was reported as similar to NTZ (EC<sub>50</sub> = 3.16–7.94 μM) under the same assay conditions.<sup>71</sup> These data again confirmed that both NTZ and TIZ are active against SARS-CoV-2 at a low micromolar range *in vitro* (we have evaluated the antiviral activity independently at Liverpool with EC<sub>50</sub> values of 5.05 μM and 4.07 μM for NTZ and TIZ, respectively, which ties in with literature observations (See Supporting Information, Figure S1). Furthermore, as described in the following section (Human PK analysis), tizoxanide glucuronide (TG) (Figures 2, 6a), the major metabolite of



**Figure 3.** Summary of viral modes of action of NTZ/TIZ.

TIZ, is another major circulating species metabolite after oral administration of NTZ in human; the *in vitro* activity of TG against the virus is yet another important factor to be considered when considering the overall antiviral efficacy of NTZ in humans but to date the activity of this metabolite has not been formally measured.

### MECHANISM OF ANTIVIRAL EFFECTS

With NTZ's demonstrated efficacy and clinical utility against a wide range of viral infections, its potential role in the treatment of SARS-CoV-2 has come into focus. Studies into the mode of action of NTZ within the different viral infections have identified significant upregulation of innate immune responses in many cases indicative of NTZ targeting one or more host factors. Specific effects on the virus have also been identified potentially due to secondary host immunomodulatory events or even direct drug action. NTZ's broad spectrum of antiviral activity is indicative of the former, but further investigations are required to definitively establish this (Figure 3). The variability of nitazoxanide's antiviral mode of action against different viruses indicates that this area requires further studies to clarify both mechanism and mode of action and to exclude nonspecific effects, such as those caused by pan-assay-interference (PAINS) properties.<sup>74,75</sup> While nitazoxanide has not been reported to act as an assay interference compound in the literature, is inert in chemical reactivity screens, and is not reported to be an aggregator,<sup>76</sup> the unusually broad spectrum of activity requires more definitive studies to define common and differential targets against its target viruses.

As noted, NTZ is also known to have antiparasitic activity against *Giardia lamblia* and *Cryptosporidium parvum*. NTZ is the mainstay of proven treatment against *Cryptosporidium* infections; however it is not effective in severely immunocompromised patients, further indicating NTZ's role in immunomodulation of the host to impart pathogen clearance.<sup>77</sup> While the focus of this review is NTZ's antiviral activity, this adds to the weight of evidence toward the immunomodulatory mode of action of NTZ. The disease specific information included below summarizes what is known within the literature, but it should be stressed that there is much that is still unknown.

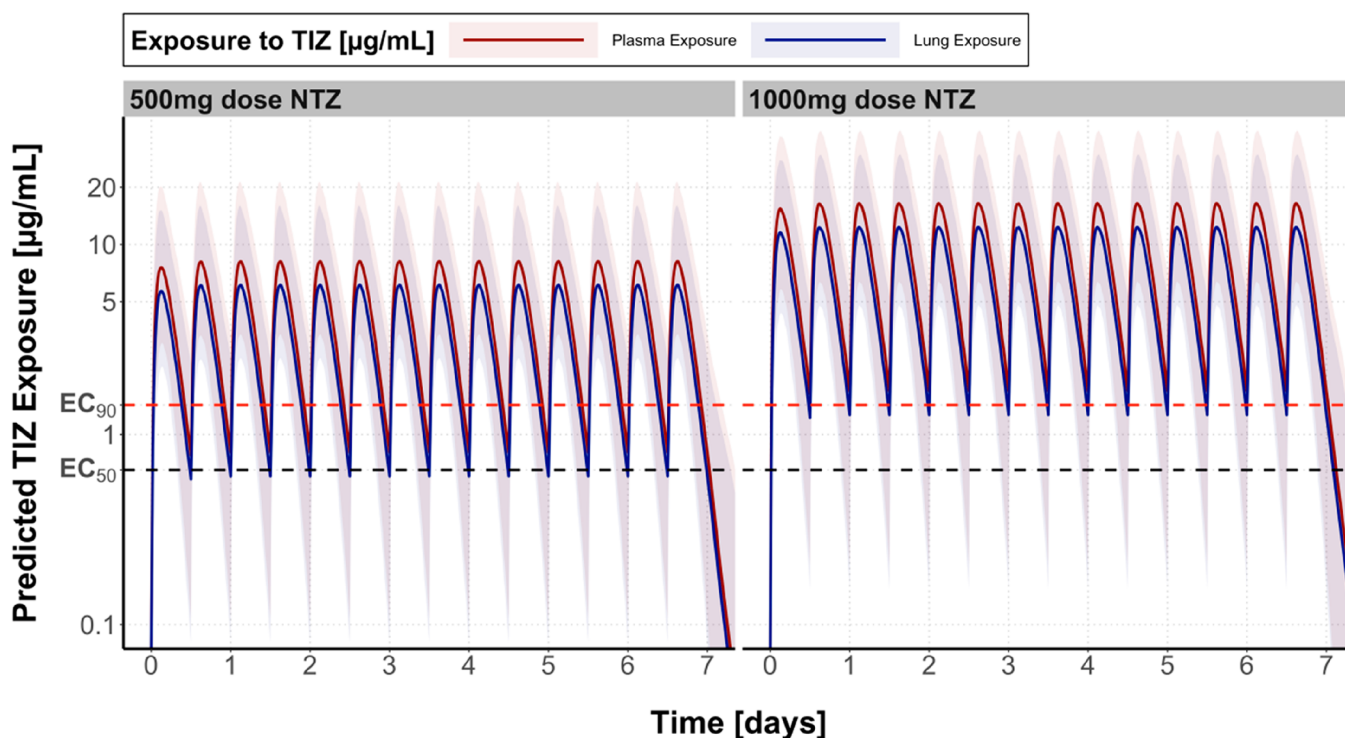
In the case of influenza A, it has been shown through *in vitro* cellular cultures (PR8-infected MDCK cells) that NTZ mediates reduced maturation of viral hemagglutinin at the post-translational stage after entry into the cell, between the endoplasmic reticulum and the Golgi apparatus.<sup>15,16</sup> Intracellular transport and insertion into host plasma membrane are

