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Synthesis of a norcantharidin-tethered guanosine: Protein phosphatase-1 inhibitors that change alternative splicing

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ABSTRACT

Phosphorylation and dephosphorylation of splicing factors play a key role in pre-mRNA splicing events, and cantharidin and norcantharidin analogs inhibit protein phosphatase-1 (PP1) and change alternative pre-mRNA splicing. Targeted inhibitors capable of selectively inhibiting PP-1 could promote exon 7 inclusion in the survival-of-motorneuron-2 gene (*SMN2*) and shift the proportion of SMN2 protein from a dysfunctional to a functional form. As a prelude to the development of norcantharidin-tethered oligonucleotide inhibitors, the synthesis a norcantharidin-tethered guanosine was developed in which a suitable tether prevented the undesired cyclization of norcantharidin monoamides to imides and possessed a secondary amine terminus suited to the synthesis of oligonucleotides analogs. Application of this methodology led to the synthesis of a diastereomeric mixture of norcantharidin-tethered guanosines, namely bisammonium (1*R*,2*S*,3*R*,4*S*)- and (1*S*,2*R*,3*S*,4*R*)-3-((4-(2-(((((2*R*,3*R*,4*R*,5*R*)-5-(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-2-(hydroxymethyl)-4-methoxytetrahydrofuran-3-yl)oxy)oxidophosphoryl)oxy)ethyl)-phenethyl)(methyl)carbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylate, which showed activity in an assay for SMN2 pre-mRNA splicing.

Spinal Muscular Atrophy (SMA) is neuromuscular disorder that results in the devastating weakness and wasting of the voluntary muscles in the extremities of infants and children.[1] The disease is caused by the homozygotic loss of the survival-of-motoneuron-1 gene (*SMN1*) that encodes a protein crucial for small nuclear ribonucleic protein (snRNP) assembly and axonal transport.[2] The absence of SMN results in the loss of spinal cord motor-neurons with tragic outcomes, resulting in death in early infancy. Humans express a related gene, survival-of-motoneuron-2 (*SMN2*) that is generated through a duplication event and that differs from *SMN1* by a silent C→T transition but that is otherwise almost identical to *SMN1*. The C→T transition (C280T) disrupts a splicing enhancer, which causes predominant skipping of exon 7, resulting in an unstable protein with a different C-terminus.[3] The near identity of the mRNA sequences of SMN1 and SMN2 with inclusion of exon 7 suggested that a therapy for SMA could be based on agents that promote exon 7 inclusion.[2] There is weak inclusion of exon 7 due to a splicing enhancer formed by three key splicing factors: tra2-beta1, SRp30c and the heterogeneous nuclear ribonucleoprotein hnRNPG.[4,5] Tra2-beta1 binds directly to protein phosphatase-1 (PP1), and the inhibition of the phosphatase by cantharidin and other PP1 inhibitors promote exon 7 inclusion in cell and mouse models[6] (Fig. 1). We previously developed cantharidin and norcantharidin analogs that blocked PP1 and promoted exon 7 inclusion.[7]

Consistent with other, known PP1 inhibitors, the cantharidin and norcantharidin derivatives lack selectivity for exon 7 inclusion because PP1 binds to an RNA recognition motif of several splicing factors where it regulates alternative splicing.[6,8]

Since PP1 has numerous regulatory partners, we sought to develop PP1 inhibitors linked at the 3′-position to a 2′-methoxy-oligonucleotide that would complement the proximal intron 7 and selectively direct the inhibitor to PP1 associated with the spliceosome. Prior work by the McCluskey group[9–13] and others[7,14] identified racemic monoamide derivatives of 3*a*,4,7,7*a*-hexahydro-4,7-epoxyisobenzofuran-1,3-dione,[11] commonly called norcantharidin (**1**), as potent PP1 inhibitors (Fig. 2). The monoamides **2** produced from secondary amines displayed good PP1 inhibitory activity[10] relative to the corresponding monoamides **3** of primary amines that were prone to cyclize to the inactive imides **4** (Fig. 2).

