Spontaneous 2′-deoxyguanosine alkylation by new generation of Top I inhibitors of camptothecin family

Journal:	*New Journal of Chemistry*
Manuscript ID	NJ-LET-12-2015-003497.R2
Article Type:	Letter
Date Submitted by the Author:	26-Jan-2016
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Journal Name

COMMUNICATION

Spontaneous 2'-deoxyguanosine alkylation by new generation of Top I inhibitors of the camptothecin family

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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We present evidence, using NMR and LC - MS techniques, that 7-ethyl-9-(*N*-morpholino)methyl-10-hydroxycamptothecin spontaneously binds covalently to 2'-deoxyguanosine at near physiological conditions. Independent on conditions, either in pure water at pH 6 or in 25 mM NaCl/25 mM potassium phosphate at pH 7, the 2-NH_2 is alkylated selectively. This is the first documented case of spontaneous covalent binding of 2'-deoxyguanosine by potential Top I inhibitor. This type of camptothecin derivatives (9-alkylamino substituted of parent SN38, 7-ethyl-10-hydroxycamptothecin) may have potential of being active *in vivo* because they belong to the class of camptothecin derivatives such as Camptosar™ or Hycamtin™ which are used in clinic for cervical, colon or breast cancer chemotherapy .

Inhibition of topoisomerases I and II, which enable proliferation of tumor tissues, is a major route in combating cancer via chemotherapy.[1] Targeted chemotherapy is a topic of longstanding interest due to its therapeutic effectiveness and chemical efficiency.[2] The role of topoisomerases I and II inhibitors involves the ternary complexes composed of enzyme/DNA/Inhibitor. Topoisomerases are essentially enzymes which, inter alia, relax the torsional strain in tumor supercoiled DNA generated in replication process.[3] The enzyme cleaves one strand, creating a nick, and thus enables the rotation around the uncleaved strand followed by ligation of the broken strand restoring the DNA duplex.[4] It is well documented that camptothecin derived Top I inhibitors require a G-C base pair in a nicked DNA duplex to bind site selectively to tumor DNA thus realizing targeted chemotherapy.[5] We have evidenced that, in a molecular complex of natural octamer $d(GCGATCGC)_2$ and nicked decamer, topotecan TPT (Hycamtin®) is stacking the 2'-deoxyguanosine in a GC base pair.[6,7] Recently we have synthesized novel camptothecin derivatives which demonstrate unusual chemical property of spontaneous covalent binding with nuclophiles. Taking into account the two mentioned properties, i.e. site specific binding of a camptothecin core in a nick of nicked DNA and covalent binding, these compounds bear properties required of medicines used in targeted chemotherapy. Binding of this type of compound in Top I induced nick in DNA will prevent ligation of a broken strand restoring the DNA duplex. It is therefore suggested that this may, in effect, result in dramatic increase of cell cytotoxic activity of a potential drug based on this structure and its mechanism of action. We have therefore chosen the 2'-deoxyguanosine as a model nucleoside to study the regioselectivity of binding various nitrogen sites. Rokita and coworkers studied time dependence of evolution of various adducts between 2'-deoxynucleosides and simple phenol derived *o*-methide (QM, *o*-methylenequinone) chemically generated in situ.[8,9,10] In general, the three possible sites, i.e. nitrogen atoms N1, N2 and N7, of alkylation in 2'-deoxyguanosine can be viewed in Scheme 1. The 2'-deoxyguanosine is of interest because we have established that TPT is stacking guanine in an terminal GC base pair in a molecular complex with a natural oligomer $d(GCGATCGC)_2$.[6] However in view of Rokita results, showing that simple phenol derived methide is able to bind the 2'-deoxyguanosine at multiple nitrogen sites, in the present contribution we were interested to establish if there is a regioselectivity of binding the nitrogen sites in guanine by SN38-aminoalkyl-9 derived methide and if the alkylation products have transient character and can undergo equilibration between various nitrogen sites. With respect to this Weinert et al.[9] documents that weak nucleophiles as N1 and N2 in dG form thermodynamic products with QM.

