Anti-neoplastic effects of aprotinin on human breast cancer cell lines: In vitro study

Saeed Soleyman-Jahi1–3,A–F, Fatemeh Sadeghi1–2,B–F, Ziba Afshari1,A,B,E,F, Tahereh Barati1,A,B,E,F, Sevil Ghasemi1,A,B–F, Samad Muhammadnejad1,A,C,E,F, Sevil Ghasemi1,A,B–F, Samad Muhammadnejad1,A,C,E,F, Saeid Amanpour1,A,E,F, Kazem Zendehdel1,A,C,E,F

1 Cancer Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Iran
2 Cancer Immunology Project (CIP), Universal Scientific Education and Research Network (USERN), Tehran, Iran
3 Division of Gastroenterology, Department of Internal Medicine, Washington University, St. Louis, USA
4 Cancer Models Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Iran
5 Research Center for Molecular and Cellular Imaging, Tehran University of Medical Sciences, Iran

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Abstract

Background. Aprotinin is a nonspecific serine protease inhibitor, which can inhibit plasminogen-plasmin system and matrix metalloproteinases. Aprotinin has been investigated as an antitumor agent. However, its antineoplastic effects on breast cancer (BC) have not been investigated yet.

Objectives. The objective of this study was to assess the inhibitory effects of aprotinin on human BC cell lines. We assessed the effects of aprotinin on local invasion and survival of human BC cell lines MDA-MB-231, SK-BR-3 and MCF-7 in vitro.

Material and methods. CHEMICON cell invasion assay kit was used to assess local invasion, and (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction (MTT) assay was used to determine the antiproliferative activity of aprotinin. Human dermal fibroblast (HDF-1) cell line was used as control normal cells.

Results. Cancer cell lines showed more invasion characteristics compared to HDF-1. Aprotinin significantly decreased the invasiveness of MDA-MB-231 in concentrations of 1 trypsin inhibitor unit (TIU)/mL, 1.3 TIU/mL and 1.7 TIU/mL in comparison with the untreated group (analysis of variance (ANOVA) p < 0.001). Treatment of SK-BR-3 with 1.3 TIU/mL aprotinin caused no significant reduction in invasiveness (p = 0.06). Treatment with different concentrations of aprotinin significantly decreased the surviving fraction and inhibited the growth of all cell lines tested in this study (analysis of variance (ANOVA) p < 0.001). Compared to cancer cell lines, normal HDF-1 cell line showed less sensitivity to antiproliferative effects of aprotinin, both in low and high doses.

Conclusions. Aprotinin significantly inhibited the growth of human breast cancer cell lines MDA-MB-231, SK-BR-3 and MCF-7, and normal fibroblast cell line HDF-1. The growth inhibitory effect was more dominant in cancer cell lines. Inhibition of local invasion by aprotinin was significant only in the case of MDA-MB-231. Future molecular studies could shed more light on mechanisms underlying antineoplastic effects of aprotinin and its potential therapeutic effects.

Key words: breast cancer, in vitro, cell proliferation, aprotinin, neoplasm invasion
Introduction

Tumor cells proceed along distinct steps to detach from their primary location, metastasize to a distant site and form a metastatic lesion. Invasion through extracellular matrix (ECM), intravasation into circulation, survival in circulation, and extravasation into the distant site are some important steps of this process.\(^1\) Metastasizing tumor cells require lytic substances to degrade the matrix and membranes on their way toward the distant site.\(^2\)

Proteolytic enzymes of the plasminogen-plasmin system play a pivotal role in the local invasion and metastasis of cancer cells.\(^3\) Plasminogen activator (PA) is a serine protease which is highly active in many types of primary tumor cells. The 2 main types of PA are the urokinase-type (uPA) and the tissue-type (t-PA). These enzymes activate plasminogen to plasmin, which is a strong proteolytic enzyme capable of digesting proteins of extracellular connective tissue matrix. Expression of uPA, its receptor (uPAR) and its inhibitor (uPAl) are reported to be involved in tumor invasion, tumor cell proliferation, metastasis, and tissue remodeling.\(^3\)\(^-\)\(^6\) Plasmin can also indirectly contribute to the spread of tumor cells by activating matrix metalloproteinases (MMPs).\(^4\) Matrix metalloproteinases are a group of endogenous metal ion-dependent proteolytic enzymes that can degrade most ECM components and regulate the activity of enzymes, chemokines and cellular receptors. These inherent properties enable MMPs to affect both the invasion and proliferation of cancer cells.\(^5\)

