International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 3, 2016

Short Communication

CETYL ALCOHOL AND OLEIC ACID SOPHOROLIPIDS EXHIBIT ANTICANCER ACTIVITY

PARUL DUBEY[1,#], PRERNA RAINA[2,#], ASMITA PRABHUNE[1*], RUCHIKA KAUL-GHANEKAR[2*]

[1]Biochemical Sciences Division, CSIR-National Chemical Laboratory, Dr. Homibhabha Road, Pune 411008, India, [1]Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University Medical College Campus, Dhankawadi, Pune 411043, India
Email: ruchika.kaulghanekar@gmail.com

Received: 15 Dec 2015 Revised and Accepted: 03 Feb 2016

ABSTRACT

Objective: Sophorolipids (SLs) are glycolipid biosurfactants that have been shown to have anticancer activity. We investigated the anti-cancer activity of cetyl alcohol sophorolipids (CAS) and oleic acid sophorolipids (OAS) in breast cancer (MCF-7, MDA-MB-231), cervical cancer (SiHa, HeLa) and non-cancerous (HaCaT and RAW264.7) cell lines.

Methods: For cell viability assay, MCF-7, MDA-MB-231, SiHa, HeLa, HaCaT and RAW264.7 cell lines were treated with different concentrations (0-160 µg/ml) of OAS and CAS for 24h. The cell viability was determined by MTT dye uptake method. Cell proliferation assay was determined by using trypan blue dye exclusion method.

Results: Our preliminary data shows that compared to OAS, CAS exhibited more significant reduction in the viability of MCF-7, MDA-MB-231 and SiHa. However, compared to CAS, OAS induced more decrease in viability in HeLa cells. Interestingly, both the types of SLs did not affect the viability of non-cancerous cells. Moreover, CAS, when used as a coating material, induced proliferation in macrophage cell line, RAW264.7.

Conclusion: The present study provides an important clue towards the anti-cancer potential of OAS and CAS derived from *Candida bombicola.* Interestingly, the ability of CAS to promote the proliferation of non-cancerous cells suggests its future application as a scaffold for enhancing the adhesion and proliferation of normal cells.

Keywords: Oleic acid sophorolipids, Cetyl alcohol sophorolipids, Breast cancer, Cervical cancer

Biosurfactants, derived from non-pathogenic organisms, are extensively gaining recognition due to biodegradability and reduced environmental impact compared to the chemical surfactants that cause the detrimental effect on the environment due to bioaccumulation and eutrophication [1]. The sophorolipids (SLs) are biosurfactants belonging to the glycolipids class, produced by many non-pathogenic yeasts such as *Candida apicola*[2], *Candida bogoriensis* [3], *Wickerhamielladomercqiae* [4], *Pichiaanomala* [5], *Candida guilliermondii* [6] and *Candida bombicola* [7, 8]. SLs show structural similarity with glycosphingolipids and gangliosides, the membrane components of mammalian cells, which are involved in processes such as signaling, oncogenesis, and differentiation [9]. SLs have been shown to have various pharmacological properties including anti-cancer activity. It has been reported that natural mixture of SLs or select derivatives [ethyl ester, methyl ester, ethyl ester monoacetate, ethyl ester diacetate, acidic sophorolipid, lactonic sophorolipid diacetate] could induce necrosis in human pancreatic carcinoma cells. SLs produced by *Candida bombicola* NRRL Y-17069 have been shown to exhibit anticancer activity against hepatocellular carcinoma (HepG2) and lung cancer (A549) cell lines [10]. SLs with one double bond in fatty acid part in their structure have been reported to have the potent cytotoxic effect on esophageal cancer cell lines (KYSE109 and KYSE450) [11]. SLs produced by *W. domercqiae* have been demonstrated to induce cell death via apoptosis in human liver (H7402) and lung cancer (A549) cell lines [12]. We have recently reported SLs of oleic and linoleic acids in pure acidic and crude forms could induce differentiation in LN-229, a glioma cell line [9].

Recently, we have reported the use of cetyl alcohol as the lipophilic carbon feed source for *Candida bombicola* (ATCC 22214) that was incorporated into the sophorolipid molecule synthesized by the yeast [13]. The effectiveness of cetyl alcohol sophorolipids (CAS) as a surfactant was shown by measuring its surface tension lowering capacity, which was found to be significantly more as compared to the oleic acid derived sophorolipids (OAS) [10]. The antibacterial activity of CAS against *Staphylococcus aureus* and *Escherichia coli* was found to be higher in comparison to OAS [13]. In the present work, we have further compared the anticancer activity of CAS and OAS on breast, cervical and non-cancerous cell lines. We have also evaluated the potential of CAS as a biocoat for tissue culture plastic ware that would help in attachment and proliferation of the normal cells.

