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Original article

Design, synthesis and structure–activity relationship of novel Relacin analogs as inhibitors of Rel proteins

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ABSTRACT

Rel proteins in bacteria synthesize the signal molecules (p)ppGpp that trigger the Stringent Response, responsible for bacterial survival. Inhibiting the activity of such enzymes prevents the Stringent Response, resulting in the inactivation of long-term bacterial survival strategies, leading to bacterial cell death. Herein, we describe a series of deoxyguanosine-based analogs of the Relacin molecule that inhibit *in vitro* the synthetic activity of Rel proteins from Gram positive and Gram negative bacteria, providing a deeper insight on the SAR for a better understanding of their potential interactions and inhibitory activity. Among the inhibitors evaluated, compound **2d** was found to be more effective and potent than our previously reported Relacin.

1. Introduction

The Stringent Response is a process crucial for bacterial viability as it activates a series of long term bacterial survival pathways including the switch into stationary phase of growth, sporulation, and biofilm formation [1–4]. This response is triggered by the accumulation of the signal molecules 5′-triphosphate-3′-diphosphate and 5′–3′-bis-diphosphate, collectively called (p)ppGpp [5] (Fig. 1A). In Gram negative bacteria (p)ppGpp is mostly synthesized by RelA and hydrolyzed by SpoT, while in Gram positive bacteria a bifunctional enzyme, Rel/Spo, both synthesizes and hydrolyzes (p)ppGpp [6,7].

RelA is a ribosome-associated (p)ppGpp synthetase activated in response to amino-acid starvation [8]. During amino-acid deprivation the binding of uncharged tRNAs to the ribosomal 'A' site stalls protein synthesis, stimulating a reaction in which RelA transfers a pyrophosphoryl group comprising the β- and γ-phosphates of an ATP donor to the 3′-hydroxy group of GTP or GDP to form pppGpp and ppGpp, respectively [8]. Despite the low abundance of RelA, up to mM levels of (p)ppGpp are rapidly produced. This is possible since (p)ppGpp synthesis evokes dissociation of RelA from the ribosome, allowing the enzyme to "hop" to another stalled ribosome and repeat the reaction [9].

Recently, the crystal structure of the catalytic N-terminal fragment of the bifunctional RelA homologue from the Gram positive bacterium *Streptococcus equisimilis* (Rel*seq*385) was determined [10]. In contrast to Gram negative bacteria, where RelA and SpoT are distinct proteins, this fragment displays both the hydrolase and synthetase active sites. The crystallographic analysis revealed two conformations for Rel*seq*385 that typify the opposing hydrolase-OFF/synthetase-ON and hydrolase-ON/synthetase-OFF states. The crystal structure also suggested a mechanism for the attack of the 3′-OH group of GDP (or GTP) onto the β-phosphorus atom of ATP. Most likely, Glu323 of Rel*seq*385 activates the hydroxyl group by proton abstraction. In addition, Glu323 also is suggested to coordinate the essential Mg^{2+} ion which is not present in the crystals and is expected to be bound to the pyrophosphate donor, ATP. Support for the critical role of Glu323 in the synthetic mechanism derives from a mutational study showing that a mutant Rel*seq*385 bearing a Glu323Gln substitution exhibited severely defective synthetase activity [10].

In the past few years, several efforts have been made toward the development of antibacterial agents based on nucleosides and their analogs. Such compounds have shown antimicrobial activity by

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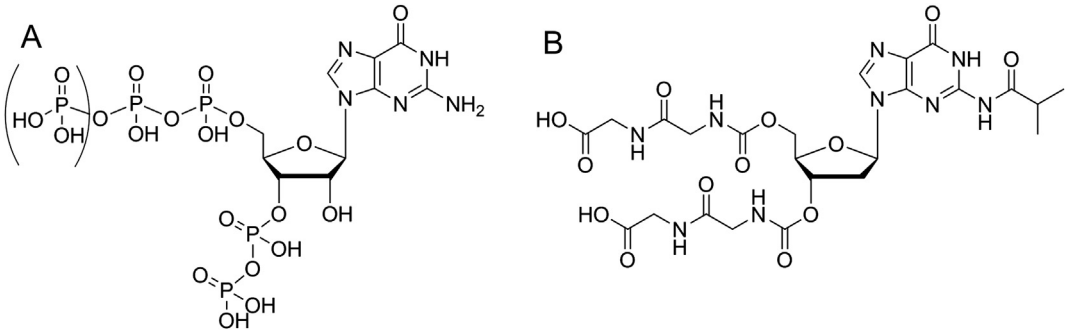


Fig. 1. Structures of (A) (p)ppGpp and (B) Relacin.

inhibiting cell wall biosynthesis [11], DNA ligases [12], riboflavin synthesis [13], polyamine biosynthesis and quorum sensing [14], nucleoside phosphorylases [15], and siderophore biosynthesis [16] among others.