Many clinical studies have demonstrated that the expression of uPA and uPAR has notable prognostic effects in many types of cancers, such as colon,\(^6\) rectum,\(^7\) stomach,\(^8\) and ovary\(^9\) cancers. There is overwhelming evidence supports of uPA-plasmin system contribution to the invasion and metastasis of breast cancer (BC).\(^10\)\(^\)\(^-\)\(^11\) Similarly, overexpression of MMPs have been associated with poor prognosis in many types of cancers, such as breast,\(^12\) colon,\(^13\) stomach,\(^14\) and ovary cancers. Therefore, blockade of these proteolytic enzyme cascades could be a promising target for cancer therapy.\(^15\)

Aprotinin is a nonspecific serine protease inhibitor. Besides reversible inhibition of kallikrein, uPA and many other serine proteases, aprotinin has antifibrinolytic and anti-inflammatory properties.\(^16\)\(^\)\(^-\)\(^17\) Recent studies have shown that a high dose of aprotinin can suppress the release of proinflammatory cytokines\(^17\) and decrease the capacity of leukocytes to pass through vascular wall.\(^18\) Historically, aprotinin has been used to reduce blood loss and the need for perioperative blood transfusion in many types of major surgeries. However, controversies have recently been raised regarding this indication of aprotinin.\(^16\)

Aprotinin has also been investigated as an antitumor agent. Its capability to inhibit important proteolytic enzymes in the process of cancer invasion and metastasis, such as uPA-plasmin system, suggests notable antitumor potential of aprotinin. However, in vitro and in vivo studies investigating the antitumor activities of aprotinin are limited in number and have reported different results, based on the type of cancer model investigated.\(^19\)\(^\)\(^-\)\(^21\) Despite the significant role of both uPA-plasmin system and MMPs in the progression and prognosis of BC, antitumor effects of aprotinin on this type of cancer have not been investigated. In this in vitro study, we aimed to investigate the effects of aprotinin treatment on human BC cell lines.

Material and methods

Effects of aprotinin treatment on survival and invasion of human BC cell lines MDA-MB-231, MCF-7 and SK-BR-3, and normal human dermal (HDF-1) cell line as control, were investigated in this study.

MTT assay

The antiproliferative activity of aprotinin was assessed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay (Sigma-Aldrich, Steinheim, Germany) in accordance with the standard protocol.\(^22\) In brief, all cell lines were seeded into 96-well cell culture plate with different cell densities. The plates were treated with increasing concentrations of aprotinin (0.4, 0.7, 1, 1.3, and 1.5 trypsin inhibitor unit (TIU)/mL) in triplicate for 72 h. Then, aprotinin was removed and the plates were fed daily with a fresh medium for 3 days. After that, the culture medium was replaced by fresh media and 50 μL of MTT (5 mg/mL). After 4 h of incubation, 200 μL of dimethyl sulfoxide (DMSO) and 25 μL of glycine buffer was added to each well, and absorbance was immediately recorded at 570 nm using enzyme-linked immunosorbent assay (ELISA) reader (Stat Fax 3200; Awareness Technology, Palm City, USA).

Invasion assay

To determine the effect of aprotinin on the local invasion of tumor cells, we performed the invasion assay using a 24-well trans-well chamber (CHEMICON Cell Invasion Assay Kit; Merck Millipore, Billerica, USA). In brief, after performing baseline invasion assays of untreated cell lines, MDA-MB-231 cell line was treated with 5 increasing concentrations of aprotinin, from 0.4 TIU/mL to 1.7 TIU/mL. After each treatment, a repeat invasion assay was performed on the corresponding cell culture. Based on the results obtained from MDA-MB-231 cell line treatment, SK-BR-3 BC cell line was treated with 1.3 TIU/mL concentration of aprotinin, followed by a repeat post-treatment invasion assay. The invasion capabilities of treated cells in duplicate wells were compared with duplicate control untreated wells. The HDF-1 normal fibroblast cell line was considered as a negative control in all procedures. We used invert microscopy for a qualitative assessment
of the invasion process and obtained photographic images of invading cells at the bottom of each invasion well. The optical absorbance of each test was measured by an ELISA reader at 570 nm wavelength.

