Differential Inhibition of Single and Cluster Type Tumor Cell Migration

REVEKKA HARISI, ISTVAN KENESSEY, JULIA N. OLAH, FERENC TIMAR, ISTVAN BABO, GABOR POGANY, SANDOR PAKU and ANDRAS JENEY

First Institute of Pathology and Experimental Cancer Research, Faculty of Medicine, Semmelweis University, Budapest, Hungary

Abstract. *For the control of tumor metastasis it is important to identify chemical compounds with antimigratory potency. Agents acting against single cell and cluster type migration are necessary for successful antimetastatic therapy. In the present study, the migration of HT-1080 fibrosarcoma cells and OSCORT osteosarcoma cells was compared in a Boyden chamber and in an extracellular matrix (ECM)-based three-dimensional cell culture (3-DCC) model system. The Boyden chamber offers a model of single tumor cell migration, whereas the 3-DCC model system demonstrates invasive growth in the form of a cluster. Since PD98059 (MEK inhibitor) exclusively reduced migration in the 3-DCC model, it may be plausible that the ERK/MAPK signaling pathway is essential for cluster type migration. Interestingly, single cell migration was stimulated upon blocking phosphatidylinositol 3-kinase (PI3K) and also p38-MAPK by treatment with LY294002 and SB203580 respectively. A remarkable reduction of single cell migration was observed following treatment with okadaic acid, a phosphatase 1 (PP1) and 2A (PP2A) inhibitor, which was rather intriguing. This study provided evidence that certain cytotoxic/cytostatic agents at appropriate concentrations were able to preferentially inhibit certain types of migration relative to cell proliferation. Single cell migration was selectively inhibited by taxol at very low subtoxic concentration, whereas 5-hexyl-2'-deoxyuridine (HUdR) exclusively inhibited the cluster type of migration. The borrelidin compound was able to inhibit both types of tumor cell migration, but single tumor cell migration was much less affected. It is interesting that migration was more reduced than proliferation by borrelidin, especially at the advanced growth stage. Taxol is recommended as an agent acting against single cell migration, as well as HUdR and borrelidin as leading compounds for developing antimetastatic drugs against cluster type migration.*

The spread of tumor cells from the primary site to distant organs represents a major pathobiological event in tumor progression. Metastasis is a multistep process involving shedding of tumor cells from the primary site, migration and attachment in a novel microenvironment to form a growing cell population. Of the various pathobiological events contributing to the complex metastatic process current interest is focused on migration and on its multiple mechanisms (1-6).

Tumor cells may migrate alone (ovarian cancer cells) similarly to lymphocytes, or granulocytes, or in the form of a cluster. There is a remarkable difference in the morphology and in the extracellular matrix (ECM)-integrin contact between tumor cells migrating alone or in a cluster (7). The reports of Friedl, Hegerfeldt, Wolf *et al*. (1-6) demonstrating various types of tumor cell migration have stimulated us to study the antimigratory action of different compounds with well known molecular targets (matrix metalloproteinases (MMPs), ECM biopolymers, integrins) contributing to migration. To this end, the Boyden chamber (8), as well as a three-dimensional cell culture (3-DCC) ECM-based model (9) making it possible to differentiate the antimigratory action of test compounds against tumor cells migrating as single cells or in clusters have been applied.

Abbreviations: ECM, Extracellular matrix; 3-DCC, three-dimensional cell culture; MMP, matrix metalloproteinase; AON, antisense oligonucleotide; HUdR, 5-hexyl-2'-deoxyuridine; PI3K, phosphatidylinositol 3-kinase; PP1 and PP2A, phosphatase 1 and 2A; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK ERK kinase.

Correspondence to: A. Jeney, MD, D.Sc., First Institute of Pathology and Experimental Cancer Research, Faculty of Medicine, Semmelweis University, H-1085, 26 Ulloi ut, Budapest, Hungary. Tel: +36 13171070, Fax: +36 13171070, e-mail: ajeney@korb1.sote.hu / revekka.harisi@oncology.hu

Key Words: Migration, extracellular matrix, three-dimensional cell-culture model, Boyden chamber, borrelidin, taxol, 5-hexyl-2'-deoxyuridine, signal pathway inhibitors, antisense oligonucleotide.