both impaired as a result. NTZ has been shown to have no effect on the other glycoprotein, neuraminidase, the target of oseltamivir and zanamivir, or the M2 protein, the target of amantadine, and it had no effect on viral infectivity, adsorption, or entry into target cells as determined by *in vitro* studies utilizing SA11-infected MA104 cells.<sup>46,61</sup>

NTZ also has effects in peripheral blood mononuclear cells (PBMCs). PBMCs from 10 healthy donors were cultured in the presence or absence of 3 different doses of TIZ (0.5, 1.0, and 10 mg/mL) in both unstimulated and flu-stimulated conditions and analyzed for T helper and cytotoxic T lymphocyte (CTL) activity as well as for toll-like receptor 7 (TLR7) and TLR8 expression and type I interferon (IFN) responses. NTZ potentiates the production of type 1 interferons (alpha and beta) produced by the host's fibroblasts.<sup>78</sup> The full significance of this is not clearly defined, but it may contribute to the antiviral activity of NTZ by interfering with maturation of the hemagglutinin glycoprotein as detailed above or as another secondary mechanism of action. Similar effects have been seen during *in vitro* HIV-1 studies utilizing PBMCs from 20 healthy donors infected *in vitro* with HIV-1<sub>BaL</sub>, where NTZ activates an innate immune response with the up-regulation of several interferon-stimulated genes (ISGs), including those involved in the cholesterol pathway, particularly the cholesterol-25 hydroxylase (CH25H). NTZ inhibition of HIV-1 replication *in vitro* could be due to its ability to stimulate potent and multifaceted antiviral immune responses.<sup>39,40,79</sup> NTZ has also been shown to decrease HIV-1 replication in monocyte-derived macrophages (MDMs) if present before or during HIV-1 infection. This NTZ effect is associated with downregulation of HIV-1 receptors CD4 and CCR5 and increasing gene expression of host cell anti-HIV resistance factors APOBEC3A/3G and tetherin.

TIZ has been studied to ascertain its mode of action in rotavirus. *In vitro* studies have demonstrated that TIZ inhibits the maturation of rotavirus viral protein 7 (VP7, a glycoprotein that forms the outer part of the virion and one of the six structural glycoproteins involved in rotavirus replication), alters viroplasm formation, and interferes with viral morphogenesis by hindering the interaction between the nonstructural proteins NSP5 and NSP2.<sup>46</sup>

With HCV in cell cultures, TIZ has been shown to activate protein kinase R (PKR). PKR plays an important role in the innate immune response. PKR is activated in cells exposed to double-stranded RNA, which subsequently results in phos-



