THE ANTI-METASTATIC POTENCY OF GNIDILATIMONOEIN, FROM *DAPHNE MUCRONATA*, COMPARED TO TWO GLYCOSYLATION INHIBITORS: CASTANOSPERMINE AND TUNICAMYCIN

MANIJEH MIANABADI, RAZIEH YAZDANPARAST

Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran

ABSTRACT

It is well known that the modification of oligosaccharide moieties of the cell surface glycoproteins modulates the adhesion and metastatic potential of several cancerous cell lines. Based on this knowledge, the anti-metastatic property of *Daphne mucronata* crude extract and one of its newly characterized active component, Gnidilatimonoein, were evaluated in wehi-164 cells by measuring their adhesion to fibronectin coated plates, relative to Castanospermine and Tunicamycin treated cells.

Twenty four hours after treatment of the cells with the plant crude extract (equivalent to 0.54 mg of the plant leaves powder/ml), Gnidilatimonoein (0.94 nM), Castanospermine (2.6 µM) and/or Tunicamycin (2.4 µM), their attachment to fibronectin-coated wells were depressed, by 24%, 30%, 26% and 58%, respectively. This data may classify the new anticancer compound, Gnidilatimonoein, as a strong glycosylation inhibitor.

Key Words: Adhesion, *Daphne mucronata*, Glycosylation inhibitor, Gnidilatimonoein.

INTRODUCTION

Metastasis constitutes a complex series of sequential interactions that enable a primary tumor to spread and colonize at secondary sites (1). Recently, it has been shown that carbohydrate residues on cell surface glyco-conjugates play an important role in the metastatic spread of tumor cells and their interaction with the environment (2). Therefore, modulation of the biosynthetic processes of protein glycosylation will affect metastatic activity of the cells (3). It has also been reported that tunicamycin and swainsonine treated B16 melanoma cells inhibit experimental metastasis (3,4).

In this report the effect of *Daphne mucronata* crude extract and its purified active component, Gnidilatimonoein (Scheme I) (5), on the tumorigenicity of wehi-164 cells were studied. The adhesive properties of wehi-164 cells treated with plant extract or its purified compound (Gnidilatimonoein) were also examined and compared with the adhesive property of wehi-164 cells after treatment with castanospermine and tunicamycin, two well known glycosylation inhibitors and anti-metastatic agents.

MATERIALS AND METHODS

Materials

The cell culture medium (RPMI 1640), fetal bovine serum (FBS) and penicillin – streptomycin were purchased from Gibco BRL (life technologies, Paisley, Scotland). The cell culture petri dishes were obtained from Nunc (Denmark). Cell line was obtained from Pasteur institute of Iran (Tehran). Chloroform, diethylether and silica gel 60 for column chromatography were obtained from Merck (Germany). Silica gel G for TLC was obtained from Fluka (Sweden). Agar and Fibronectin were purchased from Sigma Chemical Co. (USA). The [^{3}H]-Thymidine was purchased from Amersham (UK).

Plant material

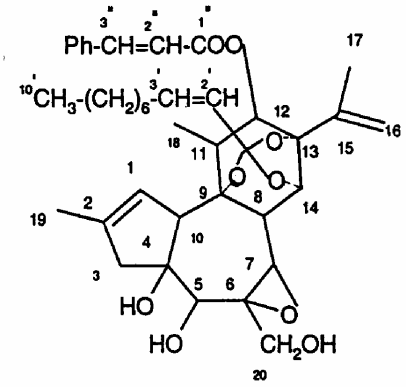
The processes of plant collection, extraction and purification of the active component (Gnidilatimonoein) have been reported previously (6,7). Each gram of the plant leaves powder contained 0.14 mg of Gnidilatimonoein.

Cell Spreding assay

The spreading assays were done according to Yamada Methods (8). The 96-well tissue culture plates were coated with 100µl aliquots of fibronectin solution and then with 100 µl of heat-denatured BSA solution (10mg/ml). Meanwhile, the wehi-164 cells were treated with a single safe dose of the plant extract (equivalent to 0.54 mg plant powder /ml), a single dose of Gnidilatimonoein (0.94nM in H2O: dimethylsulfoxide solution, 200:1 v/v), Castanospermine (2.6 µM) or Tunicamycin (2.4 µM) for 18 hrs. The treated cells, along with the

Correspondence: R. Yazdanparst, Inst. Biochemistry-Biophysic, University of Tehran, Tehran, Iran, Email: Yazdan@ibb.ut.ac.ir

untreated control cells, were detached using 0.25% trypsin in 0.02% EDTA, centrifuged, washed with PBS^- and then resuspended to 3×10^5 cells/ml of RPMI medium (9). The cells kept for 10 min at 37ºC, centrifuged and resuspended in RPMI medium. Then 100 µl aliquots of the cell suspension were added to the fibronectin coated wells. The mixtures were incubated for 60 min at 37ºC. The attached cells were then fixed with 3% formaldehyde containing 3% glutaraldehyde in PBS. The percentage of cells adapting a normal well-shaped morphology was estimated by counting 400 cells per well with randomized selected fields.



