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Research paper

Oligonucleotide transition state analogues of saporin L3

Jennifer M. Mason [a, *], Hongling Yuan [b], Gary B. Evans [a], Peter C. Tyler [a], Quan Du [b], Vern L. Schramm [b]

[a] The Ferrier Institute, Victoria University of Wellington, 69 Gracefield Rd, Lower Hutt 5010, New Zealand
[b] Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461, USA

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ABSTRACT

Ribosome inactivating proteins (RIPs) are among the most toxic agents known. More than a dozen clinical trials against refractory cancers have been initiated using modified RIPs with impressive results. However, dose-limiting toxicity due to vascular leak syndrome limits success of the therapy. We have previously reported some tight-binding transition state analogues of Saporin L3 that mimic small oligonucleotide substrates in which the susceptible adenosine has been replaced by a 9-deazaadenyl hydroxypyrrolidinol derivative. They provide the first step in the development of rescue agents to prevent Saporin L3 toxicity on non-targeted cells. Here we report the synthesis, using solution phase chemistry, of these and a larger group of transition state analogues. They were tested for inhibition against Saporin L3 giving K_i values as low as 3.3 nM and indicating the structural requirements for inhibition.

1. Introduction

Saporins are ribosome inactivating proteins (RIPs) isolated from the soapwort (*Saponaria officinalus*) [1]. Like the better known ricin A-chain (RTA), saporins bind to the sarcin – ricin loop of the 28S eukaryotic ribosomal RNA and hydrolytically depurinate adenosine 4234 [2], thereby preventing protein synthesis and causing cell death. Saporins are less specific than RTA, as they also hydrolyze adenine from other sites on RNAs.

Trial cancer therapies have exploited the toxicity of saporin and RTA - antibody constructs targeted to leukemia and lymphoma cells [3]. Off-target toxicity resulting from incomplete uptake or release of RIPs from dying target cells limits the use of these therapies. Inhibitors of RIPs could provide therapy enhancement by inhibition of unwanted RIP following the initial treatment [4]. This paper describes the synthesis and characterization of several potent inhibitors of saporin L3 (SAP) [5,6] a highly active saporin isoform originally isolated from *S. officinalus* leaves. The inhibitors are characterized with saporin L3 expressed in yeast and mutated to replace alanine 14 with cysteine (SAP A14C), a mutation remote from the catalytic site which provides a chemical attachment site for cell-recognition molecules.

The transition states for RTA and SAP-catalyzed depurination of RNAs have ribocation character from which activated adenine is largely dissociated [7]. The nonhydrolysable adenosine mimic **1** (DIA) captures, in stable form, key features of the transition state geometry. The extended bond length between leaving group adenine and ribose is mimicked by the methylene bridge, the imino-sugar is protonated at physiological pH to mimic the ribocation, and N7 of the 9-deazaadenine ring is protonated at physiological pH. DIA does not significantly inhibit either SAP or RTA, but replacement of the susceptible adenosine in RNA sequences from the reactive part of the sarcin – ricin loop with DIA gives potent inhibitors [8]. The stem loop A-10 (5′-CGCGAGAGCG-3′) along with linear and cyclized GAGA constructs are accepted as substrates by SAP [4], and these molecules provide a template for inhibitor design. SAP accepts truncated RNAs as substrates and is inhibited by the corresponding aza-sugar constructs under physiological conditions whereas RTA requires reduced pH for activity on truncated substrates and for inhibition [4,8].

The crystal structure of a cyclic G(DIA)GA tetramer bound to SAP shows a quadruple π-stack in which deazaadenine is positioned between two tyrosine phenol groups with the 3′- guanosine providing the final layer of the π-stack. These interactions, together with multiple hydrogen bonds, are proposed to provide leaving group activation in catalytic structures [9].

The RNA oligonucleotides containing DIA, **2**–**4** (Fig. 1), are potent inhibitors of SAP [4]. These oligonucleotides are stabilised

Abbreviations: RTA, Ricin A-chain; SAP, saporin L3; MTET, methyltetrazole.

* Corresponding author.
E-mail address: jennifer.mason@vuw.ac.nz (J.M. Mason).

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against ribonucleases by 2′-O-methylation [10] which, in the context of inhibitor design, is tolerated by SAP [11] and some have phosphate end groups capped with propanediol. They were synthesized in the usual manner for oligonucleotides, on solid support with stepwise addition of each nucleotide phosphoramidite, often requiring multiequivalents of each. We have found that aza -sugar phosphoramidites such as **14** (Scheme 1) give poor and non-reproducible yields under this protocol.

Using conventional solution phase chemistry, which allows for convergent strategies and more efficient use of valuable intermediates, we have synthesized a group of aza-sugar-containing 2′-O-methyl di, tri and tetra RNA nucleotides that probe both aza-sugar structure and the nucleotide context required for inhibition of SAP [12,13]. Compounds 2–4 were resynthesised for comparison and to provide sufficient material for chemical characterization.

