In Vitro Blockade of Adhesion of Breast Cancer Cells to Endothelial Cells Using Anti-inflammatory Drugs

ESTHER BISCHOFS[1], DANIEL LUBS[1], FRIEDERIKE FRITZSCHE[1], ANNE-SOPHIE MEYER[1], THOMAS BRUCKNER[2], CHRISTOF SOHN[1] and MICHAEL H.R. EICHBAUM[1]

[1]*Department of Gynecology and Obstetrics, Heidelberg, Germany and*
[2]*Institute of Medical Biometry, University of Heidelberg Medical School, Heidelberg, Germany*

Abstract. *Background: Increasing evidence suggests that a pro-inflammatory microenvironment affects distant metastasis of breast cancer cells, in particular by favoring tumor cell adhesion to endothelium. The aim of this study was to investigate the potential of different anti-inflammatory drugs to inhibit this effect in vitro. Materials and Methods: Breast cancer cells from the metastatic cell line KM22 were incubated with activated Human umbilical vein endothelial cells (HUVECs). Tumor cell adhesion was quantified by fluorescence microscopy. The anti-inflammatory drugs ibuprofen, aspirin (acetylsalicylic acid), diclofenac, and dexamethasone were used as inhibiting agents. Results: Aspirin and dexamethasone significantly reduced breast cancer cell adhesion to HUVECs (20.3%, p<0.000; and 25%, p<0.05, respectively). Ibuprofen and diclofenac did not significantly reduce tumor cell adhesion. Conclusion: Aspirin and dexamethasone seem to be able to partly inhibit adhesion of breast cancer cells to endothelium. Future studies should attempt to optimize this effect in vitro, in preparation for potential in vivo trials.*

Despite advances in the diagnosis and treatment of breast cancer, the 5-year survival rate for patients with metastatic disease in the United States was only 23.3% between 2001 and 2007 (1). Therefore, exploring the mechanisms underlying distant metastasis in order to find new potential targets for therapy represents a primary scientific goal in gynecological oncology. Further progress here, could mark a major breakthrough in the treatment of the disease. Tumor metastasis is a multi-step process. One step is the adhesion of tumor cells circulating in the bloodstream to distant endothelium. This adhesive mechanism is akin to leukocyte migration to sites of injury or infection and involves intravasation from the primary site to the bloodstream, dissemination through the circulation, cell adhesion, extravasation, and formation of metastases in the target organs (2). The initial process during which tumor cells attach to the vascular endothelium seems to be set by the interaction between sialyl–Lewis^{x}, expressed on several tumor cell types, and E-selectin, expressed on the endothelium (2, 3). The cell adhesion molecule E-selectin has been shown to mediate adhesion of colonic cancer cells (HT-29) (4-6), renal cell carcinoma (RCC) (7), and breast cancer cells (MDA-MB-231) (8, 9) to endothelial cells after being activated by pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) or interleukin-1 (IL-1), leading to tumor cell invasion and liver metastasis (2, 10). In addition to this E-selectin-mediated mechanism, the adhesion molecules intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) seem to be responsible for the formation of a firm adhesion between tumor cells and endothelium (11, 12). Recent *in vitro* models have demonstrated that a pro-inflammatory microenvironment plays an important role during the initial stages of liver metastasis from murine lung carcinoma H-59 and human colorectal carcinoma CX-1 cells (10, 13) These studies demonstrated that tumor cells trigger perisinusoidally located macrophages (Kupffer cells) to produce TNF-α, a cytokine with a known tumor-enhancing potential (14-16). Furthermore, in 2008, Kinder *et al*. showed that metastatic breast cancer cells can induce an inflammatory response from osteoblasts during bone metastasis (17) and thereby co-opt osteoblasts into creating a prometastatic microenvironment, suggesting that breast cancer metastasis may depend on the tumor's ability to activate pro-inflammatory cells. In line with these data, Eichbaum *et al*. confirmed that three different types of metastatic breast cancer cells were able to secrete

Correspondence to: Dr. med. Esther Bischofs, MD, Department of Gynecology and Obstetrics, University of Heidelberg Medical School, Voss-Strasse 9, D-69115 Heidelberg, Germany. Tel: +49 6221567856, Fax: +49 6221568599, e-mail: Esther.Bischofs@med.uni-heidelberg.de

