Zanamivir Amidoxime- and *N*-Hydroxyguanidine-Based Prodrug Approaches to Tackle Poor Oral Bioavailability

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ABSTRACT: The neuraminidase (NA) inhibitor zanamivir (**1**) is potently active against a broad panel of influenza A and B strains, including mutant viruses, but suffers from pharmacokinetic (PK) shortcomings. Here, distinct prodrug approaches are described that aimed at overcoming zanamivir's lack of oral bioavailability. Lowering the high basicity of the 4-guanidino group in zanamivir and of a bioisosteric 4-acetamidine analog (**5**) by *N*-hydroxylation was deemed to be a plausible tactic. The carboxylic acid and glycerol side chain were also masked with different ester groups. The bioisosteric amidine **5** turned out to be potently active against a panel of H1N1 (IC_{50} = 2–10 nM) and H3N2 (IC_{50} = 5–10 nM) influenza A viruses (NA inhibition assay). *In vitro* PK studies showed that all prodrugs were highly soluble, exhibited low protein binding, and were bioactivated by *N*-reduction to the respective guanidines and amidines. The most promising prodrug candidates, amidoxime ester **7** and *N*-hydroxyguanidine ester **8**, were subjected to *in vivo* bioavailability studies. Unfortunately, both prodrugs were not orally bioavailable to a convincing degree ($F \leq 3.7\%$, rats). This finding questions the general feasibility of improving the oral bioavailability of **1** by lipophilicity-increasing prodrug strategies, and suggests that intrinsic structural features represent key hurdles. J Pharm Sci 104:3208–3219, 2015
Keywords: influenza A; neuraminidase inhibitor; synthesis; drug-like properties; prodrug; pharmacokinetics; bioavailability; guanidine bioisoster; amidine

INTRODUCTION

Seasonal influenza infections account for diseases of 3–5 million people and 250,000 to 500,000 deaths worldwide caused either directly by the virus or as a consequence of secondary infections.[1] The last—fortunately rather harmless—pandemic occurred in 2009 (H1N1, "swine flu"), but in recent years, the highly pathogenic avian influenza viruses of subtype H5N1 (i.e., "bird flu", 2005) and H7N9 have been alarming as they can be transmitted from animals to humans.[2–5] Another major concern is the high mutagenic rate of the viruses, which contributes to the rapid development of resistances. For example, flu season 2007/2008 generated an oseltamivir-resistant H1N1 virus that spread globally and carried a H274Y mutation.[6] The sporadic emergence of virus strains with resistance-conferring mutations highlights the urgent need for new therapeutic agents.[7]

To date, approved drugs for the treatment or prophylaxis of influenza virus infections belong to the classes of adamantane-based M2 ion channel protein inhibitors and inhibitors of the viral surface protein neuraminidase (NA), which is a key enzyme in the life cycle of the virus and allows for its replication and spread.[8,9] As adamantane type of compounds are only effective against influenza A, suffer from several side effects and drug resistance,[8,9] current clinical practice mainly makes use of NA inhibitors (NAIs). Zanamivir (**1,** (Fig.1) represents the first marketed NAI (in 1999) and is approved for inhalative and intravenous administration.[10] Laninamivir (**2**) was introduced later as a follow-up that only differs from zanamivir with a methoxy group in the glycerol side chain, and has been approved in Japan.[11] In the form of its octanoate prodrug (CS-8958), laninamivir represents a long-acting NAI that requires only a single daily inhaled dose. This strategy emphasizes the consciousness of and necessity for the development of patient-compliant formulations of anti-flu agents.[12] In fact, oseltamivir (**3**) is the first and only orally available NAI and because of this represents the first choice NAI, stockpiled by most countries in case of a new pandemic.[13,14] Finally, peramivir (**4**) was available from 2009 to 2010 for in-patient emergency treatment of severe forms of influenza infections, despite not being approved at that time.[15–17] In 2014, it was approved as the third NAI by the United States Food and Drug Administration (US FDA) for the treatment of influenza infections (US FDA press announcement Dec/2014).

Notably, all clinically used NAIs suffer from distinct drawbacks. As indicated above, therapeutic treatment with zanamivir, laninamivir, and peramivir is restricted to inhalative or intravenous application, respectively.[18,19] Only oseltamivir is orally bioavailable but for this NAI many resistances have emerged. We recently reported on an attempt to overcome both issues at the same time, that is, breaking oseltamivir resistance while keeping oral bioavailability.[20] Because 5-guanidino substituted oseltamivir derivatives were significantly more potent and also active against oseltamivir-resistant virus strains,[10,13] we reasoned that bioisosteric replacement of this group by a 5-amidino functionality provides

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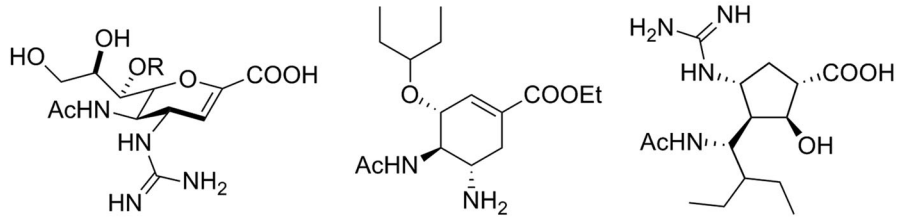

Zanamivir (**1**, R = H)
Ianinamivir (**2**, R = Me)

Oseltamivir (3)