It has been well documented that various cellular functions are controlled by ECM biopolymers and also by their cell surface receptors, the integrins (2, 5, 7, 10). Unregulated proteinases such as MMPs can remodel the ECM, resulting in aberrant binding with integrin and consequently a paradoxical cell response. Great importance has been attributed to this molecular process in tumor metastasis including the movement of tumor cells within various tissues (2, 5, 7, 10). These studies have highlighted the need to identify agents which are able to inhibit the biosynthesis or block the function of proteoglycans, integrins and MMPs. Our previous studies have shown that the inhibition of heparan sulfate proteoglycan production by 5-hexyl-2'-deoxyuridine (HUdR) reduced the metastatic capacity of some tumors (9, 11-14). The introduction and appropriate use of migratory assays has made it possible to identify the critical molecules to target for antimetastatic drug therapy.

In this study, antisense oligonucleotide (AON) against MMP-9 (AON-MMP-9), borrelidin with anti-integrin action, signal pathway inhibitors, taxol which interferes with microtubules implicated in cell motility and HUdR an alkyl-pyrimidine which reduces heparan sulfate proteoglycan synthesis without acting against nucleic acid synthesis (11) were used to test the responses of tumor cells migrating in a Boyden chamber or in a 3-DCC model system.

Materials and Methods

Chemicals. Cell culture media reagents and chemicals of analytical grade were purchased from Sigma (St. Louis, MO, USA).

Test compounds. MMP-9-AON and HUdR were synthesized by L. Otvos, L. Gruber and J. Sagi (Central Chemical Institute of the Hungarian Academy of Sciences, Budapest, Hungary). Taxol and borrelidin were kindly provided by N. Makk and G. Ambrus (Institute for Drug Research, Budapest, Hungary). LY294002, PI3K/Akt pathway inhibitor; SB203580, p38-MAPK inhibitor; and PD98059, MEK inhibitor were purchased from Sigma. Okadaic acid, PP1 and PP2A inhibitor was purchased from ICN Biomedicals Inc. (Aurora, Ohio, USA).

Cell lines. A human osteosarcoma cell culture (OSCORT culture) established in our laboratory from a primary osteosarcoma from a 17-year-old male as described previously (15) was used. The human fibrosarcoma tumor cell line (HT-1080) was kindly provided by Dr. J. Timar (Semmelweis University, Budapest, Hungary).

Assay for tumor cell migration in three-dimensional cell culture (3-DCC). For the quantitative measurement of cell migration, HT-1080 cells (10^5) were plated in a 24-well plate for 24 h in 10% fetal calf serum (FCS) containing RPMI-1640 medium. The test substances were added to the growing cell culture 24 h later and cells incubated for an additional 24 h. The culture medium with the test substance was removed and 300 μl RPMI-1640 medium containing 10 mg/ml ECM (matrigel) and 10% FCS were added. In this 3-DCC, the tumor cells migrated upwards from the monolayer to the ECM-gel during the following 24 h (Figure 1A). The number of cells in the matrigel and in the monolayer were counted in Buerker hemocytometer (9).

Assay for tumor cell migration in Boyden chamber. Single tumor cell migration was studied in a Boyden chamber as described by Albini (8). Briefly HT-1080 cells (10^6) were plated in T-25 flasks and grown in RPMI-1640 medium containing 10% FCS, at 37˚C, in a humidified 5% CO_2 atmosphere. The test substances were administered 24 h later followed by incubation for an additional 24 h, then cells were counted and 2×10^4 cells were loaded onto the 8-μm pore PVP-free polycarbonate Nucleopore filter (Nucleopore Inc., Costar, New York, NY, USA) located at the top of the Boyden chamber. Fibronectin was used as the chemoattractant for migration, after 1 h the cells were stained with Giemsa. The size of the migrated cell population was assessed by measuring the density of the filter membrane using an Eagle-Eye II system densitometer (Stratagene, La Jolla, CA, USA) (Figure 1B).

Both models include the assessment of cell growth, consequently the two elements of invasive growth, proliferation and migration, could be simultaneously monitored.

Visualization of tumor cell migration. To illustrate the difference in the movement of HT-1080 fibrosarcoma cells in 3-DCC and in a Boyden chamber, a confocal laser microscope was used. (Figure 1). Tumor cells in the 3-DCC or in the filter removed from the Boyden chamber were fixed with 1% paraformaldehyde, permeabilized with 0.1% Triton® X-100 and stained with propidium iodide (200 μl, 25 μg/ml) The cultures were examined in a confocal laser microscope. The side view of the optical sections of the gel was achieved using BIO-RAD MRC 1024 software (Bio-Rad Laboratories, Inc., Hercules, CA), applying a 3-D reconstruction model. The migrated and the non-migrated cell populations were evaluated with IMAN, version 1.4 image analysis software (MTA-KFKI-ATKI, Hungary) (Figure 1A).

