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Identification of a sulfonoquinovosyldiacylglyceride from *Azadirachta indica* and studies on its cytotoxic activity and DNA binding properties

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ABSTRACT

Chromatographic separation of the methanolic extract of the leaves of *Azadirachta indica* led to the isolation of a sulfonoglycolipid characterized as a sulfonoquinovosyldiacylglyceride (SQDG), by extensive 2D NMR and mass spectral analysis. SQDG induces apoptosis in a dose dependent manner with IC_{50} 8.3 μM against acute lymphoblastic leukemia (ALL) MOLT-4 cell lines. The compound showed significant DNA binding properties as evidenced by the enhancement of melting temperature and perturbation of the characteristic B-form in CD evidence of calf thymus DNA. The DNA binding was also characterized by isothermal calorimetry where a predominantly enthalpy driven binding to CT DNA was revealed.

Use of natural sources for the development of medicine to counter human diseases has had a long and bountiful past from ayurvedic medicine and over the counter treatment to modern ethical drugs.[1] Indeed the majority of prescription drugs and nutraceuticals have their origin in nature's products.[2–4] DNA-intercalating anti-tumor drugs constitute an important class of compounds in anti-cancer therapy.[5] Search for DNA intercalating agents from plant sources therefore continues to be an attractive field of research.

In the Indian sub-continent, Neem (Indian lilac, *Azadirachta indica* A. Juss, syn. *Melia azadirachta* L., family Meliaceae) has attracted attention for a long time due to its wide range of biological activities. Almost every part of the tree is bitter and finds application in indigenous medicine. It is reported that extracts of different parts of this plant and its constituents display a vast array of biological activities[6] such as antimalarial,[7,8] immunomodulatory,[9] spermicidal,[10,11] vaginal contraceptive,[12] antifungal,[13] antibacterial,[14] insecticidal[15,16] and antifeedant.[17,18] Till now about 200 compounds belonging to different classes have been isolated and characterized from different parts of this plant.[19] These can be broadly divided into two major classes, viz isoprenoids and non-isoprenoids. As a part of our continuous search for bioactive secondary plant metabolites,[20–22] we carried out investigation of *A. indica* leaves with emphasis on DNA binding and anti-cancer activity.

The air-dried, powdered leaves of neem[23] were defatted and repeatedly extracted with MeOH at ambient temperature. Chromatographic resolution of the MeOH extract yielded one sulfonoglycolipid (SQDG)[24] and three flavone glycosides characterized as quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside and quercetin-3-*O*-β-glucopyranoside. The sulfonoglycolipid (SQDG) was assigned the molecular formula $C_{37}H_{69}O_{12}SNa$ based on the observed pseudomolecular ion peak at *m*/*z* 817 (weak) and 839 (strong) attributed to $[M+H]^+$ and $[M+Na]^+$ ions, respectively. The preliminary study of the ^1H and ^{13}C NMR spectra pointed to the presence of the following isolated spin systems. A 6-carbon unit corresponding to a C_6 sugar with upfield carbon (54.5) and proton (2.90 and 2.55) signals for a methylene corresponded with a 6-sulphono-6-deoxy hexose unit. The chemical shifts of other carbon atoms showed it to be a sulfonoquinovopyranose unit, the anomeric configuration of which must be α from the observed $J_{1,2}$ value (3 Hz). A three carbon unit with characteristic carbon and proton chemical shifts agreed with a glycerol moiety. The rest of the signals accounted for two fatty acyl moieties attached to two consecutive carbon atoms of the glycerol unit, the third of which was attached to C-1 of the sugar unit (HMBC). The fatty acids are concluded to be mainly saturated C_{16} ones based on mass spectral evidence coupled with NMR assignments. Similar products have been reported earlier from the red alga *Chondria*

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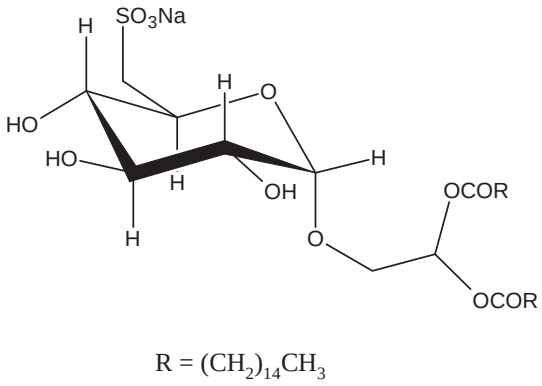

Figure 1. Sulfonoquinovosyldiacylglyceride (SQDG).