**Figure 4.** Comparison of simulated TIZ exposures at two doses with the *in vitro* potency of NTZ against SARS-CoV-2. Simulated plasma (red) and lung (blue) exposures of TIZ after receiving 0.5 or 1.0 g, bid, dose of NTZ for 7 days. The dashed lines represent the *in vitro* EC<sub>50</sub> (0.641 µg/mL) and EC<sub>90</sub> (1.43 µg/mL) levels. Simulation was based on fitting data reported in Stockis et al.<sup>91</sup> in fed volunteers to a one-compartment metabolite formation model.

phorylation of eukaryotic initiation factor 2 $\alpha$  (eIF2- $\alpha$ ), a gene known to block viral replication.<sup>53,80,81</sup> TIZ therefore has the potential to boost intracellular host antiviral activity.

*In vitro* studies using bovine viral diarrhea virus (BVDV) in MDBK cells as a surrogate for HCV infection have shown that NTZ inhibits replication of cytopathic and noncytopathic BVDV by a mechanism that is likely to involve phosphorylation of PKR and eIF2- $\alpha$ . NTZ was also found to deplete ATP-sensitive intracellular Ca<sup>2+</sup> stores resulting in mild endoplasmic reticulum (ER) stress; this in turn disrupts N-linked glycosylation of BVDV structural proteins.<sup>82</sup> NTZ also has been found to inhibit the HBx–DDB1 protein interaction in HBV. Significant suppression of viral transcription and viral protein production in the HBV minicircle system and in human primary hepatocytes naturally infected with HBV was noted.<sup>83</sup> In addition, there are reports of NTZ and TIZ having potential utility in the treatment of Ebola through broad amplification of the host innate immune response to viruses and subsequent suppression of Ebola virus replication as demonstrated *in vitro* using multiple cell lines and genome editing techniques. From an immune response perspective, NTZ enhances multiple pathways including retinoic-acid-inducible protein 1 (RIG-I)-like-receptor, mitochondrial antiviral signaling protein, interferon regulatory factor 3, and interferon activities and induces transcription of the antiviral phosphatase GADD34. RIG-I plays a particularly important role in the identification of cells infected by intracellular pathogens and helps maintain host-cell integrity. NTZ significantly inhibits Ebola replication in human cells through its effects on RIG-I and PKR.<sup>84</sup>

Inhibition of the protease complex NS2B–NS3 is the target for NTZ in Zika virus as determined by an *in vitro* screen to

identify orthosteric inhibitors that directly target flavivirus NS2B–NS3 interactions. The complex plays an essential role during flaviviral polyprotein processing and thus represents an attractive drug target.<sup>85</sup> Within Paramyxoviridae (PMV, a large family of enveloped viruses including important human pathogens such as measles, mumps, and respiratory syncytial virus (RSV)) NTZ inhibits viral replication by targeting thiol oxidoreductase ERp57 involved in the fusion protein folding process as demonstrated using multiple *in vitro* techniques on monkey kidney (AGMK) cells infected with Sendai virus (SeV).<sup>86</sup>

More recently, studies have been undertaken into the role of NTZ and TIZ in the treatment of the Middle East respiratory syndrome coronavirus (MERS-CoV).<sup>28,70</sup> NTZ inhibits the production of pro-inflammatory cytokines TNF- $\alpha$ , IL-2, IL-4, IL-5, IL-6, IL-8, and IL-10 in peripheral blood mononuclear cells (PBMCs) and results in reduced viral protein accumulation in cells, possibly due to inhibition of viral N protein expression but further work is required to fully ascertain this. While not yet studied in humans (current studies have been completed in an *in vivo* mouse model and in mouse macrophages), this data suggests that NTZ could potentially improve outcomes in patients infected with MERS-CoV/SARS-CoV-2 by suppressing overproduction of pro-inflammatory cytokines, including IL-6.<sup>87</sup> Further studies are required to fully elucidate the mechanism of action of NTZ/TIZ against coronaviruses, but the current knowledge clearly orients NTZ toward a potential role in the treatment of SARS-CoV-2.



