Proliferative effect of the phycotoxin domoic acid on cancer cell lines: a preliminary evaluation

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\section*{ABSTRACT}
Domoic acid (DA) is a neurotoxin that causes amnesic shellfish poisoning. It is produced by algae and accumulates in shellfish. Consumption of these contaminated shellfishes is suspected to produce exceptional health hazards for humans. Effect of DA on cancer cell lines remained unclear. Human endothelial cells EA.hy 927, Human leukaemia K562 cells and monkey kidney Vero cells were used to evaluate the cytotoxicity of DA. Concentrations ranging from 30 to 120 µM of DA and using Neutral Red and MTT assays demonstrated that this neurotoxin undertook promotion of cell proliferation of the three cell lines in a dose-dependent manner in contrast to untreated control cells, after incubation for 72 h. An increase in the percentage of proliferation was induced by a 120 µM of DA. The proliferation of K562 cells is encouraged by DA more than Vero and EA.hy 927 cells. The study highlighted the activity of DA \textit{in vitro}. This could be taken as reflecting the tumorigenic compound of DA. For establishment of the carcinogenic potential of DA, there is a requirement of further investigations.

\section*{1. Introduction}
Domoic acid (DA) is a potent neurotoxin produced by Algal blooms with inclusion of diatom blooms. Some of the marine phytoplankton produces these algae, such as dinoflagellates. These algae then produce phycotoxins, which are therapeutic \cite{1} and hence include toxic activities \cite{2}. The algal blooms do not only accumulate the DA in the shellfish but also in various other fishes such as anchovies and sardines. Furthermore, the food chain also becomes the reason behind transference of this DA in other sea animals such as sea lions, sea birds, and other many sea animals. This has been perceived as the reason behind illness and sometimes even death of humans and animals. This is dependent on the quantity of consumption of the toxic fish. The toxin is very effective that it could also kill large animals such as whales. This could be proved from the high rate of mortality of Minke whales in April 2007 in southern California by DA intoxication \cite{3}.

The human could only be exposed to the consumption of DA if they consume a specific amount of these contaminated shellfish, which means the shellfishes have accumulated this toxin at the time of its filter-feeding during blooms on toxigenic phytoplankton \cite{4}. The impact held by these phycotoxins on the health of the humans is divided into six types in accordance with its effects on human \cite{2}. They are vomiting, vertigo and nausea due to neurotoxic shellfish poisoning \cite{5} and, necrosis in specific regions of the hippocampus and neuronal degeneration due to amnesic shellfish poisoning \cite{6}.

Concerning the structural appearance of DA, it could be related to Kainic acid, which is soluble in water and also in the non-protein excitatory amino acid \cite{7}. DA remains stable at room temperature; however, sometimes cooking at 121°C would cause a reduction in the concentration of the DA. Although, this does not reduce the level of harm caused by the contaminated shellfish \cite{4}. Recently, there has been an establishment of monitoring of the activities of the DA globally under the water because of the enhanced number of people interested in the consumption of seafood, the pollution, the algal bloom and the threat of DA intoxication \cite{4}.

Other than the effects of DA being neurotoxin, it has further been demonstrated for the induction of apoptosis \textit{in vitro} on cerebellar granule neurons \cite{8}. The cytotoxic activity of DA to neural tissue \textit{in vitro} is undertaken over an extensive series of concentrations. At 10 µM, DA with primary cultures of cerebellar neurons caused cell death \cite{9}. There are various
studies which exposed that not only the central nervous system (CNS) but many other human organs could be adversely harmed by the DA [10–13]. A concentration-dependent cytotoxic effect was reported by Carvalho Pinto-Silva et al. after 72 h of DA exposure to colorectal adenocarcinoma (Caco-2) cells [14]. Nevertheless, when it comes to the tumour cell lines excluding neuronal cells, then there is insufficiency of information available regarding cytotoxic activities of DA. Therefore, this study aims to evaluate the effect of the DA on three distinct cell lines, which are human endothelial cells EA.hy 926, human leukaemia K562 cells and monkey kidney cells Vero by using Neutral Red (NR) and MTT assays.

2. Material and methods

2.1. Chemicals

Foetal Calf Serum (FCS), Dulbecco’s modified Eagle’s medium (DMEM), Trypsin-EDTA, Roswell Park Memorial Institute (RPMI) medium, dimethyl sulfoxide (DMSO), Phosphate Buffer Saline (PBS), mixture of Penicillin and Streptomycin were from Gibco-BCL (UK), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). DA and many other reagents were obtained from Sigma Aldrich (France).