For *in vitro* assays, Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum, penicillin/streptomycin, L-glutamine and MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide] were procured from Sigma-Aldrich (St. Louis, MO). Tissue culture plasticware was purchased from BD Biosciences, CA, USA. The cell lines MCF-7, MDA-MB-231, SiHa, HeLa, HaCaT and RAW264.7 were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2 mM L-glutamine supplemented with 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin. The cells were incubated in a humidified 5% CO2 incubator at 37 °C. Glucose was purchased from Qualigens, India. Malt extract, yeast extract and peptone were procured from Hi-media, India. Cetyl alcohol (C_{16}) was received as a kind gift from Galaxy Surfactants, India.

The yeast used in this study was *Candida bombicola* ATCC 22214. It was grown in MGYP medium (containing 0.3% malt extract, 2% glucose, 0.3% yeast extract, 0.5% peptone) at 30 °C. The culture was maintained by periodic transfer on MGYP agar slants at 4 °C. A loopful of *Candida bombicola* ATCC 22214 cells were taken from plant and seed culture was developed by transferring it to 10 ml medium [consisting of g/l: glucose, 100; yeast extract, 1.0; $MgSO_4.7H_2O$, 0.3; Na_2HPO_4, 2; NaH_2PO_4, 7; $(NH_4)_2SO_4$, 1], for 24 h at 30 °C with 180 rpm orbital shaking [14]. The seed culture was transferred to 40 ml medium for developing the starter culture and incubated for 24 h at 30 °C with 180 rpm orbital shaking. The fermentative production was further carried out by transferring the starter culture into 200 ml of above-mentioned medium in 1 L Erlenmeyer flask and incubated for 120 h at 30 °C with 180 rpm orbital shaking. The medium was supplemented with cetyl alcohol as lipophilic substrate at the concentration of 1 g/100 ml dissolved in 0.5 ml ethanol.

Candida bombicola cells were harvested from the culture broth by centrifugation at 5,000 rpm, 10 °C for 20 min. An equal volume of ethyl acetate was added to supernatant for extracting the sophorolipids as described elsewhere [15]. The aqueous layer was re-extracted until no emulsion was formed at the interface. Sodium sulphate was added to remove any traces of water. The solvent was filtered and reduced by rotary evaporation under vacuum to yield a brown colored viscous product that was stored at 4 °C.

The cell viability study was performed by using MTT dye [16, 17]. MCF-7, MDA-MB-231, SiHa, HeLa, HaCaT and RAW264.7 cell lines were grown in DMEM supplemented with 2 mM L-glutamine, 100 units/ml of penicillin/streptomycin and 10% fetal bovine serum and incubated in a humidified 5% CO2 atmosphere at 37 °C. The cells were seeded at 1×10^5cells/ml density in 96-well plates (Axygen TPP, CA, USA). After 24 h, the cells were incubated with fresh medium containing oleic acid sophorolipids (OAS), oleic acid (OA), cetyl alcohol sophorolipids (CAS) and cetyl alcohol (CA). The OAS, CAS, CA were added at concentrations ranging from 0-160 µg/ml whereas OA was added at following concentrations 2.82, 5.64, 11.29, 22.59, 45.19 and 90.38µg/ml in each well (in triplicates). The plates were incubated overnight at 37 °C in 5% CO_2 incubator. The MTT solution (5 mg/ml) was added to each well, and the cells were cultured for another 4h at 37 °C in 5% CO2 incubator. The resulting formazan crystals were dissolved in 90 µl of SDS in DMF. The intensity of colored formazan derivative was determined by measuring optical density (OD) using ELISA microplate reader (Biorad, Hercules, CA) at 570 nm (OD570–630 nm). The mean OD value was used for assessing the cell viability expressed as the percentage. From the graphs of % viability versus concentrations, IC_{50} values were obtained.

$$\% \text{ Viability} = (\text{Treated cells}/\text{Control cells}) \times 100$$

The non-treated polystyrene 35 mm dishes were coated with 0.1% of CAS, OAS and gelatin for 4 h. The excess of sophorolipids and gelatin was removed from the dishes. The plates were allowed to dry under sterile conditions. RAW264.7 cells were seeded at a density of $5x10^4$ cells/ml in the coated dishes. The plates were incubated overnight at 37 °C in 5 % CO_2 incubator. After 24 h, the cells were trypsinized and counted by using trypan blue dye exclusion method [16, 17].