In this contribution we present the evidence that *o*-methylene quinone derived from 9-alkylamino substituted SN38 binds

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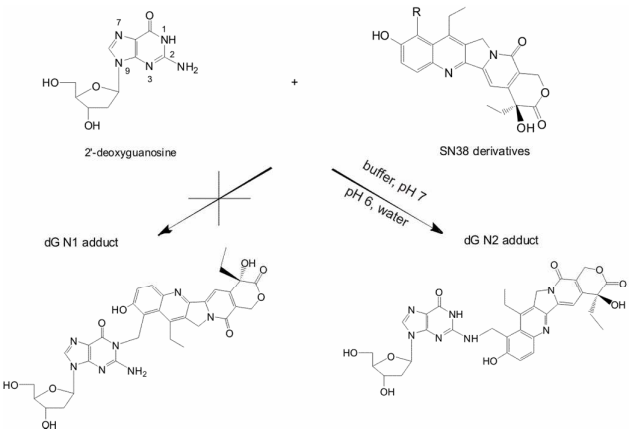
† Electronic Supplementary Information (ESI) available: Details of NMR experiments, COSY spectrum of SN38-dGN2 adduct, $^{1}H/^{13}C$ HCQC of SN38-dGN2 adduct, $^{1}H/^{13}C$ HMBC of SN38-dGN2 adduct, PFGSE spectrum of SN38-dGN2 adduct, alkylation procedure in neat water, pH 6, LC-MS run of a solid in a reaction in neat water at pH 6, 1H NMR spectrum of a reaction product in a reaction at pH 6 in neat water, ESI MS spectrum of a title compound.

spontaneously 2'-deoxyguanosine selectively to N2 nitrogen atoms independent on conditions, either in neat water at pH 6



Scheme 1 Possible binding sites of QM to 2'-deoxyguanosine considered here. Substituent R stands for alkylaminomethyl group, here N-morpholinomethyl.

(Fig 2S) or in buffered water (25 mM NaCl/25 mM K_3PO_4) at pH 7 (Fig. 1). The adducts are stable in DMSO solution.

This result contradicts the finding of Zhou et.al.[11] of selective dG-N1 alkylation in 30% aqueous DMF by quinolinyl QM but is in good agreement with the thermodynamic considerations claiming that dG-N2 alkylation in water has ΔG lower by ca. 4 kcal/mol than dG-N1 alkylation.[12]

Although nucleobase alkylation using chemically generated QMs was extensively studied during last decade and a half, to the best of our knowledge this is the first documented case of spontaneous covalent binding of 2'-deoxyguanosine by potential Top I inhibitor which may have perspective of being active *in vivo* belonging to the class of compounds used in clinic.

The alkylation reaction [§] was run in an NMR tube to allow the control of substrates and build up of the products and observation of their possible equilibration. The assignment of the binding sites in 2'-deoxyguanosine – QM adducts is straightforward; N-7 attack results in deglycosylation and free guanine base should be found in reaction products while N-1 and N-2 are easily distinguished in COSY NMR (vide infra).

The progress of the reaction was monitored by ^{1}H NMR and LC-MS techniques. The reaction was carried out at room temperature for 48 hours while solid was precipitating during that time. Reaction solution was checked by NMR and after completion of the reaction it contained unreacted 2'-deoxyguanosine and traces of SN38 and alcohol 7-ethyl-9-hydroxymethyl-10-hydroxycamptothecin (SN38-CH_2OH). The precipitated solid was checked in LC-MS (see Experimental) which disclosed presence of three products in lactone and carboxylate forms of a camptothecin core because reaction was run at pH 7 (see Fig. 1). Neither in solution nor in the solid there was free guanine base present. Reaction at N-7 nitrogen site was therefore removed from consideration. The adduct found in precipitate cannot equilibrate because it is immediately deposited from reaction solution as a solid.

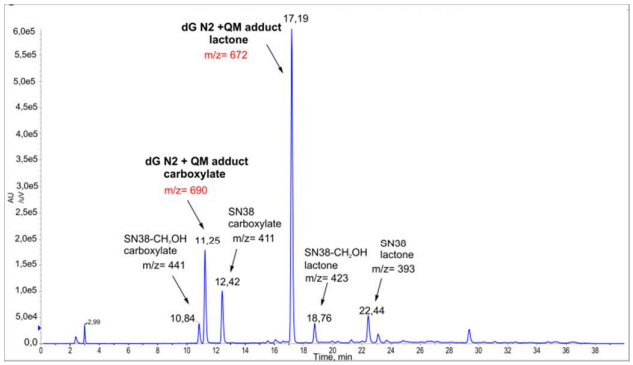


Fig. 1 LC-MS result of a precipitated solid from the reaction between 2'-deoxyguanosine and 7-ethyl-9-(*N*-morpholino)methyl-10-hydroxycamptothecin in buffered water solution at pH 7, taken immediately after dissolving the solid in a liquid phase. Retention times and m/z $(M+H)^+$ values are given for each peak.