Statistical analysis

We used mean post-treatment survival fractions given by MTT assay as a measure of survival, and mean optical densities after invasion assay as a measure of local invasion. Analysis of variance (ANOVA) test was used to compare the mean values among different cell lines. Paired t-test or repeated measure ANOVA were used to compare mean values of the same group across different concentrations of treatment when 2 or more than 2 concentrations were used, respectively. Linear regression analysis was used to investigate the correlation between aprotinin dose and response parameters. A p-value <0.05 was considered statistically significant. The statistical analyses were conducted using SPSS for Windows v. 22.0 software (IBM Corp., Armonk, USA) and GraphPad Prism v. 7 (GraphPad Software, La Jolla, USA).

Results

MTT assay

Treatment with different concentrations of aprotinin significantly decreased the surviving fraction and inhibited the growth of both normal and BC cell lines (p < 0.001) tested in this study (Table 1). Higher doses of aprotinin treatment were associated with a correspondingly further drop in the percentage of survival fraction (Fig. 1). The majority of growth inhibitory effects was achieved by the application of 1 TIU/mL of aprotinin. Compared to tumor cell lines, normal fibroblast cell line HDF-1 showed less sensitivity to growth inhibitory effects of aprotinin at 0.4 TIU/mL (p < 0.001), 0.7 TIU/mL (p = 0.003) and 1.5 TIU/mL (p = 0.02) concentrations of aprotinin. Compared to other BC cell lines, MCF-7 cell line showed higher sensitivity to growth inhibitory effects of aprotinin at a concentration of 0.4 TIU/mL (p < 0.001).

Invasion assay

As expected, cancer cell lines demonstrated significantly higher degrees (p < 0.05) of invasion compared

Table 1. Assessment of cell growth by MTT assay for normal and human breast cancer (BC) cell lines treated with different concentrations of aprotinin

| Aprotinin concentration [TIU] | Mean/SD | Survival fraction (%) | p-value |
|-------------------------------|---------|-----------------------|---------|
|                               |         | HDF-1  | MDA-MB-231 | MCF-7 | SK-BR-3 |         |
| 0                             | mean    | 100    | 100        | 100   | 100     | >0.99   |
|                               | SD      | 12.88  | 3.78       | 3.57  | 4.19    |         |
| 0.4                           | mean    | 82.56  | 60.60      | 17.32 | 60.58   | <0.001  |
|                               | SD      | 7.56   | 1.47       | 7.18  | 4.02    |         |
| 0.7                           | mean    | 43.37  | 15.57      | 4.10  | 5.60    | 0.003   |
|                               | SD      | 12.25  | 12.75      | 3.39  | 6.28    |         |
| 1                             | mean    | 5.30   | 2.49       | 2.11  | 0       | 0.31    |
|                               | SD      | 2.00   | 3.33       | 3.92  | 3.35    |         |
| 1.3                           | mean    | 7.45   | 2.19       | 1.52  | 3.25    | 0.42    |
|                               | SD      | 6.45   | 3.77       | 2.71  | 4.22    |         |
| 1.5                           | mean    | 6.98   | 0.51       | 1.36  | 0       | 0.02    |
|                               | SD      | 2.20   | 2.92       | 2.31  | 1.12    |         |
| p-value                       |         | <0.001 | <0.001     | <0.001| <0.001  | <0.001  |

HDF-1 – human normal dermal fibroblast cell line; MDA-MB-231, MCF-7 and SK-BR-3 – human breast cancer cell lines; TIU – trypsin inhibiting unit; SD – standard deviation.
to HDF-1 normal cell line as a negative control (Table 2). Compared to untreated MDA-MB-231 cells, treatment of these BC cells with 1 TIU/mL (p = 0.01), 1.3 TIU/mL (p = 0.01) or 1.7 TIU/mL (p = 0.005), concentrations of aprotinin significantly decreased the degree of invasion (Table 2, Fig. 2, 3).

