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Triazole double-headed ribonucleosides as inhibitors of eosinophil derived neurotoxin

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ABSTRACT

Eosinophil derived neurotoxin (EDN) is an eosinophil secretion protein and a member of the Ribonuclease A (RNase A) superfamily involved in the immune response system and inflammatory disorders. The pathological actions of EDN are strongly dependent on the enzymatic activity and therefore, it is of significant interest to discover potent and specific inhibitors of EDN. In this framework we have assessed the inhibitory potency of triazole double-headed ribonucleosides. We present here an efficient method for the heterologous production and purification of EDN together with the synthesis of nucleosides and their biochemical evaluation in RNase A and EDN. Two groups of double-headed nucleosides were synthesized by the attachment of a purine or a pyrimidine base, through a triazole group at the 3′-*C* position of a pyrimidine or a purine ribonucleoside, respectively. Based on previous data with mononucleosides these compounds were expected to improve the inhibitory potency for RNase A and specificity for EDN. Kinetics data revealed that despite the rational, all but one, double-headed ribonucleosides were less potent than the respective mononucleosides while they were also more specific for ribonuclease A than for EDN. Compound **11c** (9-[3′-[4-[(cytosine-1-yl)methyl]-1,2,3-triazol-1-yl]-*β*-D-ribofuranosyl]adenine) displayed a stronger preference for EDN than for ribonuclease A and a K_i value of 58 μM. This is the first time that an inhibitor is reported to have a better potency for EDN than for RNase A. The crystal structure of EDN–**11c** complex reveals the structural basis of its potency and selectivity providing important guidelines for future structure-based inhibitor design efforts.

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1. Introduction

Eosinophil derived neurotoxin (EDN) [1] is an eosinophil secretion protein stored in the matrix of the secondary granules. EDN is a member of the RNase A superfamily (also known as RNase 2) involved in the immune response system and inflammatory disorders [2,3]. EDN is one of the most abundant RNases in humans and apart from eosinophils it has been found in a variety of tissues and excretions like placenta, liver and urine. EDN was initially identified by its ability to selectively kill cerebellar Purkinje cells when injected into rabbits, a syndrome called the Gordon phenomenon [4,5] and hence it was classified as a neurotoxin. EDN displays limited cytotoxicity against helminths and respiratory epithelial cells [6,7]. EDN is a chemoattractant for human dendritic cells since it activates them inducing maturation [8] and acts as an alarmin to activate the TLR2-MyD88 signal pathway enhancing their Th2 immune responses [9]. EDN, being an RNase, displays strong activity against single stranded RNA viruses like respiratory syncytial virus (RSV) and HIV in cell culture, playing thus, a role in antiviral host defence [3,10]. The *in vivo* damage of the host tissues by EDN could be involved in the secondary effects associated with inflammatory disorders and hypereosinophilic syndromes.

EDN shares only 36% amino acid sequence identity with RNase A and its ribonucleolytic activity is ~3–30-fold lower than that of

Abbreviations: RNase A, bovine pancreatic ribonuclease A; PEG, poly(ethylene glycol); TRL2, toll-like receptor 2; EDN, eosinophil derived neurotoxin.

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RNase A [11]. Ablation of the catalytic activity of recombinant EDN through site directed mutagenesis indicated that its cytotoxic, neurotoxic and antiviral activities are all dependent on its ribonuclease activity [10–12]. The relationship between the enzymatic activity of EDN and its physiological functions has yet to be established [3]. However, it seems that there are evolutionary constraints that force EDN to display all the structural characteristics (EDN and RNase A are structurally conserved [13]) necessary for ribonucleolytic activity and hence it is has been suggested that possibly EDN has a specific endogenous substrate [3]. Nevertheless, the linkage of EDN's biological activity to ribonucleolytic activity makes it an attractive target for the rational design of specific inhibitors to suppress its activity and hence modulate its pathological actions.

The active site of all RNases is highly conserved in terms of sequence and structural architecture. The three central sites are P_1, where P-O5′ bond cleavage occurs; B_1, which interacts with the base whose ribose contributes its 3′-oxygen to the scissile phosphodiester bond; and B_2, which binds the base whose ribose provides the 5′-oxygen [14]. However, specific differences in their peripheral RNA binding sites give rise to variations in their specificity towards different substrates and may provide clues for the rational design of specific ligands for each RNase. A plausible strategy for the development of EDN inhibitors is to test RNase A inhibitors and explore chemical modifications on their structure that will give rise to their specificity for EDN. In the past, we initiated inhibitor design studies using RNase A as a template. Thus, we studied a variety of substrate analogs, including mono and diphosphate (di)nucleotides with adenine at the 5′-position, and cytosine or uridine at the 3′-position of the scissile bond [15–19], aminonucleosides [20], and morpholino, piperidino, or pyrrolidinouridine and thymidine analogs [21]. Recently, we have investigated the binding of a series of ribofuranosyl pyrimidine nucleosides and their corresponding 1,2,3-triazole derivatives to RNase A. The most potent of them was 1-[(β-D-ribofuranosyl)-1,2,3-triazol-4-yl]uracil displaying a K_i value of 1.6 μM. [22]. To improve the potency of these inhibitors we took into consideration (i) studies showing that nucleoside-dibasic acids, nucleoside–amino acid conjugates [23,24] and dinucleosides [16,25] are more potent inhibitors than mononucleosides for RNase A (K_i values in the areas of mid-μM range) [25] as well as the importance of double-headed nucleosides [26–28] and, (ii) our previous work [22] where the insertion of a triazole group between the pyrimidine base and the ribose of a ribonucleoside led to the production of potent inhibitors with K_i values in the areas of low-μM range. We now present the synthesis of a novel series of double-headed ribonucleosides with an additional purine or pyrimidine base connected at the 3′-*C*-position of the sugar ring *via* a 1,2,3-triazole linker. The inhibitory potency of these compounds was evaluated against RNase A and EDN. Furthermore, we report the crystal structure of EDN in complex with the most potent compound of this series. Comparison of the inhibitory potencies for the two enzymes provides also data to validate the approach of using inhibitors designed for RNase A, to inhibit other superfamily members like EDN.