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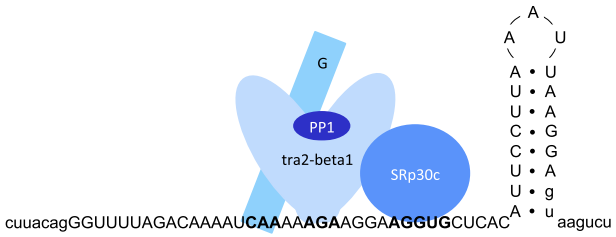

Figure 1. The SMN2 exon 7 enhancer complex. The exon is shown in capital letters; the introns are shown in small letters. Legend: G = hnRNP G, tra2-beta1 and SRp30c bind to the boldface RNA sequences.[4] PP1 binds to the PP1 binding motif in the RRM of tra2-beta1.[6]

Since we ultimately required a norcantharidin monoamide tethered to a 2′-methoxyoligonucleotide octamer **9** (Fig. 3), we planned to utilize a polymer-based synthesis in which an unsymmetrical tether **5** with a secondary amino-terminus linked to a polymer through a terephthaloyl group and with a hydroxy terminus that was suitable for the elaboration of the 2′-methoxyoligonucleotide (Fig. 3). Linkage of the tether through the commonly used *N*-phthalimidoyl group would not accommodate a secondary amine. Ammonolysis of **7** would cleave the linkage to the polymer and remove guanosine-protecting groups, and the attachment of the inhibitor to **8** would complete the synthesis of the inhibitor-tether-oligonucleotide **9**. Central to these plans was the overarching need to determine if a norcantharidin monoamide tethered to a nucleotide would retain PP1 inhibitory activity.

Synthesis of an norcantharidin-tethered guanosine proceeded from the symmetrical 1,4-bis(2,2′-hydroxyethyl)benzene (**10**) and involved the protection of one hydroxyl group in **10** as a 4,4′-dimethoxytrityl (DMT) ether and the elaboration of the hydroxyl group at the other terminus in **11** to *N*-methylamino group (Fig. 4).

The latter transformation involved conversion of **11** to the azide **13** via the tosylate **12**; addition of ethyl chloroformate; and reduction[15] of the urethane **15** to give the secondary amine **16**. Coupling of **16** to norcantharidin (**1**) and methylation furnished the norcantharidin monoamide **17,** which displayed a doubling of signals for the *N*-methyl and the methoxy groups in the ^{1}H NMR in DMSO-d_6 consistent with hindered inversion about the carboxamide nitrogen. Because the amine intermediates **14** and **16** in this sequence underwent partial O-to-N-rearrangements[16] of the DMT group during silica gel chromatography, we converted these amines without purification directly to the urethane **15** and norcantharidin monoamide **17**, respectively. Completion of the norcantharidin-tether-G construct involved DMT deprotection to afford **18** and 4,5-dicyanoimidazole-promoted coupling[17] of 5′-*O*-(4,4′-dimethoxy-

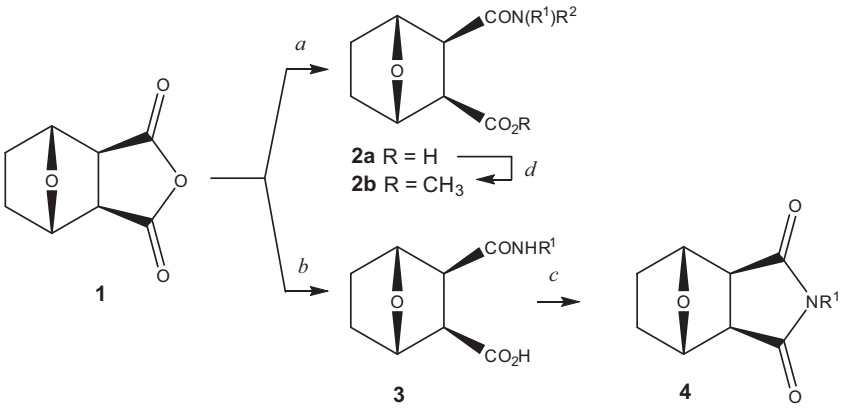

Figure 2. Monoamides and imides derived from norcantharidin (**1**). Legend: (a) R^1R^2NH; (b) R^1NH_2; (c) heat, neat or heat, toluene, reflux;[11] (d) NH_3, aqueous CH_3OH, 55 °C, pressure tube.

