Proteasome inhibitors: Their effects on arachidonic acid release from cells in culture and arachidonic acid metabolism in rat liver cells

Lawrence Levine*

Address: Department of Biochemistry, Brandeis University Waltham, MA 02454, USA
Email: Lawrence Levine* - llevine@brandeis.edu
* Corresponding author

Abstract

Background: I have postulated that arachidonic acid release from rat liver cells is associated with cancer chemoprevention. Since it has been reported that inhibition of proteasome activities may prevent cancer, the effects of proteasome inhibitors on arachidonic acid release from cells and on prostaglandin \( I_2 \) production in rat liver cells were studied.

Results: The proteasome inhibitors, epoxomicin, lactacystin and carbobenzyoxy-leucyl-leucyl-leucinal, stimulate the release of arachidonic acid from rat glial, human colon carcinoma, human breast carcinoma and the rat liver cells. They also stimulate basal and induced prostacycin production in the rat liver cells. The stimulated arachidonic acid release and basal prostaglandin \( I_2 \) production in rat liver cells is inhibited by actinomycin D.

Conclusions: Stimulation of arachidonic acid release and arachidonic acid metabolism may be associated with some of the biologic effects observed after proteasome inhibition, e.g. prevention of tumor growth, induction of apoptosis, stimulation of bone formation.

Background

The proteasome degrades many cellular proteins, several with regulatory functions. It is not surprising that proteasome inhibitors affect many biologic processes [1] including prevention of cancer [2]. The effect of proteasome inhibition on cell growth and possible cancer chemoprevention has been reviewed by Adams [3].

Epoxomicin, an \( \alpha'\-\beta' \)-epoxyketone, appears to be the most selective proteasome inhibitor. Based on its anti-tumor activity, this product was originally isolated from an actinomycetes strain C-996-17 [4]. It inhibits the chymotrypsin-like activity (cleavage after large hydrophobic residues), trypsin-like activity (cleavage after basic residues) and peptidyl-glutamyl peptide hydrolyzing (PGPH) activity (cleavage after acidic residues) of proteasomes. Activities of the Ca\(^{++}\)-dependent proteases, calpain, papain, chymotrypsin, trypsin and cathepsin are not affected by epoxomicin even at a 50 \( \mu M \) concentration [5].

The \( \beta \)-lactone, lactacystin, is relatively selective but can inhibit cathepsin A [6]. Peptide aldehydes, in addition to inhibiting proteasome activity, can also inhibit lysosomal and Ca\(^{++}\)-activated proteases [7]. The peptides containing the carboxyvinylsulfone moiety inhibit cysteine proteases [8,9].
I have shown that inhibition of proteolysis by phenylmethylsulphonyl fluoride, the peptide aldehydes carboxbenzoxyl-leucyl-leucyl-norvalinal and carboxbenzoxyl-leucyl-leucyl-leucinal (ZLLL) and lactacystin stimulate induced prostaglandin (PGI₂) production in rat liver cells [10,11]. Lactacystin stimulates arachidonic acid (AA) release from these cells [11]. Others have reported that proteasome inhibition up-regulates cyclooxygenase-2 (COX-2) and stimulates PGE₂ production in neuronal cells [12].

In this report, evidence is presented that proteasome inhibitors stimulate PGI₂ production by rat liver cells as well as the release of AA from rat liver, rat glial, human colon carcinoma and human breast carcinoma cells in culture. The stimulation of AA release from rat liver cells is partially inhibited by preincubation of the cells with actinomycin D.

**Results and Discussion**

Of the cells examined (C-9 rat liver, C-6 rat glial, HT-29 human colon carcinoma and BT-20 human breast carcinoma) the prostanoid metabolic profile has been described only for C-9 rat liver cells (95% is PGI₂ and less than 5% is PGE₂ and PGF₂α) [13]. At the low cell densities used in this study, only PGI₂, the main product of COX-mediated synthesis, can be quantitatively estimated. The rat liver cells express COX-2 both constitutively and after induction [14]. The effect of time on basal and 12-0-tetradecanoylphorbol-13-acetate (TPA) induced PGI₂ synthesis during incubation of cells with epoxomicin is shown in Fig. 1.