Key Words: Breast cancer, metastasis, anti-inflammatory drugs, endothelium, pro-inflammatory microenvironment, HUVECs, KM22 cell line.

active pro-inflammatory cytokines, in particular interleukin-6 (IL-6), IL-8, monocyte chemotactic protein-1 (MCP-1), and macrophage colony stimulating factor (MCSF) (18). In contrast, activation of nonmalignant human mammary epithelial cells did not induce a comparable secretion of proinflammatory cytokines. The same working group demonstrated that breast cancer cells can stimulate macrophages to produce TNF-α (18). Indeed, TNF-α itself can induce increased expression of endothelial E-selectin. These interrelations between metastasis and inflammation could explain why nonsteroidal anti-inflammatory drugs and cimetidine have shown prophylactic effects against the distant metastasis of certain tumor types both *in vitro* and *in vivo* (19-21) and were able to inhibit the metastatic process in various animal and *in vitro* models (22, 23). One possible explanation for these effects may be attributed to modulation of the pro-inflammatory microenvironment, in particular, the expression of endothelial E-selectin, thus reducing tumor cell adhesion to the endothelium (10, 13, 24). At present, only little information is available regarding the potential of anti-inflammatory drugs to influence the adhesion of breast cancer cells to endothelial cells. Our aim was therefore to evaluate the effect of different anti-inflammatory drugs, aspirin, dexamethasone, diclofenac, and ibuprofen, on the adhesion of the breast cancer cells KM22 to endothelium using an established, previously reported Human umbilical vein endothelial cell (HUVEC) monolayer model (18).

Materials and Methods

Cells. HUVECs were cultured using PromoCell™ kit (both obtained from PromoCell, Heidelberg, Germany). After washing and enzymatic digestion using the Detach Kit from PromoCell (including HEPES buffered salt solution, trypsin, and trypsin-neutralizing solution), the HUVECs were used between the fourth and eigth passage. KM22 is a characterized, established metastatic ductal-invasive breast cancer cell line, originally derived from human tumor biopsies at our institution, as previously described (25). Characteristics of these tumor cells have been reported elsewhere (18). KM22 cells were cultured in Dulbecco's modified Eagle's medium with L-glutamine supplemented with 6% heat-inactivated fetal bovine serum and 0.5% streptomycin/penicillin 10,000 U/ml and used in passages five to twelve. Both cell lines were maintained at 37˚C in a humidified atmosphere with 5% CO_2.

Reagents. Recombinant TNF-α and Ibuprofen® were purchased from R&D Systems, (Wiesbaden, Germany); Diclofenac Sodium® came from Sigma Aldrich (Munich, Germany); Aspisole® (aspirin) was purchased from Bayer Vital GmbH (Leverkusen, Germany) and Fortecortin Inject® (dexamethasone) from Merck Serono GmbH, (Darmstadt, Germany). The fluorescent cell tracker (CMFDA) used to label tumor cells came from Invitrogen (Karlsruhe, Germany).

Cell adhesion assay. HUVECs were grown for 36 h to confluence in 8-well Lab Tek II chamber slide systems. Dosages and incubation time duration for diclofenac, ibuprofen, aspirin, and dexamethasone were optimized in preliminary experiments (data not shown). All subsequent experiments were carried out under these conditions. For anti-inflammatory agent mediated blocking of cell adhesion, HUVECs were incubated with 200 μl (125 μg) diclofenac per well (n=24 wells) for 4 h, 1 mM ibuprofen for 8 h (n=24 wells), 0.4 mg/ml aspirin for 4 h (n=24 wells), and 0.4 mg/ml dexamethasone for 6 h (n=24 wells), at 37˚C in 5% CO_2. As a control, the endothelial cells were only treated with phosphate buffered saline (PBS) (n=24 wells). Two hours before the respective end of the incubation period, HUVECs were stimulated with 5 ng/ml TNF-α. Then the tumor cells (2.5×10^5 cells/ 200μl/ well) were prelabeled with 7.5 μM green fluorescence cell tracker CMFDA for 30 min at 37˚C and added onto the monolayer. To prevent nonspecific cell attachment, HUVECs were incubated with PBS containing 1% bovine serum albumin (BSA) for 20 min at 37˚C prior to adding the tumor cells and washed extensively with PBS after incubation with the tumor cells. After fixing the slides with methanol for 5 min, they were equipped with aquatex and a coverglass.

