Inhibitors of Angiotensin-Converting Enzyme Modulate Mitosis and Gene Expression in Pancreatic Cancer Cells (43942)

M. K. REDDY,[1] K. BASKARAN, AND A. MOLTENI
Department of Pathology, Northwestern University Medical School, Chicago, Illinois 60611-3008

Abstract. **The angiotensin-converting enzyme (ACE) inhibitor captopril inhibits mitosis in several cell types that contain ACE and renin activity. In the present study, we evaluated the effect of the ACE inhibitors captopril and CGS 13945 (10^{-8} to 10^{-2} *M*) on proliferation and gene expression in hamster pancreatic duct carcinoma cells in culture. These cells lack renin and ACE activity. Both ACE inhibitors produced a dose-dependent reduction in tumor cell proliferation within 24 hr. Captopril at a concentration of 0.36 m*M* and CGS 13945 at 150 μ*M* decreased cellular growth rate to approximately half that of the control. Neither drug influenced the viability or the cell cycle distribution of the tumor cells. Slot blot analysis of mRNA for four genes, proliferation associated cell nuclear antigen (PCNA), K-*ras*, protein kinase C–β (PKC-β) and carbonic anhydrase II (CA II) was performed. Both ACE inhibitors increased K-*ras* expression by a factor of 2, and had no effect on CA II mRNA levels. Captopril also lowered PCNA by 40% and CGS 13945 lowered PKC-β gene expression to 30% of the control level. The data demonstrate that ACE inhibitors exhibit antimitotic activity and differential gene modulation in hamster pancreatic duct carcinoma cells. The absence of renin and ACE activity in these cells suggests that the antimitotic action of captopril and CGS 13945 is independent of renin-angiotensin regulation. The growth inhibition may occur through downregulation of growth-related gene expression.**

[P.S.E.B.M. 1995, Vol 210]

Angiotensin-converting enzyme (ACE) inhibitors are used widely in the treatment of systemic hypertension and congestive heart failure (1). Captopril (D-3-mercapto-2-methylpropanoyl-L-proline) is, among them, the most extensively used compound. Like all other ACE inhibitors, captopril blocks conversion of angiotensin I (Ang I) to angiotensin II (Ang II) and inactivates simultaneously the bradykinins (2). Captopril is a thiol-containing drug and prevents accumulation of collagen and extracellular matrix *in vivo* and *in vitro* (3, 4). In addition, it has other pharmacologic properties: it is recognized as a free radical scavenger (5), as an antioxidant (6), and as a protease inhibitor (7). Some of these properties may be related to the presence of reduced thiol groups in its structure and are independent of its effect on the renin angiotensin system (3, 8).

More recently, it has been reported that captopril inhibits proliferation of human neuroblastoma cells (9), canine renal epithelial cells (10), human mammary ductal carcinoma cells (11), and human lung fibroblasts (12). Neuroblastoma is a renin-secreting tumor, and therefore the antiproliferative effect of captopril was ascribed to a decreased synthesis of Ang II by these cells, with a consequent loss of the mitogenic function which Ang II exhibits. Renin or ACE activity was also found in the other cell types (10–12), suggesting that the drug's cytostatic effect could also be explained by a possible reduction in Ang II synthesis.

We report here the effect of captopril and of another ACE inhibitor, CGS 13945 (CIBA GEIGY, Summit, NJ), on the growth and proliferation of hamster

[1] To whom requests for reprints should be addressed at Department of Pathology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611-3008.

Received February 13, 1995. [P.S.E.B.M. 1995, Vol 210]
Accepted July 10, 1995.