After completion the reaction mixture was filtered and solid was purified by HPLC on the RP-C18 LPH column (150 mm x 10 mm) using a mobile phase composed of 10 mM CH_3COONH_4 aqueous solution pH = 5.4 (A) and MeCN (B), with the following gradient: 0-30 min 10 → 35% B. The chromatography was initiated after 1 hr time allowing the equilibration of a reaction solid at pH 5.4 to a single lactone form (see Fig. 2). Flow rate of the mobile phase was 2.8 ml/min. The course of the chromatography was monitored using UV detection at a wavelength of 260 nm. Product of the reaction, N2-adduct, [§§] was collected and then lyophilized. (Yield 10 %).This product was stable in DMSO-d_6 solution, i.e. in the aprotic solvent containing some amount of water. The reaction run in neat water at pH 6 gave the same result (see ESI†for results and spectra). Both experiments prove therefore that QM formed from 7-ethyl-9-(*N*-morpholinyl)methyl-10-hydroxycamptothecin reacts with model 2'-deoxyguanosine at near physiological conditions to give a single thermodynamic product as a result of a reaction at weak nucleophilic site N2. No evidence for equilibration with other nucleophilic sites was found. This result differs from earlier disclosures of Rokita et al. [8,9, 11]However, this can be attributed to the fact that the present results were run in biological solvent and the QM has largely different electronic structure than methide derived from simple phenol.

Figure 2 documents the structure of an adduct. The definitive structure proof is given by the scalar coupling between 2-NH proton and 24-CH_2 protons and further documented by the COSY spectrum cited in ESI.† The ^{1}H/^{13}C HSQC, HMBC and PFGSE spectra are also cited in ESI.†