2.2. Cell culture and treatment

The cell lines used in this study are Human Myelogenous Leukaemia (K562), Human Umbilical Vein Cells (EA.hy926) and Vero cells, from green monkey kidney [15] (Biovalori, France). The cultivation of the cells was undertaken in the RPMI-1640 or in DMEM medium supplemented with 100 µg/mL Streptomycin, 100 U/mL Penicillin, 10% FCS and 2 mM L-glutamine as a complete growth medium. Later the cells were maintained in culture and their nurturing took place in an incubator at 37°C keeping an atmosphere of 5% CO2 – 95% air mixture. When the convergence started to reach 80%, all the cells involved in the process were sub-cultured for treatment by separating them through fresh medium at the required density. The viability was approximated by the use of the trypan blue exclusion assay.

2.3. Determination of cell viability

2.3.1. MTT assay

Cytotoxic activities of DA aligning it with cancer cell lines were estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases active in viable cells, the resulting blue formazan product can be measured spectrophotometrically. 96-well plates were used for performing the MTT colorimetric assay. The seeding of the cells was undergone at a concentration of $4 \times 10^5$ cells/well for EA.hy926 in each 96-well plate, for Vero cells concentration of $3 \times 10^3$ cells/well and for K562 cells concentration of $10^5$ cells/well. In an atmosphere enriched with 5% of CO2, the incubation of the cells was made overnight at a temperature of 37°C.

Cells which were in the phase of exponential growth were incubated with DA at concentrations ranging from 30 to 120 µM at 37°C for 72 h. Subsequently, at 37°C for 2 h, the cells were treated with 10 µL of the MTT solution (5 mg/ml stock in PBS) along with the medium being removed. Later, dissolving of the insoluble formazan crystal was required, for which the MTT solution was abandoned and an addition of 50 µL of isopropanol was made. At 540 nm, the optical density was then measured. The effects of DA on cells were estimated in terms of percentage of cell growth to the reference (untreated cells). Each experiment was performed in quintuplicate and an independent repetition of the experiment was ensured.

2.3.2. NR assay

The NR dye was used as a second cytotoxicity assay. It is based on the ability of viable cells to incorporate and bind the supravital dye NR in the lysosomes. The execution of the assay was based on the applications described by the Borenfreund and Puerner [16]. Later, the seeding of the cells was undertaken at a concentration of $4 \times 10^5$ cells/well for EA.hy926 in each 96-well plate, for Vero cells concentration of $3 \times 10^3$ cells/well and for K562 cells concentration of $10^5$ cells/well. In an atmosphere enriched with 5% of CO2 dispatching and incubation of the adherent cells was made overnight at the temperature of 37°C.

Treatment was then provided to the growing cells after the completion of the 24 h keeping the concentration of the DA to be 30 to 120 μM for 72 h. Later, the cells were cleaned with 200 μL of PBS/well, after removing the solution from the plates. The NR solution of 150 μL (50 mg/ml NR in RPMI) was given to the cells and incubated for 3 h, then again washed three times with PBS.

Through the extraction, the dye present in viable cells was released accompanying a mixture of ethanol, acetic acid and water. At 540 nm, the absorbance of NR was calculated by using a spectrophotometric microplate reader. The percentage of NR uptakes in comparison with the control was provided being proportional to the number of viable cells. The experiments were performed in quintuplicate and independently repeated at least three times.

2.4. Statistical analysis

Three independent experiments were performed for each experimental condition tested. Experimental data were expressed as a mean ± standard error (SE).
Statistical differences between the control and treated cells were determined by the ANOVA statistical test (SPSS 17.0; SPSS Inc., Chicago, IL). *P* values <.05 were considered statistically significant.

3. Results

3.1. Inhibition of cell proliferation

3.1.1. MTT assay

MTT assay was used for investigation of cell viability caused by DA. After 72 h of DA treatment with different concentrations (10–120 µM), we obtained an increase in cell viability on the three cell lines. Moreover, the proliferation was perceived to be in a dose-dependent manner. It was shown by the treatment of DA that on the three cell lines, DA had a growth promoter effect along with an increase in the number of viable cells, which is 45% for K562, 20% for EA.hy926 cells and 17% for Vero cells, keeping the concentration to be at 120 µM (Figure 1).

3.1.2. NR assay

A similar trend was seen in the result gained for NR assay, which validated that previous observation

![Figure 1](image-url)

Figure 1. Effect of increasing concentrations of DA (10–120 µM) on viability of EA.hy926 cells (a), Vero cells (b) and K562 cells (c) using the MTT assay. Results are reported as the means ± SE (*n* = 3) (*p* < .05).
regarding the cell proliferation of the cells by increasing the concentrations of DA (10–120 µM) in a concentration-dependent manner. From Figure 2 the declaration of the NR assay is perceived that when comparing with the untreated cells (control), there was an increase in K562 at 120 µM of about 38%, while in Vero cells’ proliferation and EA.hy926, it remained 16% and 37%, respectively.