The stimulation of basal PGI₂ production by epoxomicin and TPA-induced PGI₂ production by epoxomicin and lactacystin as a function of dose is shown in Fig. 2. As little as 0.3 μM epoxomicin stimulates TPA-induced PGI₂ production significantly (Fig. 2-B). It is 10 to 15 times more effective than lactacystin (compare Fig. 2-B and 2-C). Using purified bovine erythrocyte proteasomes, epoxomicin inhibits the chymotrypsin-like activity, about 4 to 5 times more effectively than does clasto-lactacystin β-lactone, the derivative of lactacystin [5]. They are almost equally effective on inhibiting the trypsin-like and PGPH-like activities [5]. Assuming that epoxomicin and lactacystin have equal access to the proteasome and that proteasome activity is regulating COX-2 in rat liver cells similarly to neuronal cells [12] then COX-2 may be degraded in the proteasome by cleavage after large hydrophobic residues.

The amplification of PGI₂ production (Figs. 1 and 2) after inhibition by epoxomicin could reflect not only stabilization of COX-2 but also an intracellular increase in the concentration of the substrate i.e. the AA that is produced during hydrolysis of the membrane phospholipids by PLase activity [15]. Extracellular and/or intracellular release of AA will depend, in part, on the localization of the phospholipids in the membrane, e.g. in its inner or outer leaflet [16]. Release of AA in response to several agonists has been described [17-20].

The effect of a 2, 4 or 6-h incubation on AA release from rat liver and rat glial cells by 1.0 μM epoxomicin was determined. Only after the 6-h incubation were the differences significant statistically. Regulation of PLase activity by the proteasome pathway appears to be a relatively slow
Figure 2
Effect of epoxomicin on (A) basal and (B) TPA-induced 6-ketoPGF\(_{1\alpha}\) production and (C) effect of lactacystin on TPA-induced 6-ketoPGF\(_{1\alpha}\) production. Cells were incubated with the reagents for 6 hours. The analyses were performed with triplicate dishes. *- statistically significant vs MEM/BSA. **- Statistically significant vs TPA.

Figure 3
Dose-response of epoxomicin, lactacystin and ZLLL on AA release from rat liver cells. After incubation for 6 hours. The analyses were performed with triplicate dishes. *- Statistically significant vs MEM/BSA.
After a 6-h incubation, epoxomicin, lactacystin and ZLLL stimulate the release of extracellular AA from rat liver cells (Fig. 3) and AA release after TPA-induction (3.7% vs 13.5% in the presence of 1.0 µM epoxomicin).

Epoxomicin also stimulates the release of AA from rat glial, human colon carcinoma and human breast carcinoma cells (Table 1). The stimulation of AA release from the rat liver cells after incubation with epoxomicin is partially inhibited by pre-incubation of the cells for 2-h with actinomycin (Fig. 4) suggesting that a fraction of the PLase is induced. As expected, the inhibition of TPA-induced PGI2 production by actinomycin D is complete (Fig. 5). Thus, some mechanisms leading to maximum AA release appear to be genomic. The induced PLase activity, probably PLA2, could reflect expression of either a secretory or cytosolic PLA2 or some combination of both enzymes [21].

The release of AA from rat liver cells, most likely resulting from PLase activation, is associated with cancer chemoprevention [14,17-19], [22-24]. In addition to its intrinsic biologic activities, AA regulates production of lipoxygen-
ase, cytochrome P-450, and epoxynogene products as well as COX activities. Prostanoid profiles differ with cell type and individual AA metabolites have different pharmacological properties [15]. COX-2 activity, as measured by PGI₂ production, is stimulated by proteasome inhibition (Fig. 1 and 2). Thus, some biologic effects of proteasome inhibition, e.g. stimulation of bone formation [25], may reflect the metabolism of the intracellular AA.

Inhibition of COX-2 activity is one possible mechanism that has been proposed to prevent colon cancer [26]. However, rather than inhibiting, tamoxifen and raloxifene, statins and epoxomicin stimulate COX-2 activity and AA release from rat liver cells [14,17-19]. As shown in Table 1, epoxomicin stimulates AA release from human colon carcinoma, breast carcinoma and rat glial cells. Tamoxifen and simvastatin also stimulate AA release from the human colon carcinoma and human breast carcinoma cells (unpublished data). These drugs have been reported to prevent cancer [27,28]. At least as measured by the COX activity of rat liver cells, tamoxifen, raloxifene, statins and proteasome inhibitors could be preventing cancer by a COX independent mechanism.

AA resulting from proteasome inhibition has many intrinsic biologic properties [reviewed in [29]]. Some of these activities may trigger PLase activity. The causal relationship of AA to cancer prevention (if any) is unclear. Production of AA by the tumor-suppressive type-II phospholipase A₂ (PLA₂G₂A) may be related to the cancer prevention [22-24]. It is not surprising that control of PLase activities present an attractive area for cancer prevention studies [30].