All the results were obtained from three independent experiments, each performed in triplicates and the values have been presented as mean±SD. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA). For multiple comparisons, Tukeys test was used. The analyses were carried out using Graph pad prism 5 software (San Diego, CA, USA). *p<0.05; **p<0.01; ***p<0.001 were considered to be statistically significant.

Recently, there have been many reports demonstrating the usage of sophorolipids as novel anti-cancer agents. The sophorolipids studied so far have been shown to exhibit cytotoxicity against liver, lung, pancreatic and esophageal carcinoma cell lines, with only a few studies on breast cancer cell lines [14]. We have for the first time evaluated the inhibitory effect of OAS and CAS on the viability of breast cancer cell lines, MCF-7 (ER+/PR+/Her2-) and MDA-MB231 (ER-/PR-/Her2-) by using MTT assay. Both MCF7 and MDAMB231 cells were treated with different concentrations (0-160 µg/ml) of CAS and OAS for 24 h, followed by MTT assay the next day. In MCF-7, CAS was found to decrease the viability of cells more significantly than OAS with IC_{50} value of 151.38 µg/ml for CAS (table 1). Contrarily, cells treated with OAS exhibited around 96% viability up to 160 µg/ml dose. A recent report has demonstrated anti-cancer activity of *Candida bombicola* derived sophorolipids against hepatocellular carcinoma (HepG2) and lung cancer (A549) cell lines with no activity towards breast cancer (MCF-7) cells [14]. In MDAMB231 cells, we found that CAS reduced the viability of the cells to a higher extent (IC50 value of 82.93 µg/ml) compared to OAS. On the other hand, cells treated with OAS exhibited around 80% viability up to 160 µg/ml dose. A recent study reported that SLs derived from *Starmerella bombicola* inhibited the cell migration of MDA-MB-231 without compromising cell viability and also increased intracellular reactive oxygen species [15]. To find out whether the activity exhibited by OAS and CAS was sole because of the sophorolipids and not because of their individual substrates (OA and CA), we tested their effect on the viability of the cancer cells (Supplementary tables 1 and 2, respectively). In response to OA and CA, both the cell types exhibited 100% viability up to 2.8 and 160 µg/ml dose, respectively. Interestingly, the amount of OA used in OAS was 0.01 µg/ml and, hence, whatever cytotoxicity was observed in MDA-MB231 was solely due to OAS. These results suggested that CAS exhibited significant anticancer activity in breast cancer cells compared to OAS and could be further explored for their detailed anti-cancer activity *in vitro* and *in vivo*.

Table 1: Effect of OAS and CAS on cell viability of MCF-7 and MDAMB231

Concentration of SLs (µg/ml)	% Viability			
	MCF-7		MDAMB231	
	CAS	OAS	CAS	OAS
0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
10	84.6 ± 7.3^{a}	100.0 ± 2.6^{b}	68.1 ± 2.2^{k}	89.0 ± 8.4^{p}
20	83.1 ± 9.5^{c}	96.4 ± 3.5^{d}	59.8 ± 2.6^{l}	84.9 ± 3.5^{q}
40	82.7 ± 10.7^{e}	92.2 ± 4.4^{f}	57.3 ± 3.8^{m}	83.2 ± 7.7^{r}
80	78.9 ± 10.5^{g}	97.6 ± 5.3^{h}	51.1 ± 2.6^{n}	82.8 ± 10.6^{s}
160	64.1 ± 19.2^{i}	95.8 ± 7.9^{j}	26.1 ± 1.9^{o}	80.3 ± 10.8^{t}

Values are represented as mean±SD of three independent experiments (n=3). OAS: oleic acid sophorolipids, CAS: cetyl alcohol sophorolipids. Tukey's multiple comparison test was done showing: "a" compared to "b", p<0.05; "c" compared to "d", p<0.01; "e" compared to "f", p<0.001; "g" compared to "h", p<0.001; "i" compared to "j", p<0.001; "k" compared to "p", p<0.001; "l" compared to "q", p<0.001; "m" compared to "r", p<0.001; "n" compared to "s", p<0.001; "o" compared to "t", p<0.001.