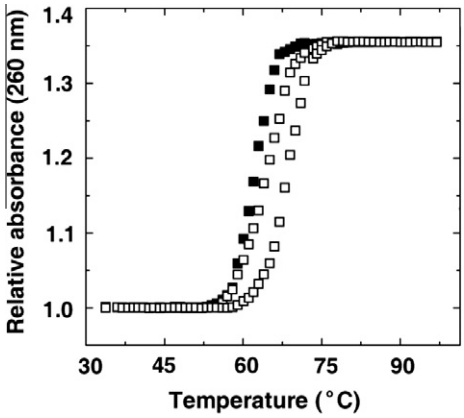

Figure 3. Thermal melting profiles of CT DNA (40 μM) (■) treated with SQDG at a drug/nucleotide molar ratio of 0.35 and 0.65 (□) in 10 mM CP buffer, pH 7.0.

crassicaulis,[25] the tropical tree *Byrsonima crassifolia*,[26] and the basidiomycete *Dictyonema glabratum*[27] among others. The NMR data of our compound compared well with that reported by Sassaki et al.[27] recorded in the same solvent. This communication is the first report of isolation of sulfonoquinovosyldiacylglyceride (Fig. 1) from neem leaves.

This compound was water soluble and was tested for its anti-leukemia activity. Acute lymphoblastic leukemia (ALL) cell line MOLT-4 was cultured[28] for 24–72 h with and without SQDG using RPMI 1640 medium supplemented with fetal bovine serum, penicillin and streptomycine. Anti-proliferative effects and induced apoptosis due to SQDG (0–50 μM) against MOLT-4 were measured by the MTT dye uptake method.[29] The effect noticed at different concentration and time period is presented in Figure 2. Maximal apoptotic effect was observed at 24 h time point, after which there was no significant change in anti-leukemic activity. This may be due to the fact that the cells become anergized after that point. It was observed that SQDG induced apoptosis in a dose dependent manner with IC_{50} 8.3 μM, which suggests that the compound is having considerable anti-leukemic activity.

For therapeutic importance in cancer treatment, compounds having anti-cancer activity as well as DNA-binding property are preferred. Consequently a series of experiments were performed to determine whether this compound had any propensity for binding with DNA.

The binding of the SQDG to double stranded calf thymus (CT) DNA was evaluated initially from optical thermal melting studies.[30] Double stranded calf thymus DNA under the conditions of the experiment melted with a T_m value of 62 °C (Fig. 3). The melting temperature of the DNA was enhanced and at saturation a ΔT_m of about 10 °C was observed. Such a large stabilization of the DNA helix appears to be due to the strong binding of the compound to the duplex DNA. The binding of SQDG to DNA was further evaluated from circular dichroic studies. Characteristic CD spectrum of calf thymus DNA displayed a canonical B-form conformation with a large positive band at 270–280 nm and a negative band at 248 nm. A small positive band at 210 nm is also apparent for the B-form structure. These CD bands of the DNA are caused due to stacking interactions between the bases and the helical structure that provide asymmetric environment for the bases. All the bands were perturbed in presence of increasing concentrations of SQDG (Fig. 4) resulting in an increase of the 275 nm band and a concomitant decrease of the 248 nm band. The 210 nm band also showed large enhancement in presence of SQDG. The changes in the CD of the DNA on binding reflect an effective coupling of the transition moments of the bound SQDG with that of the base pairs and most likely resulting from intercalative binding.