Analysis and quantification of tumor cell adhesion. A computerized, automatic Olympus BX-61 fluorescence microscope with Software Scan View version 6.0 SP3 from Applied Spectral Imaging (Edingen Neckarhausen, Germany) was used to analyze cell adhesion. A fluorescein isothiocyanate (FITC) filter had been installed in front of the ×10 objective. In total, every well was captured by 40 rectangular fields of view and the total number of cells were counted. The total number of adherent tumor cells was counted.

Statistical analysis. All data were electronically recorded. Statistical analyses were undertaken using the SAS™ software (SAS, Cary, No, NC, USA). For all continuous variables, median, and lower and upper quartiles were determined. Differences were tested using a two-sided Student's *t*-test. A value of $p<0.05$ was considered to indicate statistical significance.

Results

We evaluated the effect of four different anti-inflammatory drugs on KM22 cell adhesion to cultures of HUVECs. Figures 1 and 2 demonstrate representatively that aspirin and dexamethasone markedly inhibited KM22 adhesion to HUVECs (Figures 1 and 2).

Aspirin (Aspisole®). A median number of 18,038 adherent tumor cells were detected in HUVECs pretreated with aspirin (lower quartile 17,486; upper quartile 18,954), thereby significantly different from that of the untreated control group ($p<0.0001$). In the untreated control group, the median adherent tumor cell count was 22,628 (lower quartile 20,259; upper quartile 23,984). Compared to this, aspirin significantly reduced tumor cell attachment by 20.3%.

Dexamethasone (Fortecortin Inject®). Dexamethasone also inhibited KM22 cell adhesion to HUVEC-coated plastic wells. The median number of adherent tumor cells pretreated with dexamethasone was 17,139 (lower quartile 16,709; upper quartile 17,518). The absolute tumor cell count differed significantly from that of the untreated control group [median 22,838 (lower quartile 22,351¸ upper quartile 23,816), $p<0.05$], corresponding to a 25% inhibition of tumor cell attachment.

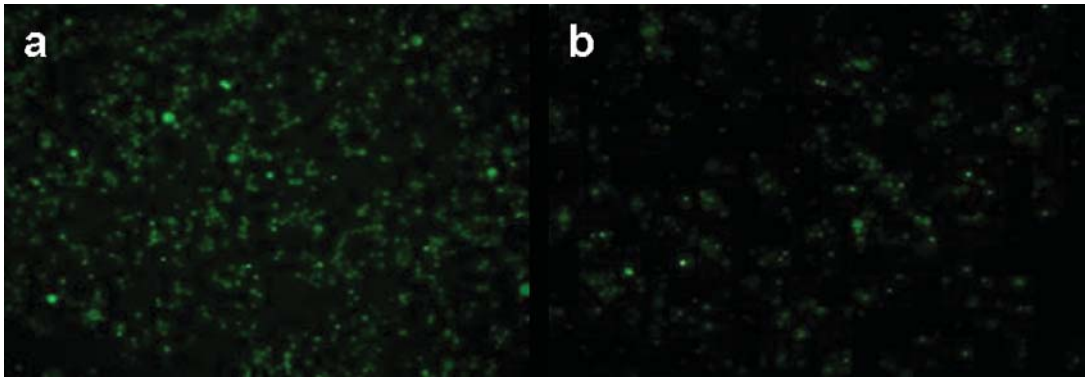

Figure 1. *Representative images of 5-chloromethylfluorescein diacetate (CMFDA)-labeled KM22 cells attached to human umbilical vein endothelial cells (HUVECs) without treatment (a) and after treatment with 0.4 mg/ml aspirin for 4 hours (b).*