Treatment of SK-BR-3 cells with 1.3 TIU/mL dose of aprotinin reduced the mean values of optical absorbance obtained for invasion solution (Fig. 4); however, the reduction was not statistically significant (p = 0.06).

**Discussion**

In this study, we found that aprotinin could significantly inhibit the local invasion of human BC cell line MDA-MB-231, an aggressive type of breast adenocarcinoma, in a concentration-dependent manner. In contrast, it showed a nonsignificant trend of inhibitory effect on local invasion of less aggressive type of BC cell line, SK-BR-3. In addition, aprotinin significantly decreased the survival fraction and inhibited the growth of normal human fibroblast cell line, HDF-1, and human BC cell lines MCF-7, MDA-MB-231 and SK-BR-3. However, normal fibroblast cells were less sensitive to growth-inhibitory effects of aprotinin compared to tumor cells.

A number of in vitro and in vivo studies have investigated the effects of aprotinin treatment on the local invasion of human cancer. In vitro studies reported that aprotinin could substantially reduce the local invasion of prostate cancer through a different mechanism. In a study conducted by Gao et al., aprotinin decreased the invasion of DU145 human prostate cancer cell line through the inhibition of tissue kallikrein. A subgroup of serine proteases that can promote cancer cell migration and invasion. Likewise, Bekes et al. demonstrated that the administration of aprotinin can have antitumor effects by reducing the local invasion and distant metastasis of PChi/diss, which is a highly metastatic type of PC-3 human prostate carcinoma cell line. They reported a different mechanism for this observation. In their experiments, aprotinin attenuated tumor cell line invasion via inhibition of plasmin, which is an activator of pro-urokinase-type plasminogen activator (pro-uPA). Pro-uPA is a serine protease pro-enzyme that, after converting to its activated form, uPA, contributes to the degradation of extracellular matrix and possibly tumor cell migration and proliferation. Similarly, in this study, we observed that aprotinin decreased the local invasion of MDA-MB-231 human BC cell line when used in concentrations of 1 TIU/mL or higher. To date, this is the first experiment that investigates the inhibitory effects of aprotinin on human BC invasion.

It is postulated that anti-invasion effects of aprotinin on MDA-MB-231 BC cell line could be mediated by the inhibition of uPAR/uPA pathway. Previous studies have

**Table 2. Quantitative results of invasion by measuring optical absorbance of solutions prepared form invasion kit wells**

| Cell line       | Optical density | p-value |
|----------------|----------------|---------|
|                | experiment 1   | experiment 2 | mean | SD |
| HDF            |                |            |      |
| untreated      | 0.63           | 0.53       | 0.58 | 0.07 | –       |
| SK-BR3         |                |            |      |
| untreated      | 1.82           | 1.86       | 1.84 | 0.03 | 0.06    |
| treated (1.3 TIU) | 1.52       | 1.66       | 1.59 | 0.09 |
| MDA-MB 231     |                |            |      |
| untreated      | 1.91           | 1.96       | 1.93 | 0.03 | –       |
| treated        | 1.87           | 1.93       | 1.90 | 0.04 | 0.99    |
| 0.4 TIU        | 1.78           | 1.88       | 1.83 | 0.07 | 0.69    |
| 0.7 TIU        | 1.48           | 1.62       | 1.55* | 0.10 | 0.01    |
| 1 TIU          | 1.52           | 1.64       | 1.58* | 0.08 | 0.01    |
| 1.3 TIU        | 1.46           | 1.54       | 1.50* | 0.06 | 0.005   |
| 1.7 TIU        |                |            |      |
| HDF – human dermal fibroblast; SD – standard deviation; TIU – trypsin inhibiting unit; NS – not significant; * p-value <0.05 for post-hoc comparisons of corresponding group with untreated group.

