AD_____

AWARD NUMBER DAMD17-97-1-7247

TITLE: Exploiting BCL-2 Overexpression in the Chemotherapy of Breast Cancer

PRINCIPAL INVESTIGATOR: Nina Schor, M.D., Ph.D.

CONTRACTING ORGANIZATION: Children's Hospital of Pittsburgh
Pittsburgh, Pennsylvania 15213

REPORT DATE: June 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1998	Annual (1 Jun 97 - 31 May 98)

4. TITLE AND SUBTITLE

Exploiting bcl-2 Overexpression in the Chemotherapy of Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7247

6. AUTHOR(S)

Schor, Nina F., M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Children's Hospital of Pittsburgh
Pittsburgh, Pennsylvania 15213

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

We have previously demonstrated the potentiation of apoptosis by overexpression of bcl-2 in pheochromocytoma cells in culture. Others have shown that estrogen treatment increases the expression of bcl-2 in MCF-7 human breast cancer cells. We have combined these findings in our hypothesis that estrogen treatment will result in increased bcl-2 expression and consequent increased susceptibility to neocarzinostatin-induced apoptosis in MCF-7 cells. Using both native MCF-7 lines and transfectants thereof, we have demonstrated the overexpression of bcl-2 in response to estrogen treatment. However, contrary to our expectations, overexpression of bcl-2 in these cells does not lead to enhancement of apoptosis induced by neocarzinostatin. Preliminary studies in our laboratory suggest that this deviation from prediction results from a difference in glutathione handling between breast cancer and neural cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	17
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

NA Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

NA For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Nina F. Schor 5/19/98

PI - Signature Date

(4) TABLE OF CONTENTS

(5) INTRODUCTION

The proto-oncogene *bcl-2* is expressed in 65-80% of human breast cancers (2,18). Approximately 50% of breast cancers are found to overexpress this gene (1-2). Overexpression of *bcl-2*, which results in overproduction of the 26 kD protein Bcl-2, has been shown to confer resistance to apoptotic cell death induced by a variety of stimuli, including chemotherapeutic-induced apoptosis in breast cancer (3-4). This is particularly important in the case of advanced, estrogen receptor-positive tumors, a circumstance in which Bcl-2 content correlates with clinical resistance to chemotherapy (4).

Recent studies in other cell lines have suggested that Bcl-2 exerts its protective effects by a mechanism that includes a shift in the redox potential of the cell to a more reduced state (5,16). Among other biochemical effects, these studies specifically implicate an increase in cellular reduced glutathione (GSH) content in this alteration in redox potential. This makes particular sense because other studies link the apoptotic process to exposure to reactive oxygen species (19-21). One potential strategy for overcoming Bcl-2-mediated chemotherapeutic resistance is to take advantage of the increase in cellular GSH and, therefore, free sulfhydryl content by using chemotherapeutic agents that require reduction by sulfhydryl compounds for their activity. We have recently explored *in vitro* the efficacy of one such group of agents, the enediynes, in pheochromocytoma cells that were genetically engineered to overexpress *bcl-2* (11).

Neocarzinostatin (NCS) is an enediyne DNA-cleaving natural product that induces apoptosis in some tumor cell lines in culture (9). Like other naturally-occurring enediynes, NCS is actually a prodrug that requires sulfhydryl activation for efficacy. As such, the cytotoxicity of NCS has been demonstrated to vary directly with the sulfhydryl content of the cell (7, 14-15). This information led to our prediction that, contrary to the case for all other chemotherapeutic agents studied, overexpression of *bcl-2* and the resulting shift in redox potential of the cell would potentiate the induction of apoptosis by NCS.

We have shown that, in PC12 rat pheochromocytoma cells that have been *bcl-2*- or control-transfected, *bcl-2* overexpression does indeed potentiate the apoptosis-inducing activity of NCS by increasing cellular levels of GSH (11). The reduction-dependent enediyne prodrugs are therefore the only class of drug that has been demonstrated to work best in those tumor cells that have become resistant to other known chemotherapeutic agents.