Methods
The rat liver (C-9 cell line) and human breast carcinoma (the BT-20 cell line) were purchased from the American Type Culture Collection (Manassas, VA, USA). The rat liver glial cells (C-6 cell line) was obtained from Dr. Elaine Lai of the Department of Biology, Brandeis University and the human colon carcinoma (the HT-29 cell line) was obtained from Dr. Basil Rigas, American Health Foundation, Valhalla, NY, USA. They were maintained in Eagle's minimum essential media (MEM) supplemented with 10% fetal calf serum, the floating cells were seeded onto 35 mm culture dishes. The plating densities varied from 0.1 to 0.5 x 10⁵ cells/35 mm dish. The freshly seeded cultures were incubated for 24-h to allow for cell attachment. After decantation of MEM containing the fetal bovine serum, 1.0 ml fresh MEM containing 10% fetal bovine serum and [³H] AA (0.2 µCi/ml) were added and the cells incubated for another 24-h. The cells were washed 4 times with MEM and incubated for various periods of time with 1.0 ml of MEM containing 1.0 mg BSA/ml (MEM/BSA) and different concentrations of each compound. The culture fluids were then decanted, centrifuged at 2000 x g for 10 min, and 200 µl of the supernate counted for radioactivity. Radioactivity recovered in the washes before the incubation was compared to input radioactivity to calculate the % radioactivity incorporated into the cells [31]. For PGI₂ production, 1.0 ml of MEM supplemented with 10% fetal bovine serum, void of [³H]AA, was added after the first 24-h incubation. The cells were incubated for another 24-h, washed three times with MEM, then incubated with the compounds in MEM/BSA for various periods of time. The culture fluids were decanted and analyzed for 6-keto-PGF₁α, the stable hydrolytic product of PGI₂, by radioimmunoassay [32].

The [³H] AA release is presented as a percentage of the radioactivity incorporated by the cells. Except for the time-course experiments which used duplicate dishes, three to five culture dishes were used for each experimental point. The data are expressed as mean values ± SEM. The data were evaluated statistically by the unpaired Student’s t-test. A P value < 0.05 was considered significant.

Acknowledgements
My thanks to Hilda B. Gjika for preparation of the manuscript and to Dr. Armen H. Tashjian, Jr., Department of Genetic and Complex Diseases, Harvard School of Public Health, for his continuing interest in these studies.

References
1. Kaselle AF, Goldberg AL: Proteasome inhibitors: from research tools to drug candidates. Chem Biol 2001, 8:739-758.
2. Mitchell BS: The proteasome – an emerging therapeutic target in cancer. N Engl J Med 2003, 348:2597-2598.
3. Adams J: Potential for proteasome inhibition in the treatment of cancer. Drug Discov Today 2003, 8:307-315.
4. Hanada M, Sugawara K, Kaneta K, Toda S, Nishihama Y, Tomita K, Yamamoto H, Konishi M, Oki T: Epoxomicin, a new antitumor agent of microbial origin. J Antibiot (Tokyo) 1992, 45:1746-1752.
5. Meng L, Mohan R, Kwok BH, Elloffson M, Sin N, Crews CM: Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. Proc Natl Acad Sci U S A 1999, 96:10403-10408.
6. Ostrowska H, Wojcik C, Omura S, Worowski K: Lactacycin, a specific inhibitor of the proteasome, inhibits human platelet lysosomal cathepsin A-like enzyme. Biochem Biophys Res Commun 1997, 234:729-732.
7. Melgren RL: Specificities of cell permeant peptidyl inhibitors for the proteinase activities of mu-calcain and the 20 S proteasome. J Biol Chem 1989, 264:18999-19003.

8. Bogyo M, McMaster JS, Gaczyńska M, Tortorella D, Goldberg AL, Ploegh H: Covalent modification of the active site threonine of proteasomal beta subunits and the Escherichia coli homolog HsIV by a new class of inhibitors. Proc Natl Acad Sci U S A 1997, 94:6629-6634.

9. Bromme D, Klaus JL, Okamoto K, Rasnick D, Palmer JT: Peptidyl vinyl sulphones: a new class of potent and selective cysteine protease inhibitors: S2P2 specificity of human cathepsin O2 in comparison with cathepsins S and L. Biochem J 1996, 315:85-89.