0037-9727/95/2103-0221$10.50/0

pancreatic ductal carcinoma cells, the WDCA (13). CGS 13945 is a 1-[4-(ethoxycarbonyl)-2-4 dimethyl-2R,4R butylrril]-2,3-dihydro-25-indole-2-carboxilic acid which does not contain -SH groups in its structure. Thus, it differs from captopril for some of its pharmacological effects. In addition, CGS 13945 has no evidence to indicate that it possesses antioxidant, proteolytic, or scavenging activities. WDCA tumor is a well-differentiated pancreatic adenocarcinoma induced in Syrian hamster by the administration of N-Nitrosobis-(2-oxypropylamine) (BOP) (13). In the present study, in addition to the proliferative function, the expression of some genes involved in the control of the growth of WDCA cells was also evaluated with or without the addition of captopril or CGS 13935 to the cultures.

Materials and Method

Pancreatic Cancer Cell Culture. WDCA cells were derived from pancreatic ductal carcinoma induced by BOP in hamsters as a transplantable ascitic tumor cell line (13). Fibroblast-free WDCA cells were grown in suspension culture in DME.F12 medium containing 10% NU-serum (Collaborative Research, Bedford, MA), supplemented with 2 m*M* L-glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 ug/ml) at 37.0 ± 0.1°C in humidified 5% CO_2-95% air.

Measurement of Growth. Ascitic cells in suspension culture were centrifuged at 200*g* for 5 min and suspended in PBS. An aliquot of trypsinized cells were counted by suspending them in 0.1% crystal violet (w/v) and 0.1% Triton X-100 (v/v) in 0.1 *M* citric acid solution. The nuclei were counted in a hemocytometer. The cell numbers were determined by incubating the cells in a 24-well chamber at the concentration of 2 × 10^4 cells per well, and by harvesting them at 24-, 48-, 72-, and 96-hr intervals in triplicates. The cell viability was determined by the Trypan blue exclusion test (14).

Cell Morphology. The cultures were examined and photographed using a Nikon inverted light microscope equipped with phase contrast optics at selected intervals of the cultures and during treatment. The cells were collected and fixed for 30 min in 1% paraformaldehyde-2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) at 4°C, post-fixed in 1% osmium tetroxide, and further processed for electron microscopy by embedding them in EMBED 812 (EM Sciences, Fort Washington, PA). Thin sections were stained with uranyl acetate and lead citrate and examined in a Jeol 100 CK electron microscope at 60 kV. The cells in suspension were fixed in 1% paraformaldehyde and processed for light microscopy by staining the sections with hematoxyline and eosine.

Cell Cycle Analysis. The cell cycle populations were determined by analyzing the DNA after staining with propidium iodide (PI) by flow cytometry. The dissociated and pelleted cells were washed twice with Ca^{2+}/Mg^{2+}-free PBS, fixed in 70% ethanol, and stored at 4°C until the analysis (usually within 24 hr). A 0.5-ml PI staining solution (50 μg/ml) containing RNAse (180 U/ml), Triton X-100 (0.1%) and polyethylene glycol (30 mg/ml) in citrate buffer (4 m*M*) pH 7.8, was added to 1–2 × 10^6 cells previously washed in Ca^{2+}/Mg^{2+}-free PBS and incubated at 37°C for 20 min. An equal volume of salt solution containing PI (50 μg/ml) Triton X-100 (0.1%) and polyethylene glycol (30 mg/ml) in 0.4 *M* NaCl solution was added. The suspensions were stored at 4°C for 6 hr before analysis in EPICS 752 cell sorter (Coulter Electronics, Hialeah, FL) (15). The data obtained by the counter were calculated by the MDADS computer.

Gene Expression. RNA was isolated by RNA Zol extraction procedure (16). Quantitation of relative mRNA in RNA samples was performed by slot blot analysis (17). Gene expression was measured after treatment with respective inhibitors for 24 hr as well as in untreated cells.

Statistical Studies. Data were analyzed by two-way analysis of variance (ANOVA), where appropriate. The collection of data per each point was carried out on triplicate samples and the averages of three different experiments were presented. Dose-response curves were generated by linear regression analysis and IC_{50} for each inhibition was determined from this data, which showed 50% reduction in cell numbers.