Detection of MMP (gelatinase) activity. The MMP activity was measured from the serum-free conditioned medium corresponding to 10^5 cells per sample. Unheated aliquots of the conditioned media were concentrated through Centricon 31 filters (Amicon, Millipore, Bedford, MA, USA), and mixed with Laemmli's sample buffer without 2-mercaptoethanol. Measurement of MMP activity was carried out as described previously (15, 16). Using gelatinase zymography, MMP-2 (gelatinase A, 72 kDa collagenase type IV) and MMP-9 (gelatinase B, 92 kDa collagenase type IV) activities were detected in the serum-free conditioned medium of the cells.

Statistical analysis. All the experiments were performed at least in triplicate and the results are presented as means±standard deviation (SD). The differences between groups were analyzed using Student's *t*-test (two-tailed); $p<0.05$ was considered statistically significant. The IC_{50} values were calculated using the Dose-Effect Analysis with Microcomputers, Elsevier-Biosoft™ software (17).

Results

Cell culture growth and cell migration. The migratory potency of HT-1080 cells at different growth stages in the Boyden chamber and in 3-DCC showed different behavior. Although tumor cells from the rapidly (2-day) and the very slowly proliferating (4-day) pre-cultures migrated equally

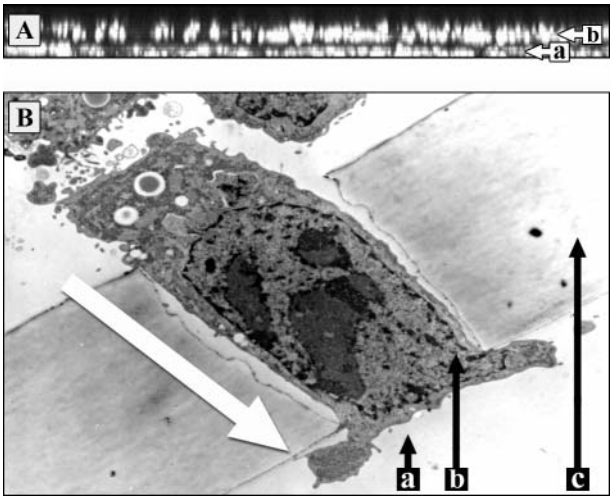

Figure 1. *Assays of tumor cell migration. A, Invasion of HT-1080 cells in matrigel (3-DCC assay), cluster type of migration; a: Cells in monolayer, b: Migrating cells. B, Migration of HT-1080 cells in a Boyden chamber, single cell migration; a: fibronectin (chemoattractant), b: migrating cell, c: filter.*

well in the Boyden chamber, the invasive growth in 3-DCC was higher in the 4-day than in the 2-day pre-cultured cells (Table I).

The slowly proliferating (4-day culture) HT-1080 cells showed an elevated level of MMP-9 (Table II). The effect of AON-MMP-9 on the invasive growth of HT-1080 cells was substantial only in the 4-day pre-cultured cell which showed almost fourfold higher MMP-9 activity than the 2-day pre-cultured cells (Table II).

Molecular targeted antimigratory treatment. Table III shows that single and cluster types of tumor cell migration were both affected by the signal pathway inhibitors used at less than cytotoxic concentration. The inhibitor of the PI3K/Akt pathway, LY294002 and the p38-MAPK inhibitor, SB203580 stimulated migration in the Boyden chamber but not in the 3-DCC away, whereas the MEK inhibitor, PD98059 reduced migration selectively in the 3-DCC. The PP1 and PP2A inhibitor, okadaic acid, exerted the most remarkable inhibitory action against migration in the Boyden chamber without affecting the proliferation (Table III).

Table IV shows that migration in the Boyden chamber was inhibited by taxol at a much lower concentration than proliferation and invasive growth in the 3-DCC model.

A concentration of 50 μg/ml of HUdR reduced the invasive growth of the HT-1080 cells in the 3-DCC by 53% , however, single cell migration in the Boyden chamber was not affected and in addition there was no inhibitory action against proliferation (data not shown).