Scheme I

Cell attachment assay

Attachment assays were conducted according to a method described by Nagata and colleaques (10). Briefly, the 24-well tissue culture plates were preincubated with various concentrations of fibronectin solutions. The adhesion factor was diluted with PBS^+ and 300 µl aliquots were added to each well. The plates were incubated for 60 min at room temperature. The non-specific adsorption sites on the plastic surface were blocked by incubation with 300 µl heat-denatured BSA (10 mg/ml) for 30 min. The [^{3}H]-Thymidine labeled wehi-164 cells, treated with the plant extract or the purified Gnidilatimonoein, as described in the previous section, were washed with PBS^-, detached with 0.25% trypsin containing 0.02% EDTA. After centrifugation for 5 min at 1000 rpm, the cells were resuspended to 3.3×10^5 cells/ml in RPMI-1640 medium and kept at 37ºC for 10 min. Then 300 µl aliquots of this cell suspension were added to the fibronectin coated wells and incubated for 20 min at 37 ºC. The unattached cells were removed and the attached cells were washed twice with PBS^+ and then solubilized using 300µl of 0.1 N NaOH.The radioactivity content of each sample was determined by a β-scintillation counter (LKB-1219 Rockbta, Germany).

Radiolabeling of acid-insoluble and DNA materials in wehi-164 cells

The wehi-164 cells (3×10^4 –1×10^5 cells) were seeded into tissue culture wells using RPMI-1640 culture medium. After 48 hrs, the cells were treated with a single safe dose of plant extract (0.54 mg plant powder /ml) or its purified component, Gnidilatimonoein (0.94 nM). The cells were incubated at 37ºC for another 18 hrs. Then L-[^{14}C]leucine and D-[^{3}H] mannose or [^{3}H] thymidine alone were added to the medium to a final radioactivity density of 0.2 µCi/ml, 5µCi/ml and 0.2 µCi/ml, respectively. The cells were incubated for another 20 min at 37ºC. Then the cell monolyers were washed twice with cold PBS^- and the acid- insoluble materials were obtained by addition of 1 ml of 5 % TCA to the cells and incubation for 1 hr at 4ºC. The acid-insoluble materials were collected by centrifugation and washed twice with 1 ml ethanol and solubilized by 0.9 ml of 0.1 N NaOH. The samples were mixed for 1 hr and their radioactivity contents were determined using a β –scintillation counter.

Soft agar test

The ability of wehi-164 cells for colony formation in soft agar, was determined by the method of Nagata and Ichikawa (11). The agar base layer (3.3%) was boiled, cooled to 45 ºC, mixed with RPMI-1640 medium (containing 10% fetal calf serum) to make a 0.5% agar solution. This mixture was then added to a 35-mm tissue culture plates and incubated at room temperature for 30 min. The second layer of 0.33% agar was prepared by boiling 1.8% agar solution followed by cooling to 41ºC, and mixing with RPMI-1640. The control and the plant treated cells in RPMI-1640 were then added to the second layer agar solution and gently mixed. Aliquots of this cell mixture were added onto the base layer (2×10^5 cells/ml). The plates were swirled quickly to spread the top agar layer evenly and then incubated for 20 min at room temperature under aseptic condition. The plates were then transferred to a CO_2-incubator (5% CO_2, 37ºC). After 8 days, the number of colonies in each plates were counted using a light microscope (Olimpus, Japan).

RESULTS AND DISCUSSION

In order to evaluate the effect of *D. mucronata* crude extract or Gnidilatimonoein on the adhesive property of wehi-164 cells to fibronectin-coated plates, it was necessary to choose a non-toxic effective dose. In an independent study, the IC_{50}

Table 1. The L-[^{14}C]-leucine and D-[^{3}H]-mannose incorporation into the acid insoluble materials of wehi-164 cells under treatment with a single dose of *D. mucronata* crude extract (equivalent to 0.54 mg plant powder per ml of the cell culture medium) or 0.94 nM of Gnidilatimnoein.