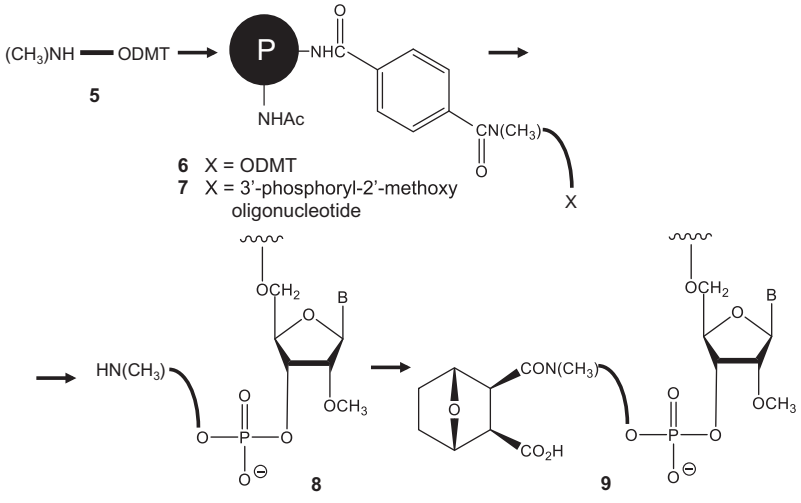

Figure 3. Synthetic plan for norcantharidin monoamide tethered to a 2′-methoxyoligonucleotide. Legend: DMT = 4,4′-dimethoxytrityl; tether displayed as solid black line; amino-substituted polymer displayed as black circle with P.

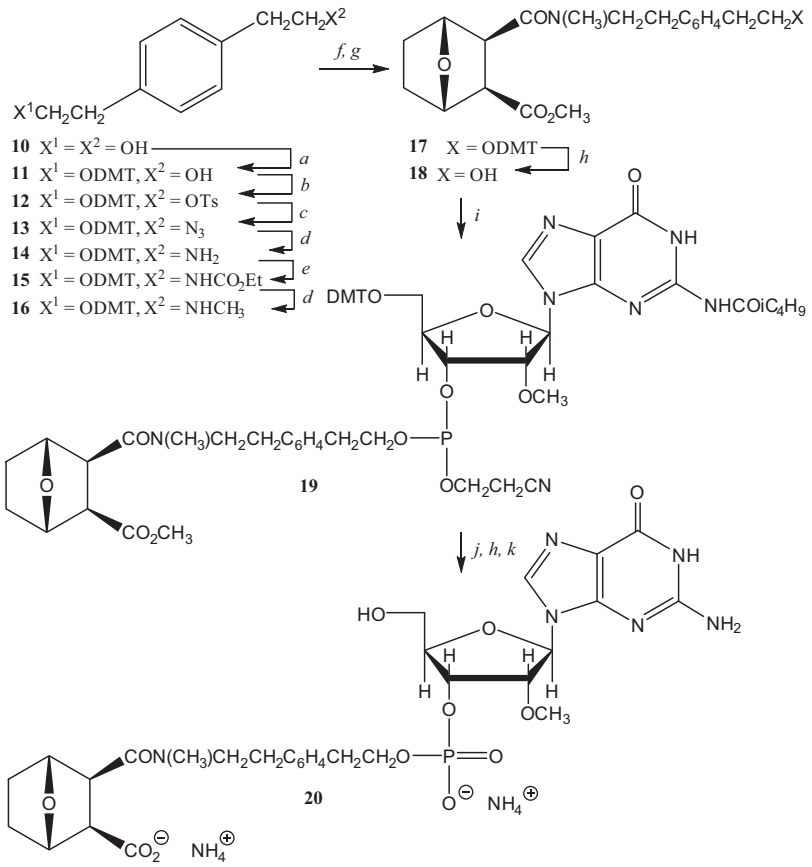

Figure 4. Synthesis of norcantharidin-linked guanosine. Legend: (a) DMTCl, Et_3N, DMAP (cat); (b) *p*-TsCl, Py; (c) NaN_3, HMPA; (d) $LiAlH_4$; (e) $ClCO_2Et$, Py; (f) norcantharidin (**1**); (g) $TMSCHN_2$; (h) TFA; (i) 4,5-dicyanoimidazole, 5′-*O*-(4,4′-dimethoxytrityl)-N^2-isobutyryl-2′-*O*-methylguanosine-3′-*O*-(2-cyanoethyl)phosphoramidite; (j) I_2, pyridine, aq THF; (k) 28% NH_4OH, 5 h, 55 °C.