Additional Measurements. The cellular activity of renin was evaluated by a radioimmunoassay (Gammacoat [^{125}I] Plasma Renin Activity Radioimmunoassay Kit; INCSTAR Corp., Stillwater, MN), which measured the amount of Ang I generated during incubation of the cells. Angiotensin-converting enzyme activity was assayed by a spectrophotometric procedure (18). The -SH group content of the medium was measured by Ellman's procedure (19).

Results

Morphology of Cells. Chemically transformed pancreatic ductal cancer (WDCA) cells are used in this study. Morphologically, these cells resemble human ductal cancer in nature and consists of columnar epithelial cells with hyperchromatic nuclei. This basic morphological appearance remained unchanged when passaged in inbred Syrian hamsters (13) and also in tissue culture throughout this investigation. These cells grew as clusters in suspension culture (Fig. 1A). Treatment with 1 m*M* captopril or CGS 13945 for 24 hr appeared to reduce the size of the clusters (Fig. 1, B and C) compared with untreated controls (Fig. 1A). It

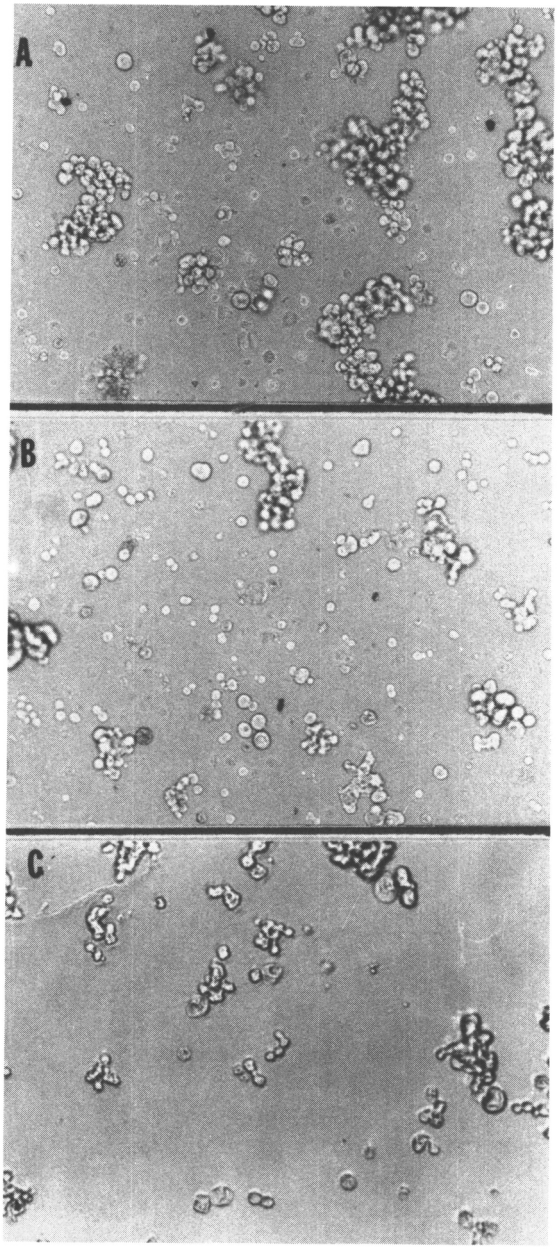

Figure 1. Photomicrograph of WDCA cells. ×63. (A) Untreated control cells, 24 hr (B) Captopril (1 m*M*), 24 hr. (C) CGS 13945 (1 m*M*) 24 hr.

is possible that the growth of WDCA cells by ACE inhibitors is mediated by inhibition of cluster formation. All the cells remained viable (95%–100%) as judged by Trypan blue exclusion test.