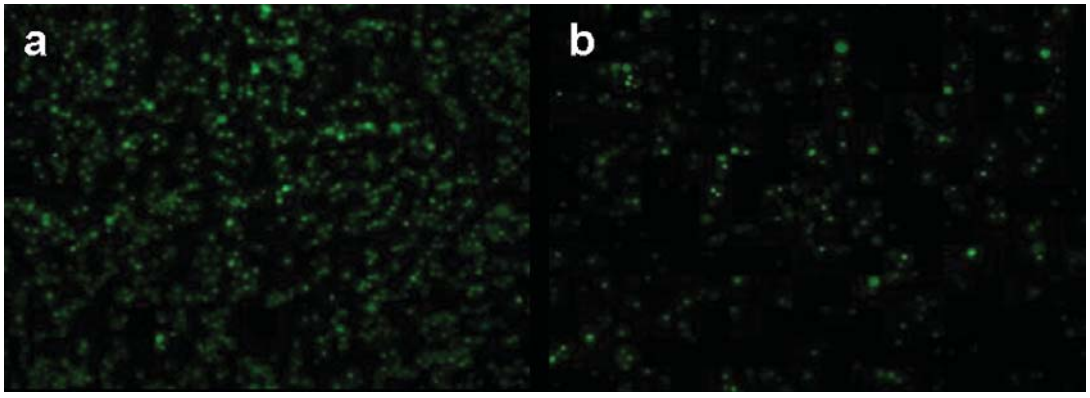

Figure 2. *Representative images of 5-chloromethylfluorescein diacetate (CMFDA)-labeled KM22 cells attached to human umbilical vein endothelial cells (HUVECs) without treatment (a) and after treatment with 0.4 mg/ml dexamethasone for 6 hours (b).*

Diclofenac (Diclofenac Sodium®). Pretreatment of HUVECs with diclofenac did not reduce KM22 cell attachment in this experimental model. The median number of adherent tumor cells in cultured of endothelial cells incubated in diclofenac was 18,856 (lower quartile 17,089; upper quartile 20,143), whereas the untreated control group had a median of 18,487 adherent cells (lower quartile 17,359; upper quartile 19,989) (p=0.99).

Ibuprofen (Ibuprofen®). Ibuprofen was not able to reduce the attachment of KM22 to HUVECs in this experimental model. A median of 20,513 adherent tumor cells were counted after pretreatment of HUVECs with ibuprofen (lower quartile 18,222; upper quartile 22,141), compared to the corresponding control group in which 20,196 cells were detected (lower quartile 17,969; upper quartile 23,445) (p=0.67).

Discussion

Increasing evidence suggests that a pro-inflammatory microenvironment favors the distant metastasis of different solid tumor entities and distinct local interactions between tumor cells, local inflammatory cells, and endothelium have been reported (1, 5, 9, 10, 20, 21, 24, 26). As previously shown, human breast cancer cells (KM22) are able to adhere to TNF-α-activated HUVECs. This adhesion is mediated by E-selectin as it can also be selectively blocked both by neutralizing monoclonal antibodies against E-selectin and by cimetidine (18). Eichbaum *et al*. developed the hypothesis that, similarly to colon carcinoma cells, breast cancer cells can initiate an inflammatory cascade that results in increased tumor cell adhesion to local endothelial cells. The present data showed that aspirin is able to significantly reduce the attachment of KM22 breast cancer cells to HUVECs by 20.3%. This is consistent with previously reported results from Bobek and co-workers, who observed an anti-adhesive effect of aspirin *in vitro* with highly invasive breast cancer cells lines BT 549 and MDA-MB-231 (23). In addition, aspirin also suppressed tumor cell adhesion to endothelium *in vitro* in a second preclinical study analyzing human prostate cancer cells (PC3) (26). Futakuchi *et al*. examined

the effect of aspirin on lung metastasis formation in an *in vivo* model of chemically induced rat hepatocellular carcinoma. This study demonstrated the potential of aspirin to inhibit lung metastasis, possibly *via* reduced attachment of tumor cells to endothelium (27). Secondly, in the present study dexamethasone inhibited tumor cell adhesion to endothelium. KM22 attachment to HUVECs was reduced by 25%. This is in line with data from Qian *et al*., who showed that in a solid-phase assay between hepG2 and endothelial cell lines *in vitro,* dexamethasone is able to block cell adhesion (28). Moreover, pretreatment of cultured human dermal endothelial cells with dexamethasone resulted in a decrease in human promyelocytic leukemia cell (HL-60) adhesion (29). Contrary to our expectations, however, the nonselective cyclooxygenase inhibitors ibuprofen and diclofenac did not show an anti-adhesive effect of KM22 breast cancer cells to endothelial cells. However, diclofenac was found to be capable of inhibiting E-selectin, ICAM-1, and VCAM-1 expression in HUVECs (30). Furthermore, ibuprofen was shown to inhibit the expression of ICAM-1 and VCAM-1 on HUVECs (31). Future experimental studies should aim to optimize the demonstrated effects of aspirin and dexamethasone on breast cancer cell adhesion to endothelium, in particular by combining these two anti-inflammatory strategies. Subsequent to these efforts, *in vivo* experiments are needed to clarify whether the results of this study can be transferred into a clinically relevant reduction of metastasis, *e.g*. in an established mouse model for hepatic metastasis.