Table I. *Migratory capacity of rapidly (48 h culture) and slowly (96 h culture) proliferating HT-1080 cells.*

Pre-culture time (hours)	Boyden chamber (optical density)	3-DCC (optical density)	
		Number of non-migrating cells ($\times 10^4$)	Number of migrating cells ($\times 10^4$)
48	89.1±1.0	9.7±0.5	10.2±1.2
96	85.5±4.3	21.4±0.3*	41.7±3.5*

Each value represents the mean±SD from three independent experiments. *Significant difference from the rapidly proliferating cells in 3-DCC ($p<0.05$) using Student's *t*-test.

Table II. *Gelatinase activity and the antimigratory effect of antisense oligonucleotide against MMP-9 (AON-MMP-9) in HT-1080 cells.*

Pre-culture time (hours)	Gelatinase activity[a]		Antimigratory action of AON-MMP-9[b] (% of control)
	MMP-2	MMP-9	
48	2.25±0.55	0.81±0.28	83
96	1.37±0.45	3.06±0.45*	46*

[a]Measured in conditioned, serum-free medium; related to 10^4 cells. [b]AON-MMP-9: 25 μg/ml. Each value represents the mean±SD from three independent experiments. *Significant difference from the rapidly proliferating (48 h pre-culture) cells ($p<0.05$) using Student's *t*-test.

Table III. *Differential action of signal pathway inhibitors on HT-1080 cell proliferation and migration in Boyden chamber and in 3-DCC model systems.*

Signal pathway inhibitor	Effect on proliferation (% of control)	Effect on migration (% of control)	
		Boyden chamber	3-DCC
LY294002 (50 μM)	104.0±3.2	134.3±15.2*	91.1±11.7
PD98059 (50 μM)	92.5±5.4	109.2±6.0	33.3±2.8*
SB203580 (5 μM)	82.0±7.3	187.0±14.5*	104.0±5.3
Okadaic acid (0.01 μM)	112.0±2.4*	15.3±1.3*	67.9±8.6*

LY294002, PI3K inhibitor; PD98059, MEK inhibitor; SB203580, p38-MAPK inhibitor; Okadaic acid, PP1 and PP2A inhibitor. Each value represents the mean±SD from three independent experiments. *Significant difference from the control ($p<0.05$) using Student's *t*-test.

The data in Table V indicate that borrelidin inhibited tumor cell invasion into the ECM, but single tumor cell migration was less affected. It is noteworthy that migration was more reduced than proliferation by borrelidin, especially in cells at the advanced growth stage (Table V).

Table IV. *Differential efficacy of taxol against tumor cell migration in Boyden chamber and in 3-DCC model systems.*

Cell culture	Effect on proliferation IC_{50} (ng/ml)	Effect on migration IC_{50} (ng/ml)	
		Boyden chamber	3-DCC
HT-1080	19.6±1.27*	2.3±0.13	36.4±1.50*
OSCORT	6.1±0.84	(–)	8.6±0.41

(–) OSCORT cells are not able to migrate in a Boyden chamber. Each value represents the mean±SD from three independent experiments. *Significant difference from the Boyden chamber ($p<0.05$) using Student's *t*-test.

Table V. *Effect of borrelidin-3-picolylamide on proliferation and migration in rapidly (48 h pre-culture) and slowly (96 h pre-culture) growing HT-1080 tumor cells.*

Pre-culture time	Effect on proliferation IC_{50} (μg/ml)	Effect on migration IC_{50} (μg/ml)	
(hours)		Boyden chamber	3-DCC
48	11.5±0.05**	>10±0.12	1.20±0.07*
96	9.4±0.05**	7±0.09	0.45±0.01*

Each value represents the mean±SD from three independent experiments. *Significant difference from the Boyden chamber ($p<0.05$) using Student's *t*-test. **Significant difference from the migration in 3-DCC ($p<0.05$) using Student's *t*-test.