Isothermal titration calorimetry (ITC) was used to thermodynamically characterize the binding of SQDG under identical buffer conditions as used for thermal melting and circular dichroic experiments. Figure 5 (upper panel) shows the representative raw heat profile resulting from a typical ITC experiment in which SQDG was titrated from the syringe into calf thymus DNA solution in the calorimetric cell. The titration resulted in a single exothermic

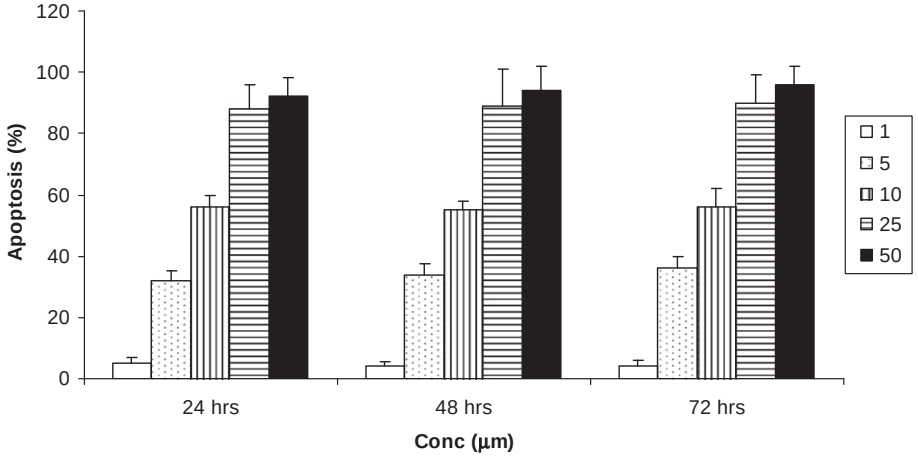

Figure 2. Effects of sulfonoquinovosyldiacylglyceride (SQDG) on MOLT-4 cell viability. MOLT-4 cells (1×10^5 cells/well) were incubated in the presence or absence of SQDG at different concentrations (0–50 μm) for different time periods (24–72 h). The bars represent ± standard deviation of $n = 6$ independent experiments.

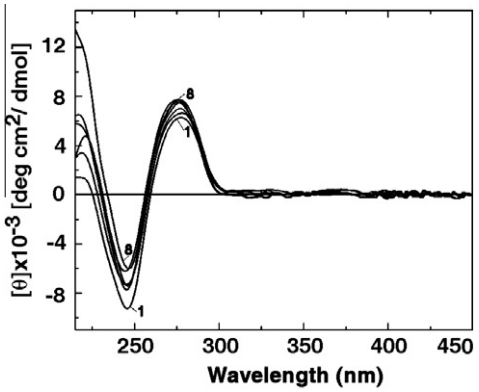

Figure 4. CD spectra resulting from the interaction of SQDG with CT DNA (60 μM). Curves 1–8 denote the interaction of DNA treated with 0, 3.2, 6.3, 8.5, 12.5, 18.3, 25.0 and 30.2 μM of SQDG.