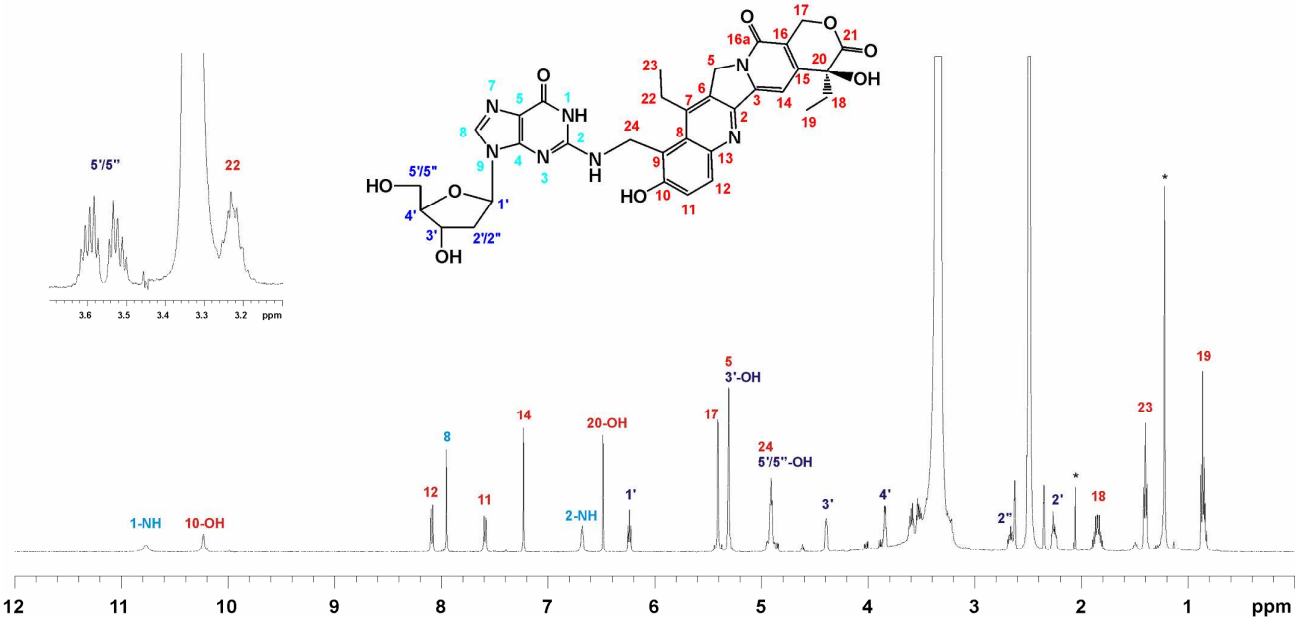


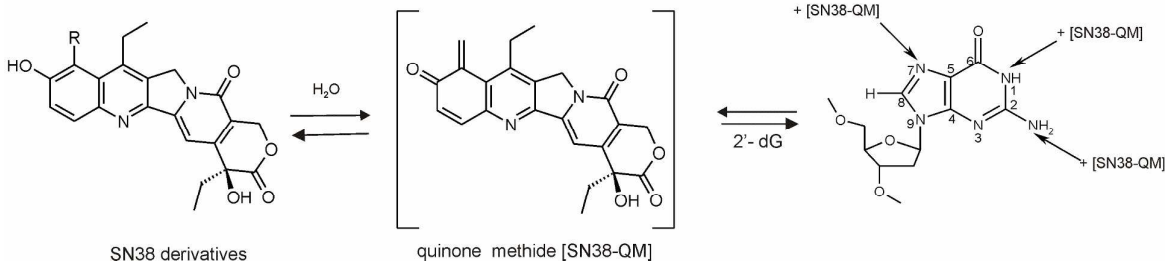
Fig. 2 ^{1}H NMR spectrum of adduct of 2'-deoxyguanosine 2-NH_2 and methide derived from alkylamino substituted SN38 in DMSO-d_6 solution without water presaturation. Insert shows the signals close to water in a spectrum recorded under weak water presaturation. The MS spectrum indicates that only lactone form is present.

o-Quinone methides (*o*-methylene quinones) attract recently wide interest in organic synthesis.[13] The application to alkylation of biological systems was presented earlier by Rokita et al. in sequence specific reactions and under control of either biomimetic reaction or near UV-irradiation.[14, 15] There are no reports on compounds which are able to alkylate DNA without assistance of chemistry inducing methide formation and therefore which can be potentially used as medicines *in vivo*. In this context we were interested in modification of camptothecin core which could enable to use this class of compounds for alkylation of the aromatic bases in Top I induced nicks in nicked DNA. We supposed that active *o*-methylene quinones can be useful. With respect to that we have recently reported that trimethylammonium salts of Topotecan (TPT) alkylate DNA oligomer under near UV irradiation.[16] Further attempts in this direction led us to a discovery of compounds which spontaneously bind to DNA oligomers and can therefore be used *in vivo* as Top I inhibitors in tumor chemotherapy. Scheme 2 shows suggested course of a reaction in water solution at near biological conditions. R substituent stands for the alkylaminomethyl group, here as N-morpholinomethyl.

The above mechanism seems plausible in view of the fact that the ESI MS spectrum discloses the presence of the methide (see ESI). However direct nucleophilic substitution at C-24 carbon atom of camptothecin core should as well be considered. The presence of parent SN38 in the products of alkylation reaction also needs explanation. These mechanistic issues are studied in ongoing research.

Using NMR and LC-MS we present the evidence that *o*-methylene quinone derived from 9-alkylamino substituted SN38 binds spontaneously 2'-deoxyguanosine selectively at N2 nitrogen atoms independent on conditions, either in pure water at pH 6 (Fig 2S) or in buffered water (25 mM NaCl/25 mM K_3PO_4) at pH 7 (Fig. 1).

To the best of our knowledge there are no reports on compounds from camptothecin family which are able to alkylate 2'-deoxyguanosine



Scheme 2 Suggested reaction course of new SN38 derivatives with model 2'-deoxyguanosine

without application of chemistry inducing methide formation and therefore which can be potentially used as medicines *in vivo.*

In this context here we report the first documented case of spontaneous covalent binding of 2'-deoxyguanosine by potential Top I inhibitor which may have perspective of being active *in vivo* belonging to the class of compounds used in clinic. We believe that the phenomenon disclosed here is a good starting point for further experiments confirming the potential use of this class of compounds[17] in clinic. Further studies regarding the binding of the title compounds to oligonucleotides are in progress.

EXPERIMENTAL

2'-deoxyguanosine hydrate was purchased from Aldrich and used as obtained (purity grade was > 95%). HPLC grade solvents were used in chromatography. Purity of bioconjugate was > 95%.

NMR Spectroscopy

The 500 MHz ^{1}H NMR spectra in H_2O were run on Varian NMR spectrometers in a 12 kHz spectral window, using the WATERGATE window sequence[18] for water suppression for H_2O/D_2O (90/10 vol %) solutions (1–2 ms soft pulses) and simple presaturation for water suppression in dmso-d6 solution. TSPA-d_4 was added to monitor chemical shifts. For more details on, $^{1}H/^{13}C$-HSQCAD, HMBC, and PFGSE spectra see ESI.