Treatment	Concentration	L-[^{14}C]Leucine incorporation (cpm/well)	[^{3}H]Mannose incorporation (cpm/well)
–	0	132.87 ± 14.67	147.13 ± 1.20
+ Crude extract	0.54 mg/ml	129.62 ± 17.2	145.13± 1.60
+Gnidilatomonoein	0.94nM	139.62 ± 18.2	152.13 ± 17.8

Each measurement represent the mean of triplicate determination ± SD.

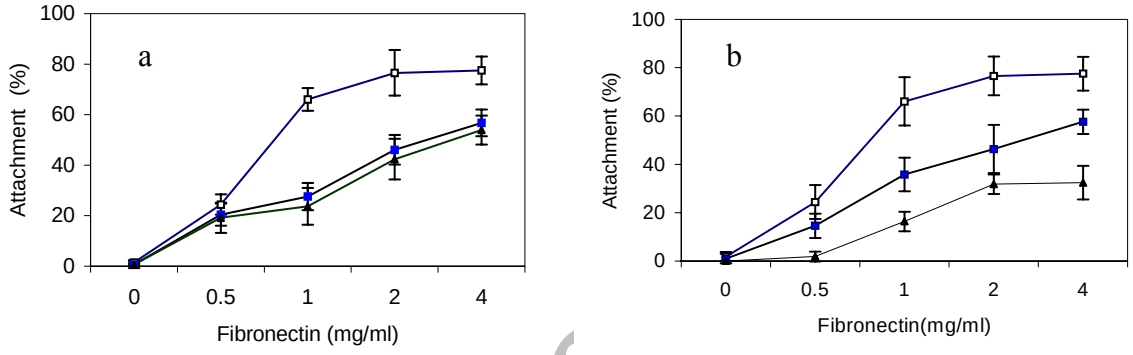


Figure 1. (a) Attachment of [^{3}H] thymidine-labeled control (□), crude extract (■) and Gnidilatimonoein (▲) treated wehi-164 cells and (b) [^{3}H] thymidine-labeled control (□), Castanospermine (■) and Tunicamycin (▲) treated wehi-164 to plastic tissue culture wells coated with the indicated amount of fibronectin. Each measurement represents the mean of triplicate determination ± SD.

for the crude extract was established to be equivalent to o.9 mg of plant leaves powder per ml of the cell culture medium. For Gnidilatimonoein, the IC_{50} determined to be 1.4 nM. Based on these data, the entire investigation, presented in this report, was done at doses lower than the IC_{50}. The non-toxicity of these doses on wehi-164 cells were assessed by measurement of the effects of *D. mucronata* crude extract and Gnidilatimonoein on the total synthesis of the cell membrane acid insoluble materials. In this respect, the extent of L-[^{14}C]-Leucine and D-[^{3}H]-Mannose incorporation into the acid insoluble materials of the treated and untreated wehi-164 cells were measured. As it is shown in table 1, the net incorporation of these two radiolabelled substances into membrane proteins is not significantly affected by the treatment of cells with a single dose of the plant extract (0.54 mg/ml) or a single dose of Gnidilatimonoein (0.94 nM), compared to the untreated control samples.

Variation in the glycosylation pattern of the cell surface molecules upon treatment, was assessed by the measurement of the extent of adhesion of the metabolically labeled wehi-164 cells to fibronectin coated plates. As it is shown in figure 1, fibronectin mediates concentration-dependant stimulation of attachment of untreated cells with maximal level of approximately 80% attachment observed at a coating concentration of 1-3 µg/ml of the adhesion factor (fibronectin). The attachment behaviour of the crude extract and Gnidilatimonoein treated [^{3}H]-labelled wehi-164 cells were comparable to those of untreated cells, concerning the coating concentration of fibronectin (figure 1). However, upon treatment of wehi-164 cells with a single dose of the plant crude extract (0.54 mg/ml) and Gnidilatimonoein (0.94nM), the attachment to fibronectin coated wells, at a density of 2 µg/ml, decreased by 27.6 % and 33.5 %, respectively (figure 1a). Similarly, at the same fibronectin concentration (2µg/ml), Castanospermine (2.6µM) and Tunicamycin (2.4 µM) inhibited the attachment of the cells to the coated plates by 39% and 58%, respectively (figure 1b). These results may provide a mechanism for the anticancer and antimetastatic properties of *D. mucronata* (6,7) and classify Gnidilatimonoein as a new and potent inhibitor of