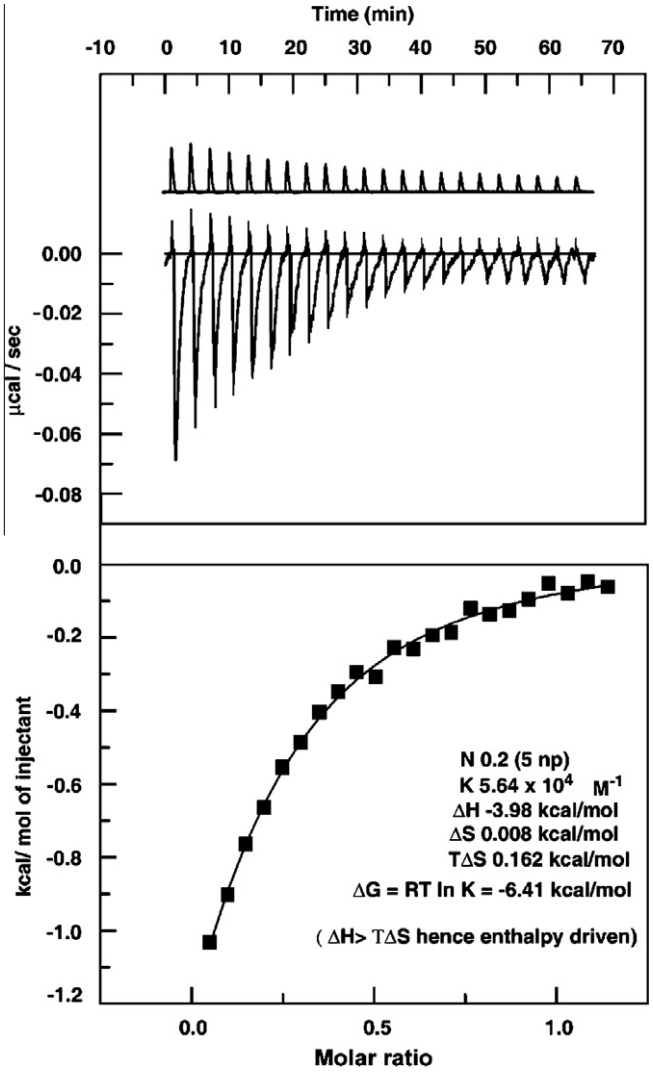

Figure 5. ITC profile for the titration of SQDG into a 50 μM solution of CT DNA in 10 mM CP buffer, pH 7.0 at 20 °C. Each heat burst curve in the upper panel is the result of a 7 μL injection from 500 μM solution of SQDG into the DNA solution. The upper panel shows the heat burst for the injection of SQDG into the same buffer as control (curves offset for clarity). Lower panel represents the corresponding normalized heat signals versus molar ratio. The data points reflect the experimental injection heat while the solid line represents the calculated fit of the data.

binding event enabling the data to be fitted to a single set of identical sites model. To extract the binding and thermodynamic parameters of the interaction, the thermogram was fitted to a single site model (lower panel) and the thermodynamic parameters were estimated from the best fit to the observed heat release. The data were analyzed with several different initial guesses and the resulting fits gave consistent values of the parameters, $K_a = 5.64 \pm 0.26 \times 10^4\ M^{-1}$, $\Delta H = -3.98 \pm 1.40$ kcal/mol, a $T\Delta S$ of 0.162 kcal/mol and a binding site size of ~5 nucleotides (1/N). The small entropy term suggested the binding of SQDG to CT DNA to be predominantly enthalpy driven. The DNA binding of several intercalators and groove binders have been shown to be largely enthalpy driven.[31] It is likely that the interaction of the compound may involve a variety of non-covalent interactions including stacking interactions from intercalation binding, all of which may contribute to the negative enthalpy.

In summary, we have disclosed for the first time the isolation of SQDG from neem leaves and its characterization by 2D NMR and mass spectral studies. It induces apoptosis of MOLT-4 cell lines in a dose dependent manner with IC_{50} 8.3 μM. The compound has strong DNA binding properties; the binding process is exothermic and enthalpy driven. The findings point to its possible usefulness as an anti-cancer agent.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.007.

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23. Matured leaves of *Azadirachta indica* were collected from the medicinal plant garden of R. K. Mission, Narendrapur, Kolkata, during January 2009 and identified by Dr. Shyamal Ghosh, Professor of Botany, Bidhan Chandra Krishi Viswavidyalaya, Kalyani, West Bengal. A voucher specimen (No. 112) was deposited in the Chemistry Department, Indian Institute of Chemical Biology, Kolkata.
24. SQGD: $[\alpha]_D$ +39.2 (*c* 0.5, MeOH); ^{1}H NMR (DMSO-d_6) 0.84 (t, *J* = 6.6 Hz), 1.22 (m), 1.48 (m), 2.25 (m), 2.31 (m), 2.55 (dd, *J* = 6.6, 13.8 Hz), 2.90 (m), 3.18 (m), 3.77 (m), 3.88 (dd, *J* = 6.0, 10.2 Hz), 4.13 (dd, *J* = 7.8, 11.4), 4.34 (d, *J* = 10.8 Hz), 4.57 (d, *J* = 3.0 Hz), 4.67 (d, *J* = 6.0 Hz), 4.78 (d, *J* = 4.2 Hz), 5.13 (m), 5.39 (m); ^{13}C NMR (DMSO-d_6) 13.9, 22.2, 24.5, 27.7, 28.2, 28.6, 28.8, 28.9, 29.0, 29.1, 29.2, 31.4, 33.5, 33.6, 40.0, 54.5, 62.7, 64.6, 68.6, 69.7, 71.6, 72.9, 74.2, 98.3, 172.4, 172.5; mass spectrum: 839.51 $[M+Na]^+$, 817.53 $[M+H]^+$.
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