10. Levine L: Proteolysis negatively regulates agonist-stimulated arachidonic acid metabolism. Cell Signal 1998, 10:653-659.

11. Levine L: Lactacystin stimulates arachidonic acid metabolism in rat liver cells: effects of cell density on arachidonic acid release, PG12 production and cyclooxygenase activity. Prostaglandins Leukot Essent Fatty Acids 2000, 63:371-375.

12. Rockwell P, Yuan H, Magnusson R, Figueiredo-Pereira ME: Proteasome inhibition in neuronal cells induces a proinflammatory response manifested by upregulation of cyclooxygenase-2, its accumulation as ubiquitin conjugates, and production of the prostaglandin PGE2. Arch Biochem Biophys 2000, 374:325-333.

13. Rigas A, Levine L: Arachidonic acid metabolism by rat liver cells (the C-9 cell line). J Pharmacol Exp Ther 1984, 231:230-233.

14. Levine L: Tamoxifen and the raloxifene analog LY170188: their effects on arachidonic acid release from cell in culture and on prostaglandin I2 production by rat liver cells. BMC Cancer 2004.

15. Smith WL: The eicosanoids and their biochemical mechanisms of action. Biochem J 1989, 259:315-324.

16. Edidin M: The state of lipid rafts: from model membranes to cells. Annu Rev Biophys Biomol Struct 2003, 32:257-279.

17. Levine L: Does the release of arachidonic acid from cells play a role in cancer chemoprevention? FASEB J 2003, 17:800-802.

18. Levine L: Tamoxifen stimulates arachidonic acid release from rat liver cells by an estrogen receptor-independent, non-genomic mechanism. BMC Cancer 2003, 3:24.

19. Levine L: Statins stimulate arachidonic acid release and prostaglandin I2 production in rat liver cells. Lipids Health Dis 2003, 2:1.

20. Levine L: Nuclear receptor agonists stimulate release of arachidonic acid from rat liver cells. Prostaglandins Leukot Essent Fatty Acids 2002, 67:453-459.

21. Diaz BL, Arm JP: Phospholipase A2 (PLA2) from rat liver. Prostaglandins Leukot Essent Fatty Acids 2000, 63:87-97.

22. Praml C, Amler LC, Dihlmann S, Finke LH, Schlag P, Schwab M: Secretory type II phospholipase A2 (PLA2G2A) expression status in colorectal carcinoma derived cell lines and in normal colonic mucosa. Oncogene 1998, 17:2009-2012.

23. Cormier RT, Hong KH, Halberg RB, Hawkins TL, Richardson P, Mulherkar R, Dove WF, Lander ES: Secretory phospholipase PLA2G2A confers resistance to intestinal tumorigenesis. Nature Genetics 1997, 18:78-84.

24. MacPhee M, Chepanik KP, Liddell RA, Nelson KK, Siracusa LD, Buchberg AM: The secretory phospholipase A2 gene is a candidate for the Mom1 locus, a major modifier of ApcMin-induced intestinal neoplasia. Cell 1995, 81:957-966.

25. Garrett IR, Chen D, Gutierrez G, Zhao M, Escobedo A, Rossini G, Harris SE, Gallwitz W, Kim KB, Hu S, Crews CM, Mundy GR: Selective inhibitors of the osteoblast proteasome stimulate bone formation in vivo and in vitro. J Clin Invest 2003, 111:1771-1782.

26. Subbaramaiah K, Dannenberg AJ: Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends Pharmacol Sci 2003, 24:96-102.

27. Jordan VC, Morrow M: Tamoxifen, raloxifene, and the prevention of breast cancer. Endocrinol Rev 1999, 20:253-278.

28. Brower V: Of cancer and cholesterol: studies elucidate anti-cancer mechanisms of statins. J Natl Cancer Inst 2003, 95:844-846.

29. Brash AR: Arachidonic acid as a bioactive molecule. J Clin Invest 2001, 107:1339-1345.

30. Laye JP, Gill JH: Phospholipase A2 expression in tumours: a target for therapeutic intervention? Drug Discov Today 2003, 8:710-716.

31. Levine L: Stimulated release of arachidonic acid from rat liver cells by celecoxib and indomethacin. Prostaglandins Leukot Essent Fatty Acids 2001, 65:31-35.

32. Levine L: Measurement of arachidonic acid metabolites by radioimmunoassay. Manual of Clinical Laboratory Immunology, 3rd edition. Edited by: Rose NR, Friedman H, Fahey JL. Washington DC: American Society for Microbiology; 1986:685-691.