LC-MS experiments

The LC-MS measurements were carried out on a High-Performance Liquid Chromatograph Prominence LC-20 (Shimadzu) coupled with a 4000 QTrap mass spectrometer. The LC separation was performed on Eclipse XDB C18 column 5 µm, 4.6 x 250 mm (Agilent) using amobile phase at pH 5.4 consisting of 10 mM CH_3COONH_4 aqueous solution (A) and MeCN (B), with the following gradient: 0-30 min 10 → 40% B, 30-35 min 40% B. Flow rate of the mobile phase was: 1.0 ml/min. The analysis was performed at 254 nm and 260 nm. The electrospray ionisation mass spectrometry (ESI-MS) spectra were recorded in the positive and negative ion mode, in the m/z range 100-1000. The source parameters set was as follows: IS 4500 V, DP 80 V, EP 10 V, CUR 25, GS1 55, GS2 45 and the source temperature was 500°C.

The financial support of a research from National Research Center by means of grant no. 2012/07/B/ST4/00566 is appreciated by the authors.

Notes and references

§ To the solution of 2'-deoxyguanosine hydrate (0.65 mg, 2.25 x 10^{-3} mmol) in buffered water (25 mM NaCl/25 mM K_3PO_4, 2.25 ml D_2O) at pH 7 was added 7-ethyl-9-(*N*-morpholinyl)methyl-10-hydroxycamptothecin hydrochloride (2.37 mg, 4.5x10^{-3} mmol). The final reaction solution contained 1.0 mM dG and 2.0 mM SN38 derivative (pH=7).

§§ HR-MS (ESI): calculated mass for $C_{33}H_{32}N_7O_9$ [M-H]- : 670.2262, measured 670.2255.

^{1}H NMR δ, ppm (DMSO) 500 MHz: 0.86 (t, J=7.3 Hz, 3H, CH_3-19), 1.40 (t, J=7.3 Hz, 3H, CH_3-23), 1.85 (m, 2H,CH_2-18), 2.25 (m, 1H,CH-2'), 2.66 (m, 1H, CH-2''), 3.23 (m, 2H, CH_2-22), 3.52 (m, 1H, CH-5'), 3.59 (m, 1H, CH-5''), 3.83 (m, 1H, CH-4'), 4.39 (m, 1H, CH-3'), 4.91 (bs, 2H, CH_2-24), 4.91 (bs, 1H, OH-5'), 5.30 (s, 2H, CH_2-5), 5.31 (s, 1H, OH-3'), 5.40 (s, 2H, CH_2-17), 6.23 (t, J=7.1 Hz, 1H, CH-1'), 6.48 (s, 1H, OH-20), 6.68 (bs, 1H, NH-2G), 7.23 (s, 1H, CH-14), 7.58 (d, J=9.0 Hz ,1H, CH-11), 7.95 (s, 1H, CH-8G), 8.08 (d, J=9.0 Hz, 1H, CH-12), 10.23 (s, 1H, OH-10), 10.77 (bs, 1H, NH-1G).

^{13}C NMR δ, ppm (DMSO) 500 MHz; HSQC/ HMBC : 8.9 (CH_3-19), 15.4 (CH_3-23), 25.5 (CH_2-22), 30.9 (CH_2-18), 38.2 (CH_2-24), 39.9 (CH_2-2'/2''), 50.6 (CH_2-5), 62.6 (CH_2-5'/5''), 65.8 (CH_2-17), 71.5 (CH-3'), 72.9 (C-20), 83.8 (CH-1'), 88.3 (CH-4'), 96.5 (CH-14), 117.5 (C-5G), 118.7 (C-16), 120.3 (C-8), 122.2 (CH-11), 131.4 (C-6), 133.2 (CH-12), 136.8 (CH-8G), 144.4 (C-7), 145.8 (C-13), 146.8 (C-3), 150.7 (C-15), 148.9 (C-2), 150.8 (C-4G), 157.1 (C-10), 157.4 (C(O)-16a), 173.2 (C(O)-21).

UV (in DMSO) nm: 271.3, 337.2, 371.2, 389.5, 452.1.

IR (in DMSO, BaF_2, d=99,8 µm) cm^{-1}: 991, 939, 964, 1154, 1181, 1295, 1462, 1604, 1663, 1873, 2016, 2159, 2540, 2562, 3232, 3384, 3545.

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Synopsis: 7-ethyl-9-(*N*-morpholino)methyl-10-hydroxycamptothecin spontaneously binds covalently to 2'-deoxyguanosine at near physiological conditions. This new phenomenon may have important significance in application of this type of new camptothecin derivatives in cancer chemotherapy.