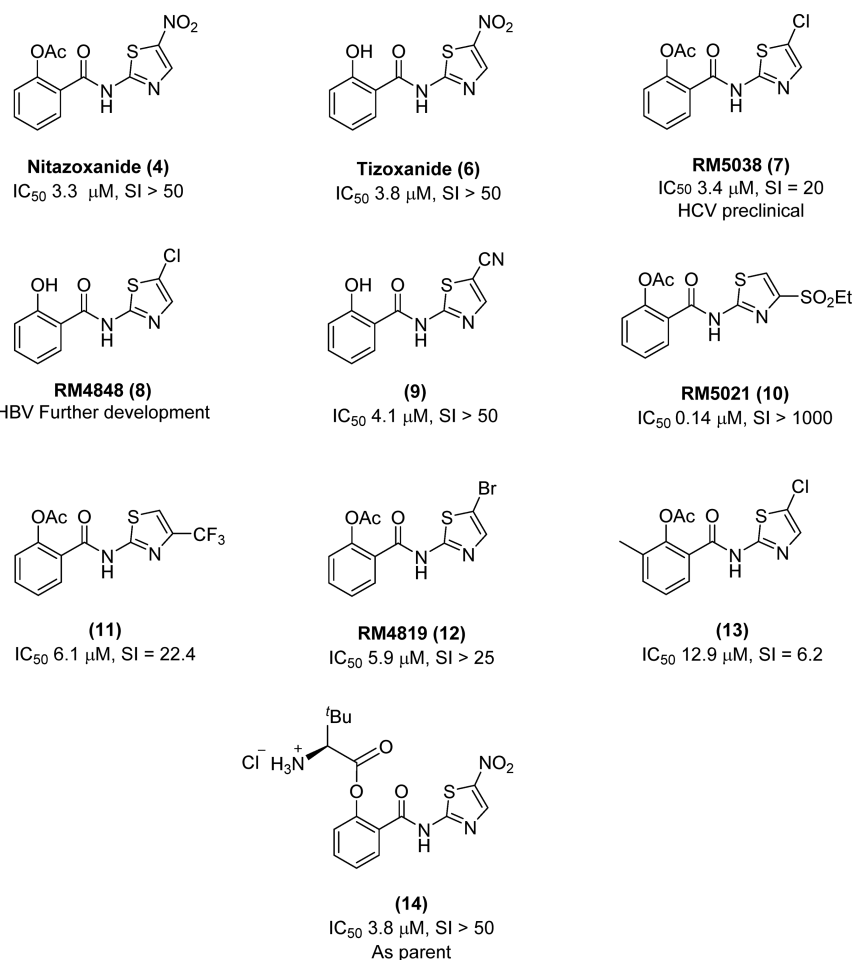


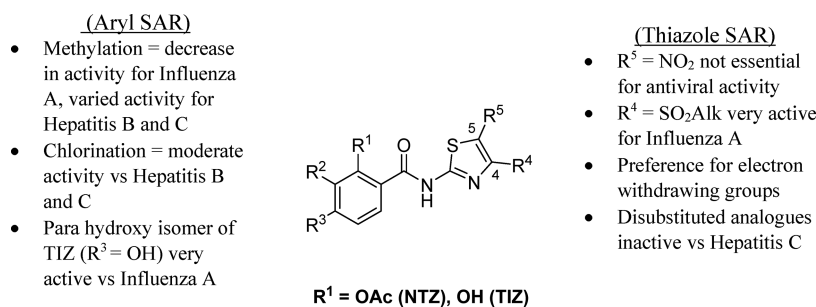
Figure 5. NTZ and various lead thiazolides: Antiviral activity of representatives vs influenza A virus.

## ■ NTZ PHARMACOKINETICS IN HUMAN AND PREDICTED ACCUMULATION IN LUNG

NTZ (500 mg, twice daily for 3 days) was approved for the treatment of diarrhea caused by *Giardia lamblia* or *Cryptosporidium parvum* in adult patients. In reported off-label uses for other indications, that is, *Clostridioides difficile* infection<sup>88</sup> or cryptosporidiosis-associated diarrhea in HIV-infected patients,<sup>89</sup> the dosage and regimen of NTZ was increased to up to 1000 mg twice daily for 14 days at most. There were a number of phase 1 clinical trials reported in the literature with extensive pharmacokinetic data in human. For example, in the paper by Stockis et al. published in 1996,<sup>90</sup> the first exploratory trial of a 500 mg single dose of NTZ in humans was described along with some basic pharmacokinetic findings of NTZ in humans, for example, the detectable species in plasma is its metabolite, TIZ. Thus, all reported PK parameters after single 500 mg oral dosing of NTZ, such as  $C_{\max}$  (1.9 mg/L),  $T_{\max}$  (2–6 h), AUC (3.9–11.3 mg·h/L), and terminal  $T_{1/2}$  (1.03–1.6 h) are based on TIZ concentrations in plasma. Once formed, TIZ primarily undergoes glucuronidation within the liver to form tizoxanide glucuronide (6a), which is excreted in the urine and bile; TIZ itself is also found to be eliminated from the body via the urine, bile, and feces.<sup>30</sup> In 2002, two back-to-back publications both authored by Stockis and co-workers reported the findings from a single ascending dose (SAD) trial (1, 2, 3, and 4 g of NTZ dosed orally) and a multiple ascending dose (MAD) trial (0.5 and 1