The electron photomicrograph of WDCA cells in culture showed pleomorphic appearance exhibiting at least three different cell types. Neither captopril nor CGS 13945 influenced the WDCA cell morphology of either the most common form of these cells or the other two cell types observed in these cultures (pictures not shown).

Cell Growth and Proliferation. The extent of WDCA cell proliferation as a function of time in relation to untreated (control) cells and the ones exposed to equimolar concentration of 1 m*M* captopril or CGS 13945 is shown in Figure 2. The cells treated with both ACE inhibitors displayed normal cell viability, and a time-dependent decrease in proliferation rate was observed with increase of time up to 96 hr of treatment (not shown here). However, the results from two-way ANOVA test for both time effect and group effect are significant (*P* values are 0.0001 and 0.002, respectively). Further, the comparisons of control with captopril- or CGS 13945–treated cells are significantly different, with *P* values of 0.001 and 0.003, respectively. In addition, a dose-dependent decrease in cell numbers was also noticed (Fig. 3). The antimitotic activity of the two drugs was shown by the decreasing numbers of WDCA cells after 24 hr of treatment, with a reduction up to 50% of the control cells (IC_{50}) with a 0.36 m*M* captopril concentration. A much lower IC_{50} of 150 μ*M* was required for CGS 13945 to exert its antiproliferative activity.

The cell cycle phase distribution of untreated cells or of the cells treated with ACE inhibitors by flow cytometry, revealed no significant changes in the distribution of cell cycle populations after 24 hr of exposure. WDCA cells did not possess either ACE activity or renin activity (Table I). However, they still exhibited antimitotic activity similar to that of the cells containing different levels of ACE and renin activities (Fig. 4). Both human lung fibroblasts and endothelial cells from bovine aorta showed measurable renin and ACE activities in culture. The activity in endothelial cells was five times higher for renin activity and 10 times higher for ACE activity compared with activities found in lung fibroblasts (Table I).

Modulation of Gene Expression. ACE inhibitors modulated the expression levels of growth-related genes: PCNA, PKC-β and two other genes: K-*ras*, and carbonic anhydrase II (CA II). Both inhibitors showed a 2-fold increase in K-*ras* gene expression and a decrease in PCNA gene expression by captopril and PKC-β gene by CGS 13945. Both inhibitors retained the normal levels of carbonic anhydrase gene expression similar to untreated cells (Table II and Fig. 5).

It appears that the inhibition observed by ACE inhibitors was not influenced by the presence of cysteine even though the medium had measurable amount of -SH groups (data not shown). This suggests that the antimitotic activity observed is not due to complete utilization of -SH groups in the cultures.

Discussion

Pancreatic cancer is the most fatal cancer in the United States, with a total 5-year survival rate of less

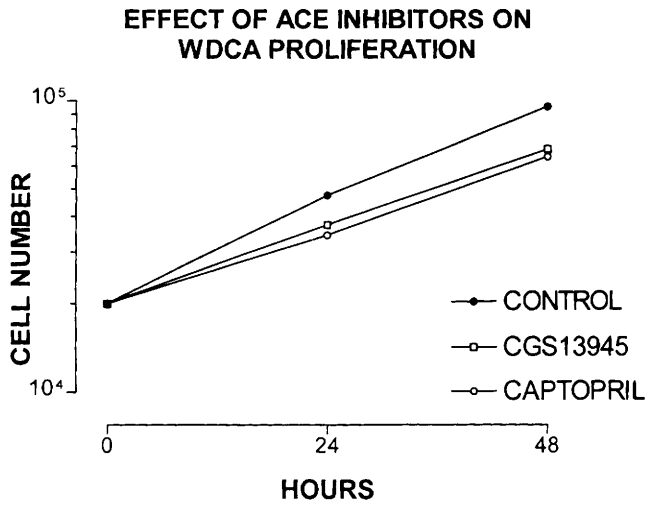

Figure 2. WDCA cell proliferation as a function of time in untreated control cells, and in cells exposed to 1 m*M* captopril or CGS 13945.