References

1 http://seer.cancer.gov/statfacts/html/breast.html

2 Kobayashi H, Boelte KC and Lin PC: Endothelial cell adhesion molecules and cancer progression. Curr Med Chem *14(4)*: 377-386, 2007.

3 Kannagi R, Izawa M, Koike T, Miyazaki K and Kimura N: Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. Cancer Sci *95(5)*: 377-384, 2004.

4 Daneker GW, Lund SA, Caughman SW, Staley CA and Wood WC: Anti-metastatic prostacyclins inhibit the adhesion of colon carcinoma to endothelial cells by blocking E-selectin expression. Clin Exp Metastasis *14(3)*: 230-238, 1996.

5 Brodt P, Fallavollita L, Bresalier RS, Meterissian S, Norton CR and Wolitzky BA: Liver endothelial E-selectin mediates carcinoma cell adhesion and promotes liver metastasis. Int J Cancer *71(4)*: 612-619, 1997.

6 Mannori G, Santoro D, Carter L, Corless C, Nelson RM and Bevilacqua MP: Inhibition of colon carcinoma cell lung colony formation by a soluble form of E-selectin. Am J Pathol *151(1)*: 233-243, 1997

7 Steinbach F, Tanabe K, Alexander J, Edinger M, Tubbs R, Brenner W, Stöckle M, Novick AC and Klein EA: The influence of cytokines on the adhesion of renal cancer cells to endothelium. J Urol *155(2)*: 743-748, 1996.

8 Narita T, Kawakami-Kimura N, Matsuura N, Hosono J and Kannagi R: Corticosteroids and medroxyprogesterone acetate inhibit the induction of E-selectin on the vascular endothelium by MDA-MB-231 breast cancer cells. Anticancer Res *15(6B)*: 2523-2527, 1995.

9 Moss MA, Zimmer S and Anderson KW: Role of metastatic potential in the adhesion of human breast cancer cells to endothelial monolayers. Anticancer Res *20(3A)*: 1425-1433, 2000.

10 Auguste P, Fallavollita L, Wang N, Burnier J, Bikfalvi A and Brodt P: The host inflammatory response promotes liver metastasis by increasing tumor cell arrest and extravasation. Am J Pathol *170(5)*: 1781-1792, 2007.

11 Neeson PJ, Thurlow PJ, Jamieson GP and Bradley C: Lymphocyte-facilitated tumour cell adhesion to endothelial cells: the role of high affinity leucocyte integrins. Pathology *35(1)*: 50-55, 2003.

12 Yamada M, Yanaba K, Hasegawa M, Matsushita Y, Horikawa M, Komura K, Matsushita T, Kawasuji A, Fujita T, Takehara K, Steeber DA, Tedder TF and Sato S: Regulation of local and metastatic host-mediated anti-tumour mechanisms by L-selectin and intercellular adhesion molecule-1. Clin Exp Immunol *143(2)*: 216-227, 2006.

13 Khatib AM, Auguste P, Fallavollita L, Wang N, Samani A, Kontogiannea M, Meterissian S, and Brodt P: Characterization of the host proinflammatory response to tumor cells during the initial stages of liver metastasis. Am J Pathol *167(3)*: 749-759, 2006.

14 Szlosarek PW and Balkwill FR: Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. Lancet Oncol *4(9)*: 565-573, 2003.

15 Sturm JW, Magdeburg R, Berger K, Petruch B, Samel S, Bönninghoff R, Keese M, Hafner M and Post S: Influence of TNFα on the formation of liver metastases in a syngenic mouse model. Int J Cancer *107(1)*: 11-21, 2003.