**Fig. 2.** Inhibitory effects of aprotinin on local invasion of MDA-MB-231 cell line. These series of images taken using invert microscope illustrate effects of treatment with increasing doses of aprotinin on local invasion of MDA-MB-231 human breast cancer (BC) cell line. Compared to untreated cells (A), treatment with 1.0 (B) and 1.5 (C) TIU/mL of aprotinin resulted in significantly (p < 0.001) more profound inhibition of local invasion in a step-wise manner. The magnification scale is x40.
demonstrated that uPAR/uPA pathway is overexpressed endogenously in MDA-MB-231 cell line. Breast cancer cells that express higher amounts of uPA have a greater proteolytic capacity at the cell surface–ECM interface, which gives them higher invasion potentials. Aprotinin can both reduce cell-surface binding to plasminogen and prevent the conversion of pro-uPA to the active uPA form. Therefore, it could be expected that aprotinin can attenuate invasive potentials of MDA-MB-231 cell line.

On the other hand, studies have demonstrated no inhibitory effects of aprotinin on melanoma and thyroid cancers. Prange et al. found that aprotinin had no anti-invasive effect on Melanom-5 and Melanom-57/12 cell lines. Likewise, aprotinin, even in high doses, had no effect on EGF-stimulated invasion of thyroid cancer. The authors proved that serine proteases, such as plasmin, had less prominent effects in cancer models used in their study.

We observed the antiproliferative effects of aprotinin on both normal and tumor cell lines. Normal human fibroblast cell line HDF-1 demonstrated less sensitivity to growth-inhibitory effects of both lower and higher doses of aprotinin, compared to tumor cell lines. Among 3 human BC cell lines investigated, MCF-7 showed slightly higher sensitivity to antiproliferative effects of low-dose aprotinin. Similar antiproliferative effects of aprotinin were seen on 3-methyl cholangthrene-induced squamous cell carcinoma in a murine model; mice treated with aprotinin had significantly smaller tumors and longer survival compared with control. Furthermore, aprotinin treatment could inhibit tumor growth in Lewis lung carcinoma, Hepatoma-22 cell and Walker-0256 carcinosarcoma model systems in mice. Therefore, the majority of current literature indicates antiproliferative effects of aprotinin on both normal and tumor cell lines, with varying degrees.

To date, few studies have reported the inhibitory effects of aprotinin on both the survival and local invasion of cancer. As seen in our study, Latner et al. showed that aprotinin treatment reduced both tumor growth and invasion when administered to hamsters bearing a highly invasive fibrosarcoma, or to mice bearing a similar malignant mammary carcinoma. Aprotinin also increased tumor necrosis. The authors attributed this effect to the enhancement of the host’s immunological system by aprotinin against the tumor cells. In addition to this mechanism, the antiproliferative role of aprotinin could be mediated by its downstream inhibitory effects on MMPs, in a similar way to mechanism underlying its anti-invasive role. Matrix metalloproteinases generally increase cell proliferation by modulating cell surface receptors as well as extracellular cytokines and apoptotic ligands. Therefore, the inhibition of MMPs by aprotinin could expectedly alter cellular proliferation. Finally, the inhibitory effect of aprotinin on the local invasion could occur, at least in part, due to its inhibitory effects on survival. However, distinct independent mechanisms should coexist for anti-invasive effects. It is inferred from our observation that despite similar inhibitory effects of aprotinin on the survival of MDA-MB-231 and SK-BR-3 cell lines, the inhibition of the local invasion was more dominant in MDA-MB-231.

Further studies utilizing in-depth and comprehensive molecular and genetic assessments are needed to provide mechanistic insights on how aprotinin inhibits the survival and invasion of BC cells. These assessments could include the expression of MMPs and uPA/uPAR system, and overexpression/silencing of other potential molecules. Furthermore, examining the type of cell death...
(e.g., necrosis, apoptosis, autophagy, or necroptosis) induced by aprotinin in cancer cell lines studied in this study could shed further light on the mechanism of inhibitory effects of aprotinin.