Although the naturally-occurring enediynes have been difficult to implement clinically because of toxicity issues (22), recent advances in pharmacology and pharmaceutics have begun to get around these problems. Modifying the NCS chromophore structure to produce other more efficacious and/or less toxic enediynes (23), masking the protein component with inert polymers (24), using enediynes adjunctively with cell-selective activating agents (7), and coupling the newer enediynes to monoclonal antibodies directed at tumor-specific antigens (25) have all proved useful in producing enediyne candidates for human therapeutics. In fact, the last of these approaches is now in Phase I/II clinical trials for ovarian carcinoma (L. Hinman, personal communication), and has shown promise in a breast cancer cell line in culture (25). The 4-fold gap in sensitivity to NCS that we observed between *bcl-2*- and control-transfected PC12 cells (11) has led us to speculate that the enediynes would be both more efficacious and safer in these patients than in those whose tumors do not overexpress *bcl-2*.

These interesting and promising preliminary results led us to predict that breast cancer cells that overexpress *bcl-2* would have a higher GSH content and therefore be more susceptible to the effects of the enediynes than are breast cancer cells that do not

overexpress this gene or normal cells. We further predicted that we could augment the difference in enediyne sensitivity between *bcl-2*-native normal and -overexpressing tumor cells by increasing the availability of cysteine, the rate-limiting substrate for GSH synthesis. Furthermore, we predicted that by increasing the Bcl-2 content of estrogen receptor-positive breast cancer cells with tamoxifen (17) or estrogen (3), we could increase the effectiveness of enediynes against these cells, raising the possibility of adjunctive treatment with enediynes and tamoxifen. This would translate clinically into an improved therapeutic index for treatment of breast cancer with the enediynes.

(6) BODY

(6)-1. Experimental Methods, Assumptions, and Procedures

All of the methods we proposed for use in these studies are currently established in our laboratory using cultured cell systems (8-12, 27-32). We describe below the specific hypotheses that were tested and the methods that were used in this first year of Department of Army funding.

(6)-1.1: General Methods Applicable to All Tasks and Technical Objectives

MCF-7 (estrogen receptor-positive) cells were obtained from the American Type Culture Collection (Rockville, MD), and were maintained as stock cultures in α-MEM (GIBCO-BRL) containing phenol red and supplemented with nonessential amino acids, 0.3% glucose, 5% fetal bovine serum, and 2 μg/ml gentamicin sulfate (37°C, 5% CO_2). Where indicated, studies were conducted under four sets of conditions known to alter *bcl-2* expression in this cell line in predictable ways: maintenance of the conditions of the stock cultures (E+; *bcl-2*-positive at "resting" levels; 3-4); addition to the stock culture conditions of 1 nM 17ß-estradiol and maintenance in the estradiol-enriched medium for 48 hr prior to study (E++; 3-fold enhancement of *bcl-2* content over resting levels; 4); maintenance for 7 days prior to study in phenol red-free DMEM (GIBCO-BRL) containing 5% fetal bovine serum stripped of steroids by absorption to dextran-coated charcoal (Sigma Chemical Corp., St. Louis, MO) for 45 min at 45°C (E-; 6-fold reduction of *bcl-2* levels relative to resting state; 3). In addition, E+ cells were studied after a four-day exposure to tamoxifen (10^{-6}M; condition T+), a condition the *in vivo* analogue of which is associated with induction of *bcl-2* (17). In all cases, the same lot of fetal bovine serum (GIBCO-BRL) was used for all conditions in each experiment, and to the extent possible, for all experiments.

In addition, clonal transfectants of MCF-7 human breast cancer cells were obtained from Drs. Charles Rudin and Craig Thompson (University of Chicago, Chicago, IL). These transfectants were produced by electroporation (300 mV, 960 mFD) with pSFFV-neo and pSFFV-bcl-2 (plasmids described in ref. 38), respectively.