g, bid, for 7 days oral dosing of NTZ).<sup>36,91</sup> All dosages (up to 4 g in a single dose) and one multiple dosing regimen (0.5 g, bid for 7 days) were well tolerated with only mild adverse events. There was increased frequency of GI side effects reported in the 1 g bid, 7 days dosing group, but no significant changes to other parameters, that is, ECGs, vital signs, and laboratory tests. From the SAD trial, it was reported that (1) plasma concentrations of both major metabolites (TIZ and TG) increased largely in a linear correlation with the dose between 1 and 4 g and (2) the food effect is significant, approximately doubling the bioavailability and the concentrations of both metabolites in all dosing groups. At the highest (4 g) dosing with food, the two metabolites, TIZ and TG, could reach  $C_{\max}$  of 70 μg/mL (TIZ) and 59.6 μg/mL (TG) and AUC of 768 μg·h/mL (T) and 832 μg·h/mL (TG), although it was also noted that at this dosing level the  $T_{\max}$  was delayed and the apparent elimination rate was noticeably lower than in the lower dosage level, particularly for the glucuronide metabolite. From the MAD trial, it was observed that at 0.5 g bid dosing level, the measured pharmacokinetic parameters of both metabolites were similar to the single dosing at the same level; on the other hand, at 1 g bid dosing, the bioavailability of both metabolites was increased noticeably (50–70%), compared with single dosing, which indicated significant accumulation at repeat dosing at this level. Also, the report data obtained from the MAD study gave further evidence of the steady state of the exposure level of both metabolites,





**Figure 6.** Antiviral SAR of NTZ/TIZ against influenza A, hepatitis B, and hepatitis C.

which can be used in human PK modeling to provide a further insight into PK profiles of NTZ at different dosing levels.

Using the pharmacokinetic data available in the public domain, it is feasible to simulate the exposure of TIZ at two dosing levels (0.5 and 1 g, bid) for 7 days (Figure 4). However, there is very limited information available concerning the partition of the major metabolites between systemic circulation and specific organs, such as the lung. As a major site of infection for SARS-CoV-2, the concentrations of the major metabolites in the lung after oral dosing of NTZ should be taken into consideration as a major contributing factor in the prediction of the antiviral efficacy for the treatment or prevention of COVID-19. In a recently published report,<sup>92</sup> a PBPK model was used to predict the partition ratios between plasma and lung for a number of repurposing candidate drugs for COVID-19. In combination of the simulation of repeat dosing NTZ and the predicted lung/plasma partition ratio, we can estimate the lung concentration of TIZ in comparison to the *in vitro* potency of TIZ against SARS-CoV-2 at the standard dosage (0.5 g, bid) and the elevated off-label dosage (1.0 g, bid) as repeated doses for 7 days (Figure 4). While at the highest off-label dose (1.0 g, bid) the simulated TIZ exposure in both plasma and the lung can be maintained above the *in vitro*  $\text{EC}_{90}$  for the majority of the dosing period. This preliminary PK/PD analysis provides some evidence to support the repurposing of NTZ for the treatment of COVID-19. Ongoing animal PK studies at Liverpool are aiming to define the pulmonary concentrations of nitazoxanide after oral administration. Furthermore, based upon our dose predictions for nitazoxanide,<sup>85</sup> a trial involving a 1500 mg bid dose of nitazoxanide has received funding from Unitaid and approval from the independent scientific advisory board for evaluation within a phase I/IIa study in the COVID19 platform trial known as AGILE ([www.agiletrial.net](http://www.agiletrial.net)).