16 Anderson GM, Nakada MT and DeWitte M: Tumor necrosis factor-alpha in the pathogenesis and treatment of cancer. Curr Opin Pharmacol *4(4)*: 314-320, 2004.

17 Kinder M, Chislock E, Bussard KM, Shuman L and Mastro AM: Metastatic breast cancer induces an osteoblast inflammatory response. Exp Cell Res *314(1)*: 173-183, 2008.

18 Eichbaum C, Meyer AS, Wang N, Bischofs E, Steinborn A, Bruckner T, Brodt P, Sohn C and Eichbaum MH: Breast cancer cell-derived cytokines, macrophages and cell adhesion: implications for metastasis. Anticancer Res *31(10)*: 3219-3227, 2011.

19 Castelao JE, Yuan JM, Gago-Dominguez M, Yu MC and Ross RK: Non-steroidal anti-inflammatory drugs and bladder cancer prevention. Br J Cancer *82(7)*: 1364-1369, 2000.

20 Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, Petrelli N, Pipas JM, Karp DD, Loprinzi CL, Steinbach G and Schilsky R: A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. N Engl J Med 348(10): 883-890, 2003. Erratum in: N Engl J Med *348(19)*: 1939, 2003.

21 Schreinemachers DM and Everson RB: Aspirin use and lung, colon, and breast cancer incidence in a prospective study. Epidemiology *5(2)*: 138-146, 1994.

22 Dianzani C, Brucato L, Gallicchio M, Rosa AC, Collino M and Fantozzi R: Celecoxib modulates adhesion of HT29 colon cancer cells to vascular endothelial cells by inhibiting ICAM-1 and VCAM-1 expression. Br J Pharmacol *153(6)*: 1153-1161, 2008.

23 Bobek V, Boubelik M, Kovarík J and Taltynov O: Inhibition of adhesion breast cancer cells by anticoagulant drugs and cimetidine. Neoplasma *50(2)*: 148-151, 2003.
24 Kobayashi K, Matsumoto S, Morishima T, Kawabe T and Okamoto T: Cimetidine inhibits cancer cell adhesion to endothelial cells and prevents metastasis by blocking E-selectin expression. Cancer Res *60(14)*: 3978-3984, 2000.
25 Zhong XY, Kaul S, and Bastert G: Evaluation of MUC1 and EGP40 in bone marrow and peripheral blood as a marker for occult breast cancer. Arch Gynecol Obstet *264*: 177-181, 2001.
26 Lloyd FP Jr, Slivova V, Valachovicova T and Sliva D: Aspirin inhibits highly invasive prostate cancer cells. Int J Oncol *23(5)*: 1277-1283, 2003.
27 Futakuchi M, Ogawa K, Sano M, Tamano S, Takeshita F and Shirai T: Suppression of lung metastasis by aspirin but not indomethacin in an *in vivo* model of chemically induced hepatocellular carcinoma. Jpn J Cancer Res *93(10)*: 1175-1181, 2002.
28 Qian SK, Chen D, Li Y, Yang XW, Deng WJ, Li Q, Lin H, Hu H, Xiao JQ and Chen SW: Effects of E-selectin and their ligands on the adhesive metastasis of hepatocellular carcinoma. Zhonghua Gan Zang Bing Za Zhi *18(6)*: 440-444, 2010 (Chinese).
29 Patel NA, Patel JA, Stins MF, Kim KS and Chang SL: Dexamethasone affects cytokine-mediated adhesion of HL-60 human promyelocytic leukemia cells to cultured dermal microvascular endothelial cells. Clin Immunol *99(3)*: 387-394, 2001.
30 Sakai A: Diclofenac inhibits endothelial cell adhesion molecule expression induced with lipopolysaccharide. Life Sci *58(26)*: 2377-2387, 1996.
31 Kapiotis S, Sengoelge G, Sperr WR, Baghestanian M, Quehenberger P, Bevec D, Li SR, Menzel EJ, Mühl A, Zapolska D, Virgolini I, Valent P and Speiser W: Ibuprofen inhibits pyrogen-dependent expression of VCAM-1 and ICAM-1 on human endothelial cells. Life Sci *58(23)*: 2167-2181, 1996.

Received January 5, 2012
Revised February 8, 2012
Accepted February 9, 2012