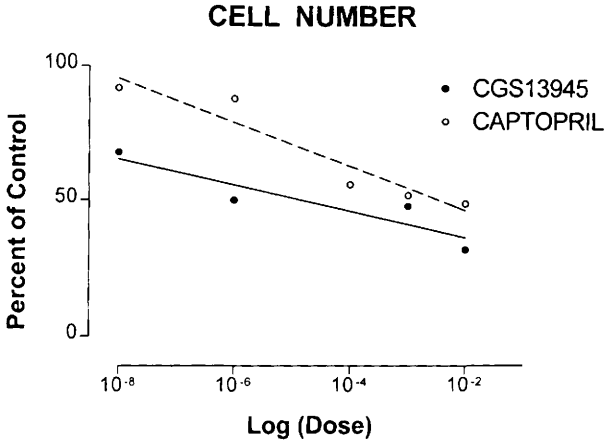

Figure 3. Dose-response relationship of captopril and CGS-13945 as antimitotic agents of WDCA cells after 24 hr of treatment.

Table I. Concentration of Renin and ACE in Different Cell Cultures

Cell line	Renin activity (ng/10^6 cells/hr)	ACE activity (% substrate hydrolyzed/10^6 cells/min × 10^{-2})
Bovine aorta endothelial cells	0.78 ± 0.12[a]	70.4 ± 15.3[b]
Human lung fibroblasts	0.16 ± 0.5[a]	6.8 ± 8.5[b]
Pancreatic cancer (WDCA)	0[a]	0[a]

Note. x, activity in human fibroblasts; o, ???.
[a] Present work. No measurable activity in pancreatic cancer cells.
[b] From Rubin *et al.* 1982 (27).

than 1%, and its incidence has increased steadily for the past two decades (20). Treatment is palliative and new therapeutic approaches are essential.

The data presented here demonstrate that captopril and CGS 13945 delay the growth of cultured cells of a pancreatic ductal cancer that displays strong similarities to human pancreatic ductal cancers. This delay occurs without interference with either cell viability or cell morphology. However, the size of the clusters, in suspension culture, was shown to decrease. This is probably due to the inhibition of growth, reflecting in lesser number of cells as a result of inhibition of cell cluster formation, or due to the interference with cell-cell interaction or to the modification of cell surface antigens. This suggests that the antimitotic activity is not due to cytotoxicity of these chemicals and is not accompanied by changes in cell morphology.

The maximum inhibition of cell growth at a concentration of 0.36 m*M* captopril was 50% of control cells. At 1 m*M* concentration, both captopril and CGS 13945 showed time-dependent decrease in cell growth and appear to be cytostatic rather than cytotoxic. Although captopril is more potent than CGS 13945 as an ACE inhibitor, this trend does not reflect in its ability for cytostatic action. Similar results were reported on human lung fibroblast proliferation (10) and on endo-