A number of registered clinical trials (Please refer to Table 3 for a list of NTZ and COVID-19 related trials.)<sup>93</sup> in several countries (e.g., the U.S., Brazil, Mexico, South Africa, India and Egypt etc.) related to NTZ will provide further evidence to support the nitazoxanide repurposing effort; however, further detailed investigation through PK/PD modeling, including provision of *in vitro* activity of TIZ and its glucuronide against SARS-CoV-2 in the most physiological relevant conditions (selection of host cell line, assay media, and multiplicity of infection, etc., could significantly affect the outcome of *in vitro* antiviral activity for the same drug; this was observed and analyzed in some recent reports<sup>73</sup> concerning the *in vitro* screening of SARS-CoV-2) and simulation of the concentrations of both metabolites in the lung at different dosages and regimen will provide critical information to guide better design of clinical trials and understanding of trial results.

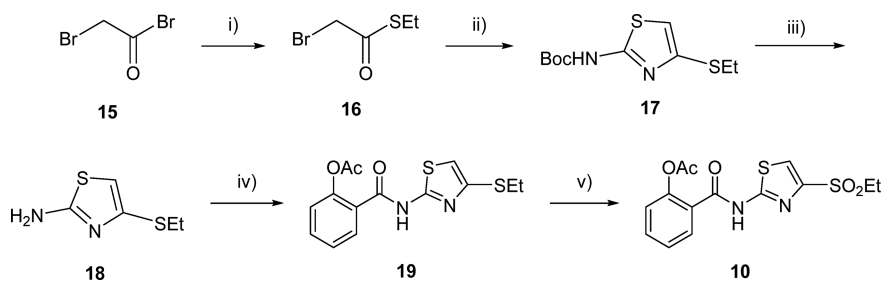
## ■ THIAZOLIDINE ANALOGUES: BRIEF OVERVIEW OF LITERATURE EXAMPLES AND ANTIVIRAL SAR

With NTZ/TIZ demonstrating clear potential in treatment of SARS-CoV-2, it is also important to consider the multiple thiazolidine analogues that are present within the literature as they could facilitate improved SARS-CoV-2 therapy.

Figure 5 depicts antiviral potency of various thiazolidines versus the H1N1 strain of influenza A virus.<sup>16</sup> Both NTZ and RM4848 (8) are being evaluated versus HBV, the former is in clinical trials, and the latter is in preclinical development. In general, the  $\text{IC}_{50}$  values for the parent prodrug acetates, for example, NTZ (4), RM5038 (7), and RM5021 (10), are similar to their phenolic metabolites. This includes other prodrug variants such as the *tert*-butyl amino prodrug (14) listed in Figure 5. In terms of development status, RM5038 and RM5021 are currently being evaluated in animal preclinical studies.

There are no reported SAR studies on analogues of nitazoxanide where an attempt is made to tease out direct antiviral versus host mediated effects in *in vitro* assays by traditional medicinal chemistry, and so moving forward phenotypic screening will remain key to expanding SAR in this field. For potential future lead optimization chemistry versus coronaviruses, SAR trends across other viruses provide valuable informative data on starting points. With this in mind, we have examined available data as a guide for future thiazolidine lead optimization campaigns. Currently, the SAR for NTZ/TIZ's antiviral activity is relatively limited and is summarized in Figure 6 (with representatives included in Figure 5). Functionalization of both the thiazole and phenyl rings has been performed against hepatitis B, hepatitis C, and influenza A.<sup>16,27,29</sup> One significant difference between NTZ's antiviral versus its antibacterial activity is the importance of the nitro group attached to the C5 position of the thiazole ring. Previous SAR studies into NTZ's anaerobic antibacterial activity have shown the nitro group to be essential for activity, with deletion or modification completely abolishing activity.<sup>94–96</sup> One study demonstrated the importance of the nitro group being a result of its ability to be reduced into a hydroxylamine, forming a key intermediate in its mechanism of action against anaerobes.<sup>94</sup> Moreover, previous work evaluating the SAR of NTZ against *Mycobacterium tuberculosis* has also shown that removal of the nitro group results in inactivity.<sup>24</sup>

Conversely, a number of thiazolidine analogues (Figure 5) have shown the nitro group to not be essential for antiviral activity. In particular, direct replacement of the nitro group with other electron withdrawing substituents has provided analogues with good antiviral activity. That the nitro group is not essential for antiviral activity is significant since it rules out a redox based mechanism of action (bioactivation by reduction

Scheme 1. Synthesis of a 4'-(Ethylsulfonyl)thiazole<sup>a</sup>

<sup>a</sup>Conditions and yields (i) NaSEt, Et<sub>2</sub>O, 86%; (ii) BocNHCSNH<sub>2</sub> **10**, <sup>i</sup>PrOH, 4 Å MS, 55%; (iii) dil. TFA, CH<sub>2</sub>Cl<sub>2</sub>, then aq. NaHCO<sub>3</sub>, extract with Et<sub>2</sub>O, 96%; (iv) *O*-acetylsalicyloyl chloride, CH<sub>2</sub>Cl<sub>2</sub>, NMM, 81%; (v) mCPBA (2.2 equiv), CH<sub>2</sub>Cl<sub>2</sub>, 95%.