Discussion

As illustrated in Figure 1, the Boyden chamber offered a model for single tumor cell migration, whereas the 3-DCC model demonstrated invasive growth in the form of clusters. In these complex model systems, several features of single cell and cluster type migration were observed, including their differential responses to signal inhibitors, and chemical compounds with well-defined molecular targets. It was noteworthy, however, that the HT-1080 cells were able to migrate to a similar extent in the Boyden chamber irrespective of the cells' pre-culture time. In contrast the invasive growth (*i.e.* migration into the ECM) in the 3-DCC showed cell growth stage dependency. Since the single tumor cells exerted their migratory potential at the early rapid growth stage, it is conceivable that certain factors were produced enabling ECM invasion at the advanced slowly proliferating growth stage. The elevated level of MMP-9, a target molecule responsible for the cluster type of migration (5), seemed to correlate with the higher invasive growth of the tumor cells originating from the 4-day pre-culture. The contribution of MMP-9 to the invasive growth of the HT-1080 cells received further support from the effect of AON-MMP-9 treatment.

As it has been reported that tumor cell migration in single and in cluster forms is directed by different molecular mechanisms (1-6), the question of whether migration in the Boyden chamber and in the 3-DCC could be modified by signal pathways inhibitors was addressed. Using signal inhibitors, remarkable differences were observed between the two types of migration, suggesting that variant regulatory mechanisms are responsible for these two processes. Using PD98059 (50 μM), the migration in 3-DCC was reduced exclusively, therefore it may be plausible that the ERK/MAPK signaling pathway is required for the cluster type migration. Interestingly, single cell migration was stimulated upon blocking PI3K (phosphatidylinositol 3-kinase) and also p38-MAPK molecules by treatment with LY294002 and SB203580 respectively. The very remarkable reduction of single cell migration by okadaic acid was rather intriguing. Okadaic acid, a selective and specific inhibitor of PP1 and PP2A, was recommended for use in studies on the phosphorylation of transcription factors in gene regulation (18). Consequently the presented data, showing reduced migration in the presence of okadaic acid, but stimulation after treatment with either LY294002 or SB203580, suggested that abrogation of the phosphorylation of PI3K and p38-MAPK may be important for single cell migration. However, it must be remembered that okadaic acid has been reported to enhance collagenase and stromelysin-1 and has been assumed, but not demonstrated, to have an important role in metastasis (18). Taking these data together, it seems most probable that enhanced collagenase expression mediated through jun-transcription factors does not participate in tumor cell migration. All that can be concluded at this time is that further studies are needed to elucidate the role of signal pathways in tumor cell migration.

Drugs developed against tumor growth are widely used at the advanced, metastatic stage of human tumors, however, limited information is available concerning their efficacy against tumor cell migration. This study provided evidence that certain cytotoxic/cytostatic agents at certain concentrations preferentially inhibited certain types of migration relative to proliferation. In this respect, taxol is worth a mention because single cell migration was selectively inhibited by this currently widely used anticancer drug when used at a very low subtoxic concentration. The well-documented critical role of the ECM and integrin in metastasis stimulated the investigation of drugs such as HUdR acting on the interaction between tumor cells and certain ECM biopolymers. In this respect, certain analogs of borrelidin are noteworthy because the adhesion of tumor cells to fibronectin and also to vitronectin could be prevented

by them. HUdR was reported to control metastasis formation in our preclinical models (9, 11, 12) and borrelidin-3-picolylamide was shown to reduce tumor metastasis, which may be related to its effect on the expression of αvβ3 integrin and on actin polymerization (9). The cluster type of migration was inhibited by HUdR and more extensively by borrelidin in the present study.

The significance of individual agents with well documented mechanisms is reinforced by the concept of tumor cell plasticity operating during metastasis as proposed by Friedl and Wolf (3). Depending on the microenvironment or the therapy applied, tumor cells are able to change their migratory mechanism and escape from the effect of the therapy. Thus successful antimetastatic therapy will be achieved when agents acting against single cell and cluster type migration become weapons in the arsenal of clinical oncology. The present study recommends HUdR and borrelidin as leading compounds in the development of antimetastatic drugs against cluster type migration. In addition, the selection of agents acting on phosphatases for testing against both of types of tumor cell migration seems promising. The present study suggested that taxol may be used against single cell migration. Since doxorubicin was reported previously (19) to inhibit cluster type migration at subtoxic concentrations, the combination of these two drugs offers a possibility of acting against both single cell and cluster type tumor cell migration.

References

1 Friedl P, Noble PB, Walton PA, Laird DW, Chauvin PJ, Tabah RJ, Black M and Zanker KS: Migration of coordinated cell clusters in mesenchymal and epithelial cancer explants *in vitro*. Cancer Res *55*: 4557-4560, 1995.