To our knowledge, there are no reports demonstrating the cytotoxicity of SLs against cervical cancer. We have for the first time evaluated the cytotoxicity of OAS and CAS in cervical cancer cell lines, SiHa (HPV16+) and HeLa (HPV18+). Both the cell lines were treated with different concentrations (0-160 µg/ml) of CAS and OAS for 24 h, followed by MTT assay the next day.

SiHa cells, upon treatment with CAS and OAS, exhibited around 81 and 96% viability, respectively, at 160 µg/ml concentration (table 2). On the other hand, HeLa cells, upon treatment with CAS and OAS, exhibited 53.6 and 3.7% viability at 160µg/ml concentration, respectively. Interestingly, IC_{50} values of CAS and OAS were 100.55and 22.84µg/ml, respectively, in HeLa cells suggesting that OAS was more toxic to HeLa cells at lower doses. Further, to find out whether the activity exhibited by OAS and CAS was not because of their individual substrates (OA and CA), we tested their effect on the viability of the cervical cancer cells (Supplementary tables 1 and 2, respectively). We found that SiHa and HeLa cells showed95 and 70% viability up to 160µg/ml concentration of CA, respectively, and100% viability up to 5.64 µg/ml dose of OA (Supplementary table 2). As discussed before, the amount of OA used in OAS was 0.01µg/ml and hence, the cytotoxicity observed in SiHa and HeLa cells was solely due to OAS.

Thus, from the above preliminary results, it could be concluded that CAS effectively reduced the viability of both the breast and cervical cancer cell lines and thus could be further explored for its antineoplastic potential to understand the underlying signal transduction mechanisms at in-vitro and *in vivo* levels. After evaluating the effect of SLs on the viability of cancerous cell lines, we wanted to find out whether they were toxic to the non-cancerous

cells or not. We treated HaCaT cells with different concentrations (0-160 µg/ml) of CAS and OAS for 24 h, followed by MTT assay the next day. Both the SLs were non-toxic to HaCaT cells up to 160 µg/ml dose (fig. 1).

Table 2: Effect of OAS and CAS on cell viability of SiHa and HeLa

Concentration of SPLs (µg/ml)	% Viability			
	SiHa		HeLa	
	CAS	OAS	CAS	OAS
C	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
10	96.4±2.7[a]	100.0±15.2[b]	95.1±4.6[k]	89.3±3.8[p]
20	87.3±8.6[c]	100.0±3.7[d]	93.5±4.9[l]	62.3±10.5[q]
40	84.4±11.3[e]	100.0±7.5[f]	92.6±1.8[m]	19.6±7.7[r]
80	83.9±9.1[g]	100.0±3.6[h]	71.7±0.8[n]	15.3±3.5[s]
160	81.1±12.4[i]	96.0±2.1[j]	53.6±2.5[o]	3.7±3.1[t]

Values are represented as mean±SD of three independent experiments (n=3). OAS: oleic acid sophorolipids, CAS: cetyl alcohol sophorolipids. Tukey's multiple comparisons tests was done showing: "a" compared to "b", p>0.05NS; "c" compared to "d", p<0.01; "e" compared to "f", p<0.05; "g" compared to "h", p<0.01; "i" compared to "j", p>0.05NS (Not significant); "k" compared to "b", p>0.05NS; "l" compared to "q", p<0.001; "m" compared to "r", p<0.001; "n" compared to "s", p<0.001; "o" compared to "t", p<0.001

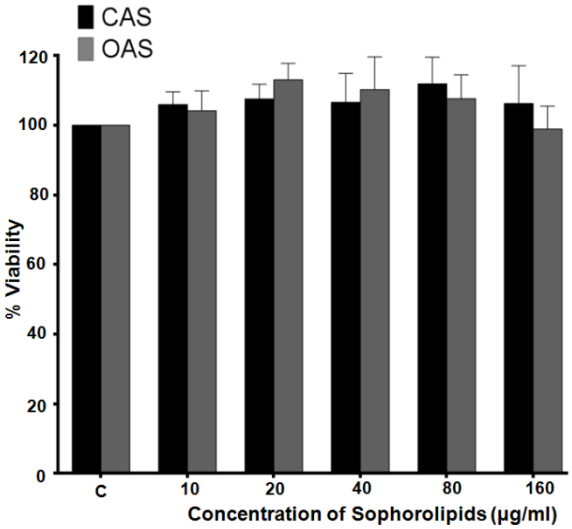

Fig. 1: Effect of OAS and CAS on the viability of HaCaT cells. All the data are presented as mean±SD of five independent experiments (n=5) at p<0.001, indicating statistically significant differences compared to the control untreated group. OAS: oleic acid sophorolipids, CAS: cetyl alcohol sophorolipids

Upon treatment of the cells with individual substrates, OA and CA, they exhibited 100% viability up to 22.59and 160µg/ml doses, respectively (Supplementary Tables 1 and 2). Since CAS showed more promising results in both breast and cervical cancer cell lines, we further evaluated its effect on the viability and proliferation of non-cancerous macrophage cell line, RAW 264.7.