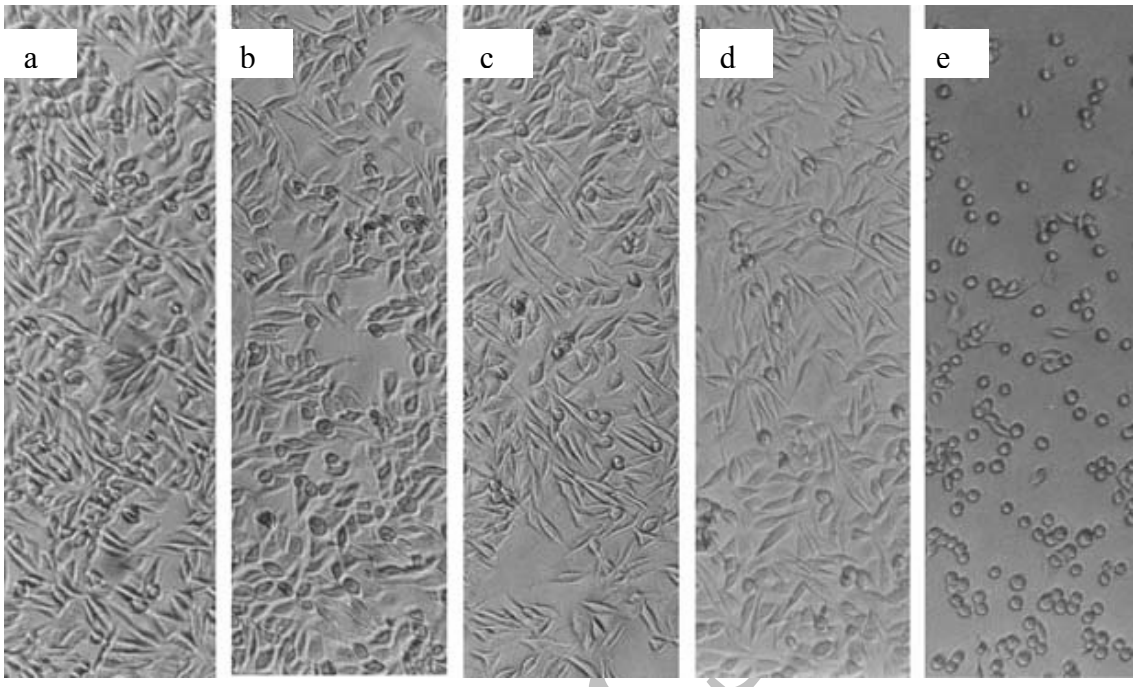


Figure2. Microscopic views of the morphology of untreated control wehi-164 cells (a), cell treated with crude extract of *D. mucronata* (0.54 mg/ml, b), Gnidilatimonoein (o.94 nM, c), Castanospermine (2.6 μM, d), and Tunicamycin (2.4 μM, e).

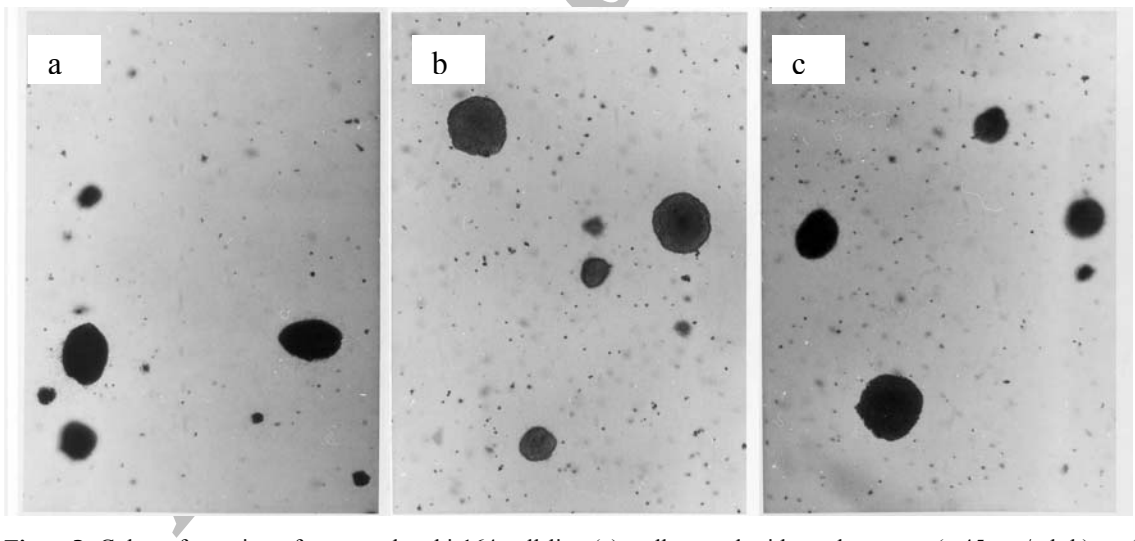


Figure3. Colony formation of untreated wehi-164 cell line (a), cell treated with crude extract (o.45 mg/ml, b), and cell treated with Gnidilatimonoein (0.94 nM, c) on agar surface.