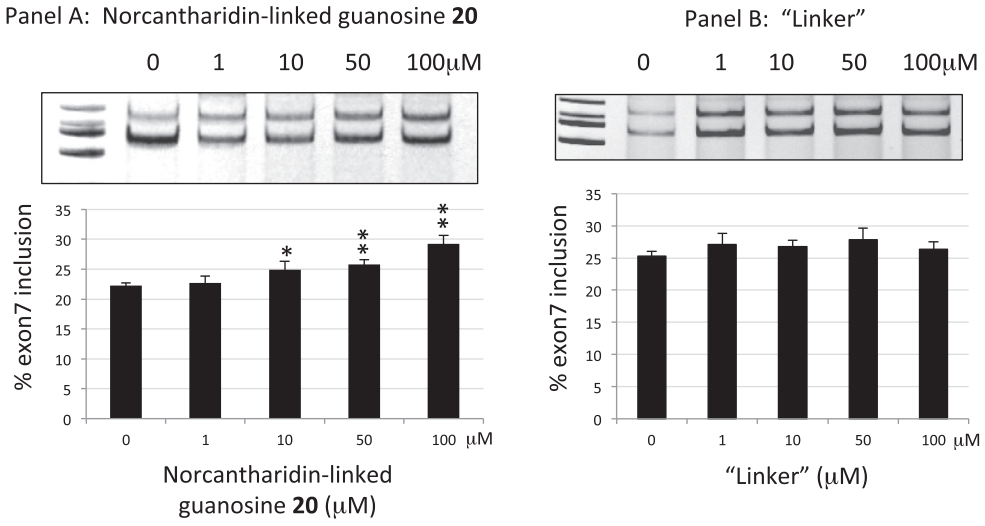

Figure 5. (Panel A) Norcantharidin-tether-guanosine **20** alters the SMN2 exon 7 splice-site selection. HEK293 cells were transfected with an *SMN2* splicing reporter, consisting of exons 6–8, as well as the included introns. After 1 h, the cells were treated with **20** at the concentrations indicated. Total RNA was isolated after 14–16 h, and the *SMN2* mRNA was amplified. The *p*-values for 10, 50 and 100 μM concentrations were 0.0355, 0.0028 and 0.0012, respectively. (Panel B) 'Linker' or 2-(4-(2-(methylamino) ethyl)phenyl)ethan-1-ol does not alter the SMN2 exon 7 splice-site selection.

trityl)-N^2-isobutyryl-2′-*O*-methylguanosine-3′-*O*-(2-cyanoethyl) phosphoramidite to give the phosphite adduct **19**. Oxidation of **19** to the phosphate, deprotection of the 5′-DMT ether with trifluoroacetic acid, and ammonolysis removed the β-cyanoethyl group and hydrolyzed the norcantharidin methyl ester and the guanosine N^2-isobutyryl amide to provide the bisammonium salt of the norcantharidin-tether-G construct **20** as a mixture of diastereomers.

We tested norcantharidin derivatives using an in vivo minigene assay.[18] The monoamide **2a** of norcantharidin and *N*-methylphenethylamine, which represented a portion of the

tethered norcantharidin without a nucleotide, was added to HEK293 cells cotransfected with an SMN2-reporter minigene.[6] Because the monoamide **2a** displayed activity, we *synthesized* the norcantharidin-tether-guanosine construct **20** (Fig. 4) and determined that **20** increased exon 7 inclusion in a concentration-dependent manner and increased exon inclusion by about 25% (Fig. 5A) with statistical significance at the 10–100 μM concentrations. As a control, the tether or 'linker', namely 2-(4-(2-(methylamino)ethyl)phenyl)ethan-1-ol, which connected the norcantharidin and guanosine, showed no effect on inclusion (Fig. 5B).

In summary, methodology for the synthesis of a guanosine-tethered norcantharidin was developed that could accommodate the synthesis of a norcantharidin-tether-oligonucleotide. Although the stage was now set for the synthesis of such a construct, the modest change in exon inclusion seen with **20** did not warrant the investment of effort in this undertaking.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.12.054.

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