The cells exhibited around 91% viability in response to CAS up to a higher dose of 160 µg/ml (fig. 2).

Interestingly, in the proliferation assay, we found that compared to either uncoated or gelatin coated wells, CAS treated wells increased the proliferation of RAW264.7 cells significantly (fig. 3A). However, upon coating of cancerous cell lines, MCF-7 (fig. 3B) and SiHa (fig. 3C), on CAS coated wells, they showed a slight decrease in proliferation, compared to either uncoated or gelatin coated wells.

Recently, we have demonstrated the use of silk fibroin sophorolipids (SF-SL) as novel scaffolds for promoting the growth of mouse fibroblast cells *in vitro* [18].

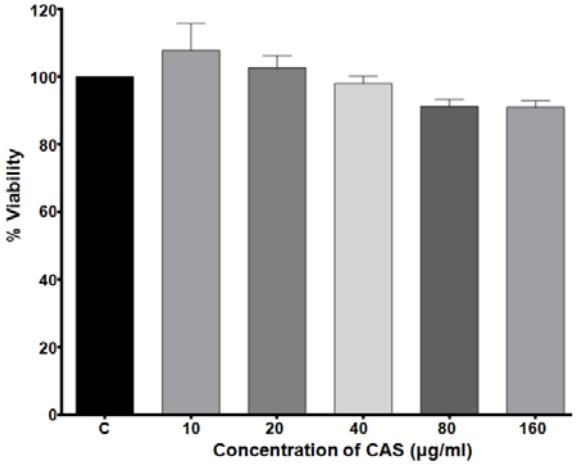

Fig. 2: Effect of CAS on the viability of RAW264.7 cells. All the data are presented as mean±SD of five independent experiments (n=5) at p<0.001, indicating statistically significant differences compared to the control untreated group. CAS: cetyl alcohol sophorolipids

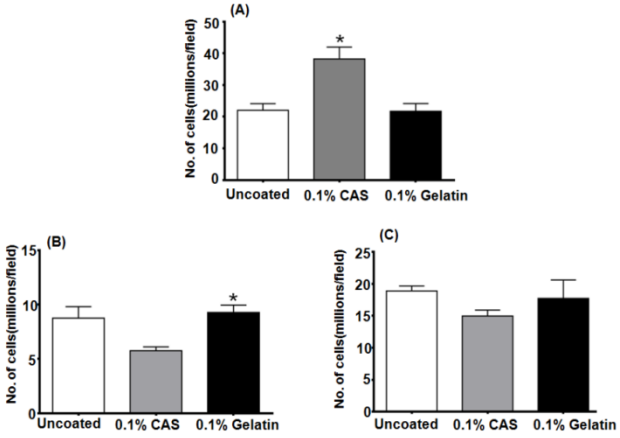

Fig. 3: Effect of CAS on the proliferation of (A) RAW264.7, (B) MCF-7 and (C) SiHa. All the data are presented as mean±SD of three independent experiments (n=3), indicating statistically significant differences compared to the untreated control group. *p<0.05, **p<0.01, *** p<0.001 indicated statistically significant differences. CAS: cetyl alcohol sophorolipids

However, since this is a preliminary work, it requires extensive experimentation and comparisons with other coating materials to ascertain its future application as scaffolds for growing of primary cell cultures. The present study has explored the anti-cancer potential of oleic acid and cetyl alcohol sophorolipids derived from *Candida bombicola* that could be studied in detail in the future. The selectivity of these sophorolipids towards the cancerous cells invites attention towards their usefulness as adjuncts to current therapeutic modalities in cancer. Moreover, the property of cetyl alcohol sophorolipids in promoting the growth of non-cancerous cells could find applications as scaffolds in tissue culture, tissue regeneration, and wound healing.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENT

The authors would like to thank NCL and IRSHA for supporting this work.

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