and redox cycling) and increases the drug-like properties of the thiazolidine analogue. While nitazoxanide has been shown to be a well-tolerated drug clinically, it is well evidenced that drugs containing nitro groups can induce severe toxicity with the nitro aromatic or heterocycle being classed as a metabolic alert. Thus, analogues removing this liability while maintaining potency will have a clear potential advantage.<sup>97</sup>

Overall, the antiviral SAR work carried out so far against hepatitis B, hepatitis C, and influenza A has shown a clear preference for electron withdrawing groups attached to not only the C5 but also the C4 position of the thiazole ring.<sup>16,27,29</sup> As shown in Figure 5, a significant improvement in influenza A activity can be achieved with the incorporation of alkylsulfone groups at the C4 position.<sup>16</sup> Only two 4',5'-disubstituted analogues have been reported so far (4'-Me/5'-Br and 4'-Ph/5'-Br), however both proved to be inactive against hepatitis C.<sup>29</sup>

In addition to investigation of the thiazole ring, substitution on the phenyl ring also has been of interest in determining the overall antiviral SAR of NTZ/TIZ analogues. Methylation at the different positions around the phenyl ring typically causes a decrease in antiviral activity against influenza A.<sup>16</sup> However, for hepatitis B and C, methylation around the phenyl ring has a much more varied effect on antiviral activity. Halogenation of the phenyl ring has also been explored for hepatitis B and C. Chlorination of TIZ and the 5'-Br analogue results in moderate activity.<sup>27,29</sup> Interestingly for influenza A, the para hydroxy isomer of TIZ shows a 10-fold increase in activity.<sup>16</sup> The free phenol, or its precursor the acetate (or other prodrug ester), is essential for activity. Even methylation to the *O*-methyl ether virtually abolishes all activity.<sup>98</sup> It does appear that a free NH and a free OH are both required for active thiazolidines.

## SYNTHETIC CHEMISTRY OF NITAZOXANIDE, PRODRUGS, AND IMPROVED THIAZOLIDES

One of the attractive features of NTZ and other thiazolidines is that a general synthesis is available for a wide range of analogues starting from commercially available *O*-acetylsalicyloyl chloride by reaction with an appropriate 2-amino-5-substituted thiazole. In the case of NTZ **4** itself, the use of Et<sub>3</sub>N as base and THF as solvent is necessary; 2-amino-5-nitrothiazole is poorly soluble and a weak nucleophile. In cases where the aminothiazole is a better nucleophile, a two-phase procedure, viz. CH<sub>2</sub>Cl<sub>2</sub> or EtOAc/aq. NaHCO<sub>3</sub>, may be a good and simple alternative. This is effective for the HCl salt of 2-amino-5-chlorothiazole, leading to the 5-Cl analogue **7** in high yield. The Liverpool group studied many coupling

methods, for example, HATU and other uronium reagents, which we have summarized previously.<sup>27,29</sup>

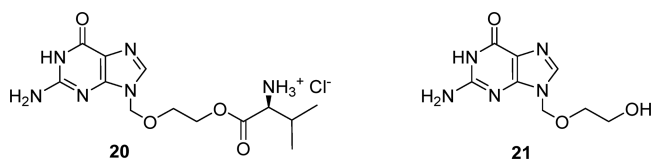
A related series of molecules are 4'-substituted thiazolidines. In general, 2-amino-4-X-thiazoles, with the exception of X = Ph or Br/Cl, are not readily available, and multistep syntheses may be required to access the corresponding thiazolidines. An important example is the 4'-ethylsulfonyl thiazolidine **10**, which showed outstanding activity against an H1N1 strain of influenza A virus, IC<sub>50</sub> = 0.14 μM.<sup>16</sup> This analogue was synthesized by a five-step route from bromoacetyl bromide **15** (Scheme 1).<sup>99</sup>

As noted, an important point when considering the potential of NTZ as an antiviral is that it behaves *in vivo* as a prodrug for the free phenol, TIZ (**6**); the acetate ester is readily cleaved by esterases in the blood.<sup>36</sup> A number of alternative acyl groups have been studied, but none showed a significant advantage over **4**.<sup>27</sup> If TIZ **6** itself is needed, the acetate in **4** may be cleaved using mild acid (aq. HCl, 60 °C) or base (aq. NH<sub>3</sub>, THF, 20 °C then HCl to pH 1). In regards to biological screening, for any particular thiazolidine, the acetate and free phenol are essentially equiactive. The free phenol is invariably the active circulating metabolite *in vivo*.