In the search for novel strategies to combat multi-drug resistant bacteria, we have explored the Stringent Response as a potential target for the development of a new generation of antibiotics [17,18]. We have recently reported on Relacin [18] (Fig. 1B), a novel ppGpp analog that prevents the activation of the Stringent Response by inhibiting (p)ppGpp production. Relacin perturbs the switch into stationary phase in Gram positive bacteria and leads to cell death. Furthermore, Relacin inhibits sporulation and biofilm formation; additional bacterial long term survival strategies used by such bacteria. In this report we present the synthesis of a series of symmetrically and asymmetrically substituted analogs of Relacin, designed at gaining a deeper understanding on the importance of the moieties at positions 5′ and 3′ on the deoxyribose ring and their potential interactions within the active site of Rel Proteins. The inhibitory potential of the prepared compounds was biochemically evaluated on the (p)ppGpp synthetase activity of RelA and Rel/Spo purified from *Escherichia coli* (*E. coli*) and *Deinococcus radiodurans* (*D. radiodurans*), respectively.

2. Results and discussion

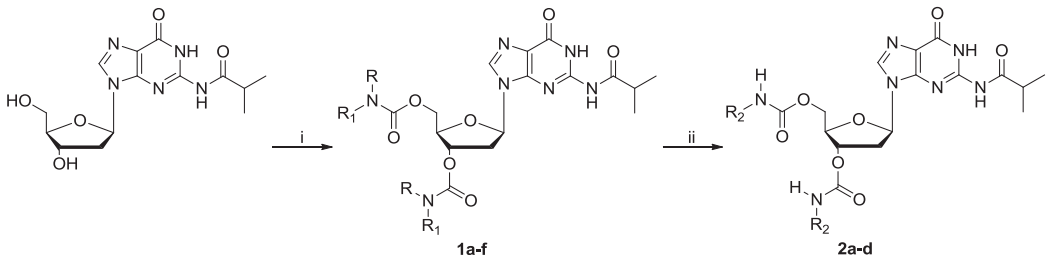
2.1. Design of ppGp(p) analogs

Relacin, our model compound, has a di-peptide (Gly-Gly) at both 3′ and 5′ positions of the deoxyguanosine. This active analog has the following features: (1) symmetry in the choice of the di-peptide added to both 3′ and 5′ positions and, (2) two negative charges (at physiological pH) from the two acidic C-termini.

Our first goal was to compare the activity of Relacin to other symmetrically substituted compounds. As shown in Scheme 1, such analogs were prepared by mixing 2-isobutyryl-2′-deoxyguanosine with an excess of carbonyldiimidazole (CDI) in acetonitrile overnight. After the addition of CDI, the suspension completely dissolved and eventually the di-imidazolide derivative precipitated. After stirring overnight, the precipitate was filtered and resuspended in dichloromethane (DCM). The different amino substituents (Table 1) were dissolved in DCM containing an excess of diisopropylethylamine (DIEA) and added to the former suspension. The mixture was stirred at room temperature until the reaction ended (according to TLC analysis). The final products were obtained after hydrogenolysis of the benzyl ester group to afford compounds **2a**–**d**.

As RelA may accommodate different size substituents at the 3′ and 5′ positions of deoxyguanosine, we decided to explore the activity of a family of Relacin analogs that are asymmetrical. Scheme 2 depicts the synthesis of the asymmetrically substituted compounds. The first step was the formation of an imidazolide intermediate at position 3′ of the suitable DMT-protected 2′-deoxyguanosine (**3**). This intermediate was prepared adapting the procedure reported by Korshun et al. [19]. Next, the benzyl esters of either glycine or glutamic acid were added to a solution of the freshly prepared imidazolide in DCM containing DIEA. The mixture was stirred overnight. After acidic workup and column chromatography, the dimethoxytrityl (DMT) group was cleaved from compounds **4a**,**b** to yield compounds **5a**,**b** and the free hydroxyl moiety at position 5′ was reacted with a slight excess of CDI in DCM. When all the starting material was consumed, (typically after 15 h) an excess of the benzyl esters of either glycine or glutamic acid was added together with DIEA and the mixture was stirred overnight until the completion of the reaction to afford compounds **6a**,**b**. After a further chromatographic purification step, the benzyl protecting groups were cleaved as described for the symmetric analogs resulting in the final products **7a**,**b**.

The potential inhibitors were tested *in vitro* on purified Rel proteins from both Gram positive and Gram negative bacteria using reported methods [18]. The results, summarized in Table 2, show



Scheme 1. Synthetic route for the preparation of symmetric compounds. Reaction conditions: i) a) CDI, acetonitrile, RT, overnight; b) R,R_1-NH, DCM, DIEA, RT, 2–20 h, 17–77%; ii) H_2-10% Pd/C, methanol, RT, 3 h, 30 psi, 80–90%.