In contrast to local antiproliferative and anti-invasive effects, aprotinin treatment could increase the survival of metastatic cancer cells in some in vivo studies. This was attributed to its antifibrinolytic effects. The spread of metastatic cells through the vasculature of secondary tissue is enhanced by the association of these cells with microthrombi. Aprotinin and other antifibrinolytic agents could enhance the association between metastatic cancer cells and microthrombi, resulting in increased survival of these cells. Therefore, it could be inferred that aprotinin would be more beneficial in the early stages of cancer, before metastasis occurs. Furthermore, it seems mandatory to individually investigate the summary effects of aprotinin on each specific type of cancer by weighing local antiproliferative and anti-invasion effects against the potential harmful effects attributable to improving the survival of metastatic cells.

Beneficial effects of aprotinin in cancer patients have also been reported in clinical studies. Aprotinin administration could increase the post-pneumectomy survival of patients with mesothelioma, whether due to its anti-inflammatory, antiserine protease, or anti-angiogenesis effects. This beneficial role of aprotinin in survival was independent from its positive effects attributable to reducing bleeding. Such clinical evidence further supports potential indications of aprotinin as an antitumor drug. Similar to other recently introduced agents with potential antitumor effects, aprotinin could be considered as an adjunct therapy to complement current chemotherapeutic regimens. In addition to enhancing the efficacy, this combination could decrease the dose of chemotherapeutic drugs needed and limit exposure to their numerous unfavorable toxic effects.

Clinical studies have reported some adverse effects of systemic administration of aprotinin. High doses of aprotinin resulted in allergic and anaphylactic reactions, as well as immunoglobulin G (IgG) formation in 50% of patients receiving aprotinin in the context of cardiac operations. In addition, systemic administration of aprotinin could increase the risk of renal failure, myocardial infarction, stroke, and encephalopathy. Therefore, if confirmed by future experimental and clinical studies as promising anticancer drug, it would be safer to consider aprotinin as a local agent. For example, tissue-engineering techniques could be exploited to develop scaffolds that locally release aprotinin in a specific timely manner. Surgeons could use these scaffolds to invest the resection site of primary tumor bulk (e.g., after mastectomy) in order to locally inhibit the proliferation and invasion of remaining tumoral cells in the resection margin. An almost similar method of anticancer drug delivery is currently used in the treatment of glioblastoma multiform.

The MTT assay used in this study is a simple, rapid and reproducible method to investigate cytotoxicity of anticancer agents. Its results have high correlation with more labour-intensive and time-consuming method, clonogenic assay. Longer recovery phase used in this study could diminish the limitation of MTT assay in differentiating the reduction in cellular metabolic activity from the reduction in number of cells.

We investigated 3 human BC cell lines in this study. Selection of these cell lines was carried out in order to include different subtypes of human BC cell lines with respect to the expression of estrogen receptor (ER), progesterone receptor (PR) and HER2. MCF-7 is positive for ER and PR and negative for HER2. SK-BR3 is negative for ER and PR and positive for HER2. MDA-MB-231 is negative for all 3. Future studies could consider other BC cell lines to investigate whether current findings could be generalized to other types of BC cell lines. Furthermore, we used HDF-1 as a control group in this study. It is a non-transformed, non-malignant normal human fibroblast cell line with high sensitivity to cytotoxic agents. Although findings on this cell line could be extrapolated to other normal cell lines, using purely normal human breast cell line as a control could have provided more robust evidence in this topic. Finally, in vitro behavior of aprotinin may not be a precise predictor of its behavior in in vivo or in situ settings. Animal model and clinical studies, if indicated after further confirmatory in vitro studies, could provide more robust evidence.

In conclusion, our study showed that aprotinin significantly inhibited the growth of normal fibroblast and BC cell lines. Compared to normal cell line, tumor cell lines showed higher sensitivity to antiproliferative effects of both low- and high-dose aprotinin. Furthermore, aprotinin administration at doses equal to or above 1 TIU/mL, significantly inhibited the local invasion of MDA-MB-231 BC cell line. Our findings encourage further confirmatory in vitro studies to be conducted with a more comprehensive mechanistic approach. They can determine if investigations of aprotinin as anticancer drug could advance to in vivo or clinical studies.

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