2. Results and discussion

2.1. Chemistry

Tetramer **4** (G*p*DIA*p*G*p*A) is an inhibitor construct corresponding to the GAGA substrate of SAP. It was synthesized in a linear fashion by stepwise addition to the 3′-terminal adenosine (Scheme 1). Reaction of the 3′-hydroxyl of commercially available 5′-*O*-dimethoxytrityl-6-*N*-benzoyl-2′-*O*-methyladenosine **6** with chloroacetyl chloride followed by hydrolysis of the dimethoxytrityl (DMTr) group with aqueous acetic acid gave 5′-OH adenosine **7** in good yield. The phosphate linkages were protected as benzyl esters as they provide the additional stability over the cyanoethyl group required for solution phase work. Benzyloxydiamidite **5**, synthesized from phosphorus trichloride in one pot and purified by partition between hexane and acetonitrile [14], was coupled with guanosine **8** to give the amidite building block **9** which was coupled with adenosine **7** using 5-methyltetrazole (MTET) as the activator. To the best of our knowledge, MTET has not previously been used as an activator in phosphoramidite coupling reactions. It's predicted pK_a of 5.1 [15] is close to that of the commonly used 4,5 − dicyanoimidazole (DCI, pK_a 5.0) and it is not subject to the same shipping restrictions as tetrazole. It was effective in many of the reactions reported herein. Oxidation of the intermediate phosphite ester with aqueous 70% *t*-butylhydroperoxide afforded the benzyl protected phosphate ester **10** in excellent yield as a mixture of diastereomers at phosphorus. After purification by silica gel chromatography the DMTr group was removed with acetic acid to give **11** in good yield.

A Mannich reaction [16] between 6-*N*-benzoyl-9-deazaadenine, (3*R*, 4R)-4-(hydroxymethyl)-pyrrolidin-3-ol [17] and formaldehyde gave benzoyl-protected DIA **12** which was selectively tritylated on the primary hydroxyl in good yield to give **13**. Preparation of amidite **14** was initially problematic as it was readily hydrolysed (to an H-phosphonate) during purification on silica gel. However, optimization of the chromatography to give rapid elution of **14** as well

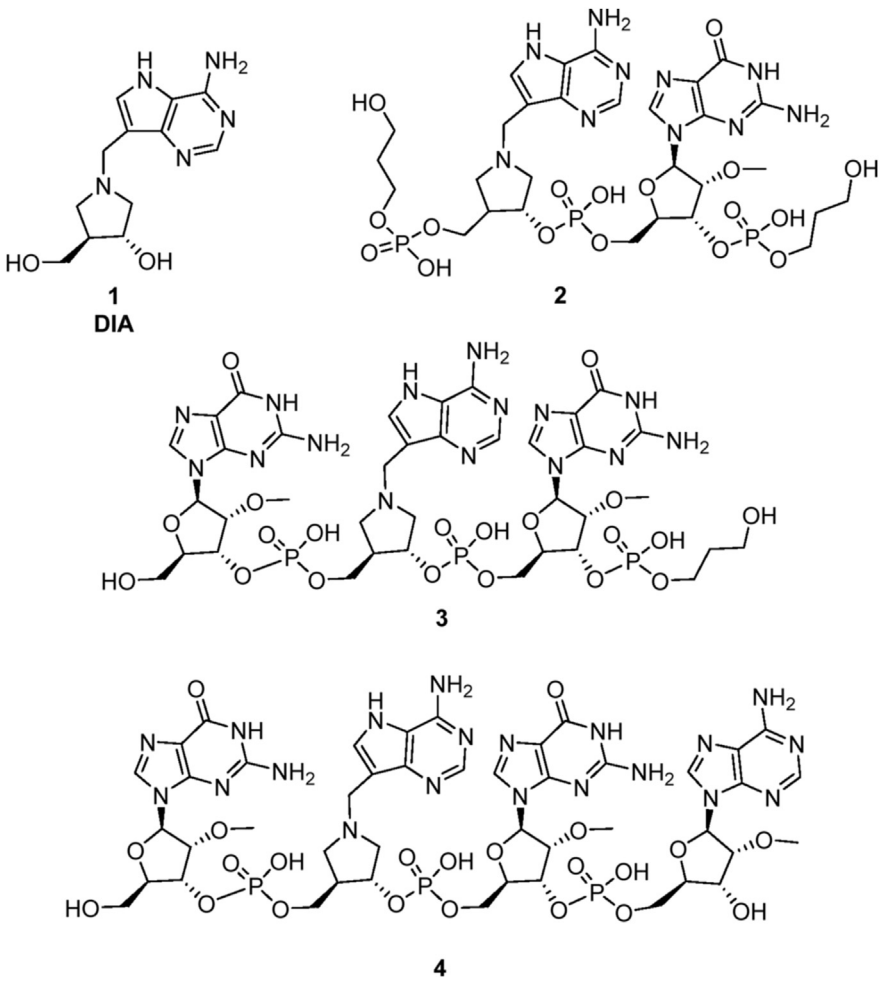

Fig. 1. Adenosine mimics **1** and some inhibitors of SAP.

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