2. Results and discussion

2.1. Chemistry

The main goals of the current study were: (1) the attachment of an additional adenine at the 3′-*C*-position of uracil, thymine and fluorouracil ribonucleosides *via* a 1,2,3-triazole linker and (2) the introduction of an additional uracil or thymine at the 3′-*C*-position of adenine and N^6-benzoyladenine ribonucleosides through a 1,2,3-triazole linker. The synthesis of the desired 1,2,3-triazole double-headed nucleoside analogs **5a–c**, **11a–c** and **12a,b** is outlined in Scheme 1.

As our first example of this class of double-headed nucleosides, analogs **5** with the additional adenine connected at the 3′-*C*-position of deoxyribonucleosides *via* a 1,2,3-triazole linker were synthesized. Condensation of the known azido ribofuranose **1** [29] with silyl-protected uracil, thymine and 5-fluorouracil in the presence of trimethylsilyltrifluoromethane-sulfonate ($Me_3SiOSO_2CF_3$) as the catalyst in acetonitrile (CH_3CN) gave exclusively, due to the participation of the 2′-acetoxy group, the corresponding 3′-azido-3′-deoxy-β-D-ribonucleosides **2a–c**, in good yields (60–71%). Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) between key intermediates **2a–c** and propargyl adenine (**3**) [30], performed with catalytic amounts of copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and sodium ascorbate in refluxing tetrahydrofuran/water (THF/H_2O, 1:1), afforded the protected 3′-*C* substituted double-headed ribofuranonucleosides **4a–c**. Finally, removal of all *O*-acetyl groups of **4a–c** with saturated methanolic ammonia furnished the target derivatives **5a–c**, in good yields (61–78%).

Alternatively, the synthetic efforts were focused on the preparation of compounds **11** and **12** containing an adenine at 1′-*C*-position of the deoxyribose unit and a pyrimidine base at 3′-*C*-position connected through a 1,2,3-triazole linker. By the same manner, reaction of the azido sugar **1** [29] with silylated N^6-benzoyladenine *via* the Vorbrüggen coupling method gave ribonucleoside **6**, which was then "clicked" with propargyl uracil (**8a**), [30], thymine (**8b**) [30] and N^4-acetylcytosine (**8c**) [31] until formation of the protected bisheaded analogs **9a–c**. Treatment of **9a–c** with ammonia/MeOH (saturated at 0 °C), afforded only the fully deprotected derivative **11c** as well as the benzoylated **11a,b**, while attempts to further remove the benzoyl group either with sodium methoxide [32] or guanidine [33] resulted in a mixture of intractable and inseparable materials. In order to access all fully deprotected nucleosides **12a,b**, N^6-benzoyladenine was originally replaced by the non-commercially available N^6-acetyladenine [34]. Thus, conjugation of silyl-protected N^6-acetyladenine with anomeric sugar **1** [29] led to ribonucleoside **7**, which upon "click" chemistry reaction with propargyl uracil (**8a**) [30] and thymine (**8b**) [30] was readily converted to the corresponding bisheaded nucleosides **10a,b**. Finally, deacetylation with ammonia solution in methanol, yielded the desired free nucleosides **12a,b**.

The newly synthesized triazole cycloadducts were well characterized by NMR, UV spectroscopies, mass spectrometry and elemental analysis. According to ^{1}H NMR spectra of the "click" products (**4**, **5**, **9**, **10**, **11** and **12**) the newly formed triazole proton was observed at 8.08–8.25 ppm. It should also be mentioned that in all cases, the transformation into the triazole cycloadducts was quantitative and occurred with full regioselectivity, resulting in the formation of the corresponding 1,4-disubstituted 1,2,3-triazoles (**4**, **9** and **10**) as the sole products, since their triazole proton appeared at low field [35].

2.2. EDN production and purification

The recombinant EDN was expressed in *Escherichia coli* and purified by a method similar to the one reported for ECP [36]. A synthetic gene for human EDN was cloned into the pET11c expression vector and the protein was purified from inclusion bodies. This method has been reported previously in crystallography studies [13,37,38]. However, a detailed protocol including purification yield has not been described yet. We report here a step-by-step method for the efficient expression and production of EDN. This procedure produces 2.5 mg per liter of bacterial culture of pure EDN as judged from SDS–PAGE.

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