the cellular glycosylation pathway. Additionally, variation in the morphology of the treated cells with respect to the untreated control cells provided further support for the activity of *D. mucronata*. As it is shown in figure 2, the untreated wehi-164 cells spread with characteristic polygonal morphology on fibronectin substrate. However, the crude extract and the Gnidilatimonoein treated cells showed poor adhesion besides of exhibiting extensive rounding up of the cell bodies similar to Castanospermine and Tunicamycin treated cells (figure 2). Regardless of these significant variations, the anchorage-independence growth of wehi-164 cells was not affected upon treatments.

According to our data, treatment of the cells with each of these agents, at the specified doses of this report, did not significantly changed neither the

number of colonies in each test, nor the average diameter of the colonies (figure 3). Based on these observations, it may be concluded that *D. mucronata* or its active component, Gnidilatimonoein did not affect the tumorigenicity of wehi-164 cells similar to castanospermine action (7). However, the metastatic activity of the treated wehi-164 cells has most probably changed due to the modification of the cell surface glycoproteins. Further work, for the extensive characterization of these glycoproteins, is going on and the results will be published soon.

CONCLUSION

Using cell culture techniques, the effect(s) of *D. mucronata* crude extract and its major active component, Gnidilatimonoein, on the adhesive property of the cells to fibronectin coat was evaluated. According to data, the plant is capable of reducing the adhesive property of the cells probably through changes in the carbohydrate moieties of the cell surface glycoproteins and in that respect, these are similarities between Gnidilatimonoein and two well known glycosylation inhibitors, Castanospermine and Tunicamycin. Further research is required to find the exact enzyme(s) involved in the glycosylation pathway which is under the plant extract influence.

ACKNOWLEDGEMENT

The authors are thankful to the research council of the University of Tehran for the financial support of this investigation.

REFERENCES

1. Fidler IJ, Gersten DM, Hart IR. (1978) The biology of cancer invasion and metastasis. Adv Cancer Res 28: 149-250.
2. Bretscher MS. (1973) Membrane structure: some general principles. Science 181:622-629.
3. Irimura T, Gonzalez R, Nicolson GL. (1981) Effects of tunicamycin on B16 metastatic melanoma cell surface glycoproteins and blood-borne arrest and survival properties. Cancer Res 41: 3411-3418.
4. Humphries MJ, Matsumoto K, White SL, Olden K. (1986) Oligosaccharide modifications by swainsonine treatment inhibits pulmonary colonization by B16-F10 murine melanoma cells. Proc. Natl. Acad. Sci. USA 83: 1752-1756.
5. Mianabadi M, Rivera E, Yazdanparast R. (2003) A new diterpene ester from the leaves of *Daphne mucronata.* J Trop Med Plants 4:1-4.
6. Hedayati M, Yazdanparast R, Fasihi H, Azizi F. (2003) Anti-tumor activity of *Daphne mucronata* extract and its effects on TNF-α-receptors and TNF- α release in cultured human monocytes. Pharmeceutical Biol 41: 194-198.
7. Sadeghi H, Yazdanparast R. (2003) Anti-tumor activity of cell cycle arrest of a new diterpene ester from *Daphne mucronata* using K562 cells. IBJ 7: 127-131.
8. Yamada KM, Kenny DW. (1984) Daulistic nature of adhesive protein function: fibronectin and its biologically active peptide fragments can auto-inhibit fibronectin function. J Cell Biol 99: 29-36.
9. Freshney RI. (1990) Culture of animal cells. New York: Weily-Liss(3rd edition) .
10. Nagata K, Humphries MJ, Olden K, Yamada KM. (1985) Collagen can modulate cell interactions with fibronectin. J Cell Biol 101:386-394.
11. Nagata K, Ichikawa Y. (1979) Requirments for RNA and protein sythesis in the induction of several differentiation-markers in a myeloid leukemia cell line. J Cell Physiol 98: 167-175.