The human metabolism of thiazolidines has been studied in detail only for NTZ **4** and RM5038 **7**. Once the ester has been cleaved, the majority of the circulating dose is cleared as the *O*-glucuronide; *O*-sulfation is only a minor pathway. Compounds **4** and **7** are not significantly bioactivated by a panel of CYPs; it appears that the electron-withdrawing substituent at C(5) prevents oxidative metabolism.<sup>30</sup> It has yet to be determined whether a 4'-electron withdrawing substituent as in **10** is equally effective. By contrast, 4,5-unsubstituted thiazoles are typically CYP-oxidized leading to toxic thiourea metabolites.<sup>100,101</sup>

The solubilities of the thiazolidines, especially as the free phenols, are not optimal for good systemic absorption for the treatment of respiratory viruses. One widely used method of improving the oral absorption of a number of drugs is the use of a pro-drug ester. A relevant example is the valine derivative valcyclovir **20**,<sup>102,103</sup> which increases the oral absorption of its parent, the antiviral agent acyclovir, **21** from 20% to 54%. A similar amino-acid derivative of TIZ (**14**) (Figure 5), expected to show superior solubility and bioavailability to the acetate esters NTZ **4** and **7**, has been prepared and is currently undergoing investigation.<sup>19</sup>

While it appears that thiazolidines discussed in this review (e.g. NTZ and RM5038/4848) can both affect terminal glycosylation and transport of viral hemagglutinin to the cell plasma-membrane, additional studies on analogues have not been performed to correlate this activity with measured



antiviral effect in culture, and this will be important in future target validation studies. Another aspect of research that should follow would be to examine the propensity of an analogue series to affect key host factors. Very recent studies have demonstrated that NTZ inhibits host ERp57 activity, causing newly synthesized F-protein misfolding and F-aggregate formation and halting F-trafficking to the host plasma membrane. Additional studies in multiple viruses implicate additional host targets, but to date there are no SARs to relate drug structure to function in these host mediated effects; again this should be the subject of additional research.

## CONCLUSIONS

NTZ has a broad spectrum of anti-infective activity mediated through modulation of host innate immune responses as well as direct activity against multiple viral targets. NTZ has been shown to improve clinical outcome when used against multiple respiratory viruses and has been shown to have *in vitro* activity against the coronaviruses, including SARS-CoV-2. This, combined with its ease of synthesis, established human pharmacokinetics, favorable preliminary PK/PD simulations of plasma and lung accumulation, and extensive pharmacological safety testing in both animals and humans highlights the therapeutic potential of NTZ.

While there are still questions to be answered with respect to the mode of action of NTZ within SARS-CoV-2, the utility of thiazolidine analogues and pro-drugs, and more detailed PK/PD modeling in order to inform further clinical trials, NTZ and its second generation thiazolidines without doubt have the potential to be appropriate choices for repurposing versus SARS-CoV-2 either as monotherapy or as part of combinations. With combination therapy in mind Bobrowski and co-workers have completed a very recent *in silico* guided *in vitro* analysis of possible drug combinations for SARS CoV-2.<sup>104</sup> From the 16 synergistic cases, combinations of nitazoxanide with three other compounds (remdesivir, amodiaquine, and umifenovir) were the most notable, all exhibiting significant synergy against SARS-CoV-2. Based on this observation, it would seem logical to consider clinical trials of remdesivir with nitazoxanide given the FDA approval of the former for use in COVID-19 infection. Using our online drug–drug interactions tool, we would not expect any significant drug–drug interactions between remdesivir and nitazoxanide.<sup>105</sup> Clearly, to realize the full potential of NTZ against COVID-19, further assessments of safety and efficacy at current and elevated doses should be investigated in ongoing and future clinical trials.<sup>106,107</sup>

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscinfecdis.0c00478>.

Methods of SARS-CoV-2 antiviral determination, dose response curves for nitazoxanide and tizoxanide against

SARS-CoV-2 *in vitro*, summary of clinical trials conducted with NTZ (PDF)

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### Notes

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