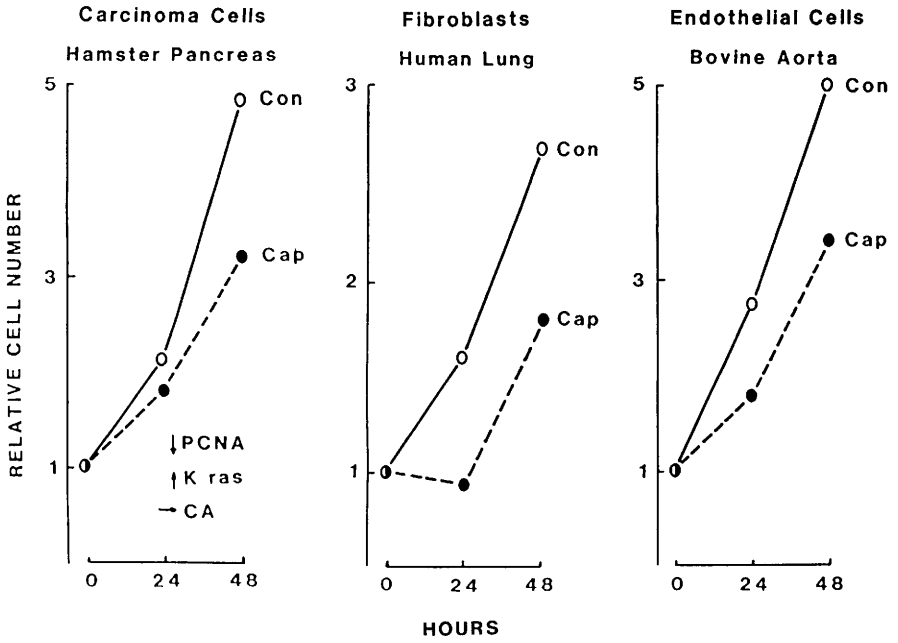

Figure 4. Relative cell number as a function of time in control, captopril- or CGS 13945–treated cells (1 m*M*/24 hr) (A) WDCA cells, hamster pancreas. (B) Human lung fibroblasts. (C) Endothelial cells from bovine aorta.

Table II. Modulation of Gene Expression in WDCA Cells by ACE Inhibitors[a]

Group	PCNA	PKC-β	K-*ras*	CA II
Control	1.00	1.00	1.00	1.00
Captopril (1 m*M*)	0.27[b]	1.03	2.04	1.34
CGS 13945 (1 m*M*)	0.87	0.32[b]	2.13	0.83

[a] Slot blot analysis of total mRNA normalized to poly A content.
[b] Different from control $P < 0.05$.

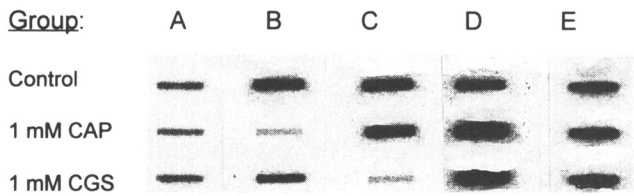

Figure 5. Slot blots showing pancreatic gene expression levels in total RNA using respective probes. (A) poly T probe to measure poly A content. (B) PCNA. (C) PKC-β. (D) K-*ras*. (E) Carbonic anhydrase II (CAII). Concentrations of 1 m*M* of captopril and CGS 13945 were used on WDCA cells for 24 hr. Twenty micrograms of total RNA of each sample was loaded in respective slots.

thelial cell proliferation (4), the two cell lines that are associated with varying amounts of renin and angiotensin activities (Table I and Fig. 4). Pancreatic ductal cancer cells should therefore be added to the list of normal or other neoplastic cells, including human mammary carcinomas, human lung fibroblasts, and bovine aortic endothelial cells, where captopril shows significant growth inhibition (10–12, 21). They require a relatively higher dose of the inhibitors than that used for therapeutic purpose. *In vivo,* captopril treatment reduces the incidence and growth of radiation-induced cutaneous squamous cell carcinomas and sarcomas as well as the growth of hepatomas (22, 23).

The gene expression analysis of cell cycle–related genes showed that PCNA and PKC-β are downregulated both by captopril and CGS 13945. CGS 13945 exposure in particular specifically decreased PKC-β expression to a greater extent than captopril, and its cytostatic activity required relatively lesser concentrations (150 μ*M*) than that of captopril (0.36 m*M*). All the data point to the modulation of cell cycle gene expression as the possible cause of the drugs' antimitotic property. However, CA II expression is not effected by either ACE inhibitors, thus suggesting that the nonspecific nature of this inhibition. The 2-fold increase in K-*ras* expression also seems to be a nonspecific interaction in these cells.

The distribution of different phases of cell cycle did not reveal any changes by these chemicals after a 24-hr exposure period, suggesting that these inhibitors imparted no blocks in cell cycle parameters. Several anticancer drugs are shown to interfere with cell progression (cell division and growth) at discreet points in the cell cycle (22).