2 Hegerfeldt Y, Tusch M, Bröcker EB and Friedl P: Collective cell movement in primary melanoma explants: plasticity of cell-cell interaction, beta1-integrin function, and migration strategies. Cancer Res *62*: 2125-2130, 2002.

3 Friedl P and Wolf K: Tumour cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer *3*: 362-374, 2003.

4 Wolf K, Wu YI, Liu Y, Geiger J, Tam E, Overall C, Stack MS and Friedl P: Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. Nat Cell Biol *9*: 893-904, 2007.

5 Friedl P and Wolf K: Tube travel: the role of proteases in individual and collective cancer cell invasion. Cancer Res *68*: 7247-7249, 2008.

6 Friedl P and Wolf K: Proteolytic interstitial cell migration: a five-step process. Cancer Met Rev *28*: 129-135, 2009.

7 Arboleda MJ, Lyons JF, Kabbinavar FF, Bray MR, Snow BE, Ayala R, Danino M, Karlan BY and Slamon DJ: Overexpression of AKT2/protein kinase beta leads to up-regulation of beta1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. Cancer Res *63*: 196-206, 2003.

8 Albini A: Tumor and endothelial cell invasion of basement membranes. The matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms. Pathol Oncol Res *4*: 230-241, 1998.

9 Jeney A, Kenessey I, Timar F, Olah J, Pogany G, Babo I and Harisi R: Study of drugs against neoplastic metastasis. Magy Onkol *50*: 93-100, 2006 (in Hungarian).

10 Milner R, Edwards G, Streuli C and Ffrench-Constant C: A role in migration for the alpha V beta 1 integrin expressed on oligodendrocyte precursors. J Neurosci *16*: 7240-7252, 1996.

11 Pogany G, Raso E, Peterszegi G, Keresztfalvi M, Lapis K and Jeney A: Alterations in nucleoside monophosphate concentrations in 3LL tumours after combined treatment with tiazofurin and 5-hexyl-2'-deoxyuridine. Anticancer Res *12*: 281-284, 1992.

12 Otvos L, Sagi J, Sagi G, Szemzo A, Toth FD and Jeney A: Enzymatic hydrolysis and biological activity of oligonucleotides containing 5-substituted pyrimidine bases. Nucleosides Nucleotides *18*: 1665-1666, 1999.

13 Timar J, Jeney A, Kovalszky I and Kopper L: Role of proteoglycans in tumor progression. Pathol Oncol Res *1*: 85-93, 1995.

14 Timar J, Diczhazi Cs, Bartha I, Pogany G, Paku S, Raso E, Tovari J, Ladanyi A, Lapis K, Kopper L and Jeney A: Modulation of heparan-sulphate/chondroitin sulfate ratio by glycosaminoglycan biosynthesis inhibitors affects liver metastatic potential of tumor cells. Int J Cancer *62*: 755-761, 1995.

15 Harisi R, Dudas J, Timar F, Pogany G, Kovalszky I, Szendroi M, Timar J and Jeney A: Invasive growth and topoisomerase switch induced by the tumorous extracellular matrix in osteosarcoma cell culture. Cell Biol Int *11*: 959-967, 2005.

16 Babo I, Bocsi J and Jeney A: The site-dependent growth characteristics of a human xenotransplanted basaloid squamous cell carcinoma. J Cancer Res Clin Oncol *125*: 35-41, 1999.

17 Chou J and Chou TC: Dose-effect analysis with microcomputers: quantitation of ED_{50}, LD_{50}, synergism, antagonism, low-dose risk, receptor liganci binding and enzyme kinetics. IBM-PC Series, Elsevier-Biosoft: Cambridge, United Kingdom, 1988.

18 Westermarck J, Lohi J, Keski-Oja J and Kahari VM: Okadaic acid-elicited transcriptional activation of collagenase gene expression in HT-1080 fibrosarcoma cells is mediated by JunB. Cell Growth Differ *5*: 1205-1213, 1994.

19 Harisi R, Dudas J, Timar F, Pogany G, Paku S, Timar J, Kovalszky I, Szendroi M and Jeney A: Antiproliferative and antimigratory effects of doxorubicin in human osteosarcoma cells exposed to extracellular matrix. Anticancer Res *25*: 805-813, 2005.

Received March 1, 2009
Revised May 22, 2009
Accepted May 26, 2009