4. Discussion

The DA is a toxin that holds a dramatic influence on the economy’s health [17], which directed the interest of the researchers towards it. The research presented in the literature in this context report the neurotoxic properties of DA and also its potential toxic impact on diverse biological systems [2]; however, there is not much information available concerning the DA’s

Figure 2. Effect of increasing concentrations of DA (10–120 µM) on viability of EA.hy926 cells (a), Vero cells (b) and K562 cells (c) using the NR assay. Results are reported as the means ± SE (n = 3)(p < .05).
**in vitro** effects on the growth of cancer cell. Hereby, this has been aimed in this paper to evaluate and compare the impact of the high concentration of DA in different cell lines such as K562, EA.hy926 and Vero cells.

Two different tests were undertaken for assessing the impact of DA on the cell viability of cancer cells after 72 h of treatment. Among these assays, the NR assay is based on the incorporation of the NR dye by lysosomes of viable cells. This means the toxin involved in damaging the plasma or lysosomal membrane also reduce the uptake and subsequent retention of the dye [18]. The application of the NR assay in the present study for analysing the impact of DA on the viability of cell lines remained successful.

A differential mode of action of the DA on cells has been reflected by the differences accomplished by each assay on each cell line. Moreover, the MTT assay determines the mitochondrial activity while the NR assay reflects the integrity of the lysosome. Hereby, higher sensitivity could be obtained through the NR assay and this could be explained by detailing that there is a primary effect of the studied marine toxin followed by the mitochondria or other organelles on the lysosomes. While taking into consideration the cell lines, the neural cell lines could be counted as one of the cell line which has been effected by DA toxicity. However, for the other cell line, the role of DA remained as a growth promoter of cells.

It is evidenced from several scientific studies that DA is a neurotoxin [8,13,19,20]. For example, in major cultures of murine cortical neurons, the neurotoxicity was exposed to doses ranging from $10^{-6}$–$10^{-3}$ M (equal to 0.3–300 mg/ml) and while in major cultures of cerebellar neurons at the dose 10 mM (equal to 3 mg/ml). Moreover, structural similarity of the DA with the excitatory neurotransmitter glutamic acid and its analogues explain the mechanism of toxicity of this toxin, which has more potential receptor affinity [4].

Therefore, a constant influx of cations into the cell and excessive activation has been caused by the interaction of DA with the glutamate receptors (GluR), which takes place on nerve cells terminals [21], while the excess intracellular Ca$^{2+}$, along with cell swelling and eventually cell decrease, causes disruption of cellular function, which could lead to a permanent loss of neurological function. Controversial and varying results from the actual significance effect of the cytotoxic activity to the growth promoter were obtained from the studies based on the effect of DA on cell lines which are other than neural cells.

Doses ranging from 0.3 to 20 mg/ml via Small-Follicle Granulose Cells (SMGC), were found with no effect on the proliferation of the cells. Large-Follicle Granulosa Cells (LGGC), which is considered in the **in vitro** models for evaluating the DA effects on proliferation of ovarian cell and production of steroid, also showed no significant effects [22].

All cell lines were used (including Vero, EA.hy926 and K562 cells) for a demonstration of the significant cell proliferation at concentrations ranging from 10 to 120 µM after 72 h of exposure. Pizzo et al. [22] suggested that the differential response demonstrated by different cell types could be due to the level of a specific subtype of glutamate receptors present on cells.

Accordingly, cancer cells may express a particular subtype of glutamate receptors and DA may act through metabotropic glutamate receptors (mGluR) as a structural analogue to glutamate where emerging evidence suggests a role of glutamate and its receptors in the biology of cancer. The high affinity of DA for the glutamate receptors suggest that this biotoxin may also play a role in the biology of cancer with oncogenic properties. The review is previously dome of the communications of mGluRs, their ligand, glutamate, in processes which are promoting the growth of tumours of neuronal and non-neuronal origins [23]. While promoting the spreading of many tumours, the glutamate elevates cancer-associated matrix metalloproteinases (MMPs) inducer (Ganor et al., 2009 and Ganor Y1, Levite M. 2014).

### 5. Conclusion

The DA’s effect on the proliferative of the three cancer cell lines suggests that the role similar to that played by glutamate on cancer cells could be played by DA, in fact with a probably higher potential due to the receptor affinity being stronger. However, there is a need of further investigation in order to clarify effects on different cancer cells, molecular interactions of DA with glutamate receptors, keeping the emphasis on its analogies to glutamate, which is also known tumour promoter. Hereby, the people suffering from cancer should give more attention to the food, especially to the seafood.

### Disclosure statement

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