The mechanism(s) by which either captopril or CGS 13945 inhibits WDCA cell growth itself is not clear at present. Several modes of action are possible. It appears that inhibition of cell proliferation by these compounds is not related to cell toxicity because treated cells exhibited 95%–100% cell viability. Antimitotic activity cannot be attributed to ACE inhibition since none of the pancreatic cell types (acinar, cen-

troacinar, ductal, etc.) or the pancreas *per se* have been shown to contain renin-angiotensin activities. The depletion of thiol groups in the cultures may not be the reason for this cell inhibition, because addition of -SH groups containing compounds such as cysteine to the medium does not influence the cell numbers in presence of ACE inhibitors.

It is well known that the growth of cancer cells is arrested by compounds that turn on or off specific genes that control these processes by (i) binding to DNA or (ii) binding to messenger RNA, or (iii) competing for nuclear binding factor(s), as seen in the case of double-stranded oligonucleotides which competitively bind with them (24–27). Which one of the above mechanisms is operative with this growth inhibition by ACE inhibitors needs to be evaluated. However, it appears that the interference with the expression of growth-related genes PCNA and PKC-β could be the pathway of inhibition in pancreatic cancer cells. Further research to delineate the mechanism(s) associated with growth inhibition by these compounds is needed to understand this process. The anticarcinogenic properties of these compounds are not evaluated in these cells at present. It will be interesting to test if the treated cells will display either delayed growth of tumors in nude mice or lose the characteristics of anchorage independency in culture, or both.

Whatever the mechanisms of delayed cellular growth may be, the set of experiments presented here adds another cancer cell line to the list of normal and cancer cells, and of experimental tumors in which ACE inhibitors delay their cellular growth.

Authors would like to express their appreciation to Dr. D. G. Scarpelli for providing WDCA cells, Dr. W. F. Ward for ACE inhibitors, and Drs. Zain and R. A. Bambara (Department of Biochemistry, University of Rochester Medical Center, NY) for rat PCNA (cDNA) and Dr. A. W. Rodemaker for the statistical evaluation. We thank K. Stenson for the preparation of this manuscript. (Part of the data has been presented at the 1992 FASEB meetings.)