Peramivir (4)

Figure 1. Chemical structures of clinically used neuraminidase inhibitors (NAIs).

access to similarly active analogs that are amenable to specific prodrug concepts. Indeed, the oseltamivir amidine turned out potently active against a large panel of H1N1 and H3N2 influenza A virus strains and effective against an oseltamivir-resistant A/H1N1 mutant. Strikingly, a simple amidoxime prodrug strategy for this compound enabled an oral bioavailability (31%, rats) that was comparable to oseltamivir (36%, rats).[20]

Moreover, several distinct prodrug approaches have been attempted to overcome pharmacokinetic (PK) issues of zanamivir and the more potent oseltamivir guanidine, including intra- and intermolecular ion pairing strategies or targeting of active transporters with specific amino acid conjugates.[21,22] In particular, the latter concept—that has been proposed by the Amidon group—showed some success as the oral bioavailability of oseltamivir guanidine could be increased to 23% (mice, fed state) and 48% (mice, fasted state) with a carrier-mediated L-valinate prodrug.[23] The same concept was realized for zanamivir. Amidon group could demonstrate at least *in vitro* a fourfold higher uptake of a zanamivir L-valine prodrug in transfected hPept1/HeLa cells and an improved permeability in Caco-2 cells (ninefold) compared with zanamivir.[24] Moreover, in an *in situ* rat perfusion model, the valine prodrug exhibited an effective permeability (P_{eff}) that was comparable to the well-absorbed metoprolol, whereas zanamivir was not permeable at all. Therefore, it will be interesting to see how these L-valine prodrug conjugates perform *in vivo*.

Here, we describe a prodrug approach for zanamivir that builds on our recent success with the orally bioavailable, highly potent oseltamivir amidoxime prodrug outlined above. The guanidine group in zanamivir was bioisosterically replaced by an acetamidine (zanamivir amidine, **5**) (Fig. 2). For both NAI drugs, prodrug strategies were realized that make use of an *N*-hydroxylation of the amidine (i.e., amidoxime) and the guanidine (i.e., *N*-hydroxyguanidine), a concept that overcomes the high basicity and thus (almost) permanent positive charge of the compounds. The resulting less basic *N*-hydroxylated derivatives would later be bioactivated *in vivo* by *N*-reduction. The underlying metabolism as well as scope and limitations of this prodrug concept have been previously demonstrated.[25–28] However, as the carboxylic acid and glycerol side chain in zanamivir significantly contribute to a high polarity of the parent drug and impair cellular uptake by passive diffusion, we also masked these groups by simple ester prodrugs. Therefore, for both the amidine (**5**) and guanidine (**1**) drug of zanamivir, two prodrugs were prepared that contain either a preacetylated glycerol side chain (**6**, **8**) or an unsubstituted glycerol moiety (**7**, **9**) along with a carboxyl ester and the respective *N*-hydroxylated amidine or guanidine. All prodrug candidates were extensively profiled for their *in vitro* and *in vivo* PK properties.

EXPERIMENTAL

Chemistry

General

^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were obtained on a Bruker ARX 300 spectrometer (Bruker, Bremen, Germany) at 300 K. Low-resolution mass spectra were recorded on a Bruker-Esquire-LC with electrospray ionization (ESI). Purity of synthetic products was ≥98% as determined by LC–MS. Recordings of high-mass resolution spectra (HRMS) were conducted on a Bruker 7.4 Tesla FTICR mass spectrometer BioApex II (Bruker, Bremen, Germany) equipped with an ESI-ion source (Agilent, Waldbronn, Germany); purification of synthesized compounds was performed by column chromatography using silica gel (particle size 40–63 μm). Flash chromatography on a RP18 column (43 g, Redi*Sep*®) was performed with the CombiFlashRETRIEVE® system. Starting materials were commercially available and used without further purification. All solvents were distilled and dried according to standard procedures. Zanamivir amine (**10**) and zanamivir hydroxyguanidine ethylester (**9**) were purchased from InnoChemie GmbH (Würzburg, Germany).

5-Acetylamino-7,8,9-Tri-O-Acetyl-4-(N-Acetimidamido)-2,6-Anhydro-3,4,5-Trideoxy-D-Glycero-D-Galacto-non-2-Enonic Acid Methyl Ester Hydrobromide (11)

Protected zanamivir amine precursor 10 (860 mg, 2 mmol) was dissolved in 6 mL abs. EtOH and cooled to 0°C. *S*-naphthylmethyl thioacetimidate hydrobromide (1.18 g, 4 mmol) was added portion wise over 60 min, and the reaction was stirred for 2 h at room temperature. The mixture was concentrated in a vacuum, taken up with approximately 30 mL water and washed thrice with approximately 20 mL Et_2O. The water phase was concentrated under reduced pressure and the crude product purified twice by column chromatography on SiO_2 (CH_2Cl_2/MeOH, 9:1, R_f = 0.42) to obtain a fine, white solid. Yield: 663 mg (60%). ^{1}H NMR (DMSO-d_6, 300 MHz): Amidine 11 appeared as rotamers/isomers predominantly observable at H-3, the amidine moiety and the NHAc group (87:13 ratio referring to the H-3 signal). Chemical shifts of the major rotamer/isomer are listed only. δ/ppm = 1.77 (s, 3H), 2.00, 2.01(3 × s, 9H), 2.10(s, 3H), 3.74 (s, 3H), 4.11 (m_c, 2H), 4.35 (dd,