1. Franciosa JA. The use of ACE inhibitors in congestive heart failure. In: Kostis JB, DeFelice EA, Eds. Angiotensin Converting Enzyme Inhibitors. New York: Liss, pp123–148, 1987.
2. Ward WF, Molteni A, Ts'ao CH, Hinz AM. Captopril reduces collagen and mast cell accumulation in irradiated rat lung. Int J Radiat Oncol Biol Phys **19:**1405–1409, 1990.
3. Ward WF, Kim YT, Molteni A, Solliday NH. Radiation induced pulmonary endothelial dysfunction in rats. Modification by an inhibitor of angiotensin converting enzyme. Int J Radiat Oncol Biol Phys **15:**135–140, 1988.
4. Mastsubara T, Saura R, Hirohata K, Ziff M. Inhibition of human endothelial cell proliferation in vitro and neovascularization in vivo by D-penicillamine. J Clin Invest **83:**158–167, 1989.
5. Westlin W, Mullane K. Does captopril alter reperfusion-induced myocardial dysfunction by scavenging free radicals? Circulation **77**(Suppl 1):130–139, 1988.
6. Roberts MA, Robinson PA. Copper chelates of antirheumatic and anti-inflammatory agents and their superoxide dismutase-like activity and stability. Br J Rheumatol **24:**128–136, 1985.
7. Kennedy AR. Antipain, but not cycloheximide, suppresses radiation transformation when present for only one day at five days post-irradiation. Carcinogenesis **3:**1093–1095, 1982.
8. Molteni A, Ward WF, Ts'ao CH. Effect of Captopril on experimentally-induced pulmonary fibrosis. Med Thorac **14:**129–142, 1992.
9. Chen L, Re RN, Prakash O, Mondal D. Angiotensin-converting enzyme inhibition reduces neuroblastoma cell growth rate. Proc Soc Exp Biol Med **196:**280–283, 1991.
10. Bauer H, Lametschwandtner A, Steiner M, Bauer HC. Influence of angiotensin converting enzyme inhibitor (captopril) on kidney epithelial cells in vitro: Studies on potassium (^{86}Rb) influx and cellular proliferation. Clin Chim Acta **187:**47–54, 1990.
11. Small W, Ward WF, Kim YT, Molteni A, Goolsby CL. Captopril inhibits proliferation of human mammary ductal carcinoma cells in culture. FASEB J **8:**p5, A672, 1994.
12. Nguyen L, Ward WF, Ts'ao CH, Molteni A. Captopril inhibits proliferation of human lung fibroblasts in culture: A potential antifibrotic mechanism. Proc Soc Exp Biol Med **205:**80–84, 1994.
13. Scarpelli DG, Rao MS. Transplantable ductal adenocarcinoma of the Syrian hamster pancreas. Cancer Res **39:**452–458, 1979.
14. Hubchak S, Mangino MM, Reddy MK, Scarpelli DG. Characterization of Syrian golden hamster pancreatic duct cells maintained in extended monolayer culture. In Vitro **22:**889–897, 1990.
15. Blomjous ECM, Schipper NW, Baak JPA, Vos W, De Voogt HJ, Meijer CJLM. The value morphometry and DNA flow cytometry in addition to classic prognosticators in superficial urinary bladder carcinoma. Am J Clin Pathol **91:**243–248, 1984.
16. Chemezynski P, Sacchi N. RNA Zol™ Method. Bulletin No. 1, 1988 from Cinna/Biotecx Laboratories Internal Inc. Anal Biochem **162:**156–159, 1987.
17. Hollander MC, Fonnance AJ Jr. Estimation of relative mRNA content by filter hybridization to a polythymidylate probe. Biotechniques **9:**174–179, 1990.
18. Cushman DW, Cheung HS. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol **20:**1637–1648, 1971.
19. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys **82:**70–77, 1959.
20. Wingo PA, Tong T, Bolden S. Cancer statistics. Am Cancer Soc **45:**8–31, 1995.
21. Ward WF, Kim YT, Molteni A, Solliday NH. Radiation-induced pulmonary endothelial dysfunction in rats: Modification by an inhibitor of angiotensin converting enzyme. Int J Radiat Oncol Biol Phys **15:**135–140, 1988.
22. WF, Molteni A, Ts'ao CH, Hinz CM. The effect of captopril on benign and malignant reactions in irradiated rat skin. Br J Radiol **63:**349–354, 1990.
23. Ward WF, Molteni A, Kim YT, Ts'ao CH, Taylor JM. Captopril inhibits mitosis and growth of diethylnitrosamine-induced preneoplastic hepatic foci in F344 rats. (abstract) FASEB J **6:**A1932, 1992.
24. Weinstein IB. The origins of human cancer: Molecular mechanisms of carcinogenesis and their implications for cancer prevention and treatment. Cancer Res **48:**4135–4143, 1988.
25. Holcenberg JS, Wu HP. Pharmacologic control of specific gene expression. In: Muggia FM, Ed. New Drugs, Concepts and Results in Cancer Chemotherapy. Boston: Kluwer Academic, pp1–18, 1992.
26. Jaskulski D, DeRiel JK, Mercer WE, Calabretta B, Baserga R. Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin. Science **240:**1544–1546, 1988.
27. Rubin DB, Mason RJ, Dobbs LJ. Angiotensin-converting enzyme substrates hydrolysed by fibroblasts and vascular endothelial cells. Exp Lung Res **3:**137–45, 1982.