(6)-1.2: Task 1 [Verification by Western blotting of effects of manipulations of estrogen exposure (i.e., conditions E++, E+, E-, T+) on *bcl-2* content of MCF-7 cells]

For all studies, relative *bcl-2* content of native MCF-7 cells maintained under each condition was assayed by Western blotting as we have previously described (11). In the case of MCF-7 transfectants, stably transfected clones were screened for Bcl-2 production by Western blot analysis using the N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology).

(6)-1.3: Task 2 [Measurement of concentrations of GSH, GSSG, and total glutathione (GSH+GSSG) in E++, E+, E-, and T+ MCF-7 cells]

To determine the GSH and total glutathione (GSH+GSSG) contents of MCF-7 cells containing different amounts of *bcl-2*, E+, E++, E-, and T+ cells (10^7 of each) were washed free of medium, and suspended in 1 ml of phosphate-buffered saline (PBS). The suspension was homogenized and assayed for GSSG and GSH+GSSG by the method of Tietze (33). The rate of change in the OD_{412} was measured spectrophotometrically over a period of 3 min. This rate was converted to the total glutathione concentration by plotting on a simultaneously run standard curve for ΔOD_{412} versus total glutathione concentration (constructed using GSH standard solutions). The concentration of GSSG alone was determined by an identical procedure performed on samples that were treated with N-

ethylmaleimide prior to assay, to eliminate the reaction of GSH with DTNB (33). The cellular contents of GSH and GSH+GSSG were then calculated for each condition. Each determination was performed in triplicate. Values so obtained for each experiment were compared between E+ and E++, E-, or T+ cells using Student's t test, as we have done in our published work (27-28).

(6)-1.4: Task 3 [Performance of concentration-response studies for E++, E+, E-, and T+ MCF-7 cells and MCF-7 transfectants exposed to neocarzinostatin]

An NCS concentration-response study was conducted for E+, E++, E-, and T+ MCF-7 cells and MCF-7 transfectants, as we have previously described for pheochromocytoma cells (11). Cells plated in 6-well tissue culture plates were treated with a range of concentrations of NCS (0-0.5 μg/ml) for 1 hr at 37°C. Cultures were then washed free of NCS, and adherent cell number was determined daily in control- and NCS-treated E+, E++, E-, and T+, and MCF-7 transfectant cultures as we have previously described for neuroblastoma and pheochromocytoma cells (6,8,11). The statistical significance of differences between E+ and E++, E-, and T+ cells, in turn, was assessed for each concentration of NCS using Student's t test, with $p \leq 0.05$ being considered significant.

(6)-1.5: Task 4 (Preparation of a manuscript describing the findings relative to Tasks 1-4)

A manuscript co-authored by Drs. Schor, Kagan and his laboratory colleagues, and Thompson and his laboratory colleagues is in the final stages of preparation. The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript.

(6)-2. Results and Discussion

(6)-2.1: Task 1 [Verification by Western blotting of effects of manipulations of estrogen exposure (i.e., conditions E++, E+, E-, T+) on *bcl-2* content of MCF-7 cells]

We have measured the effects of addition to and subtraction from the medium of estradiol to the Bcl-2 content of MCF-7 cells in culture. Our first experiments involved using complete medium as the control, and adding various amounts of estradiol to the medium, as originally described in the proposed Methods. These studies, all using the same batch of serum for constitution of the medium, demonstrated no change in the Bcl-2 content of the cells. Our assumption was that the serum-replete medium contained suffient estradiol that neither our addition of small amounts of this compound nor our partial depletion of estradiol with charcoal treatment made a significant change in the Bcl-2 content. We surmounted this problem in two ways. First, we obtained estrogen-free medium commercially stripped (by column chromatography) of estrogen, and performed our studies by adding back various amounts of estradiol. This study conclusively demonstrated the estrogen concentration-dependent increase in concentration of Bcl-2 in the cells (See Figure 1). In addition, we have obtained from the laboratory of Dr. Craig Thompson (University of Chicago, Chicago, IL) two clones of MCF-7 cells that have been transfected with the *bcl-2* gene, and their mock-transfected counterparts. We have confirmed over-production of Bcl-2 in the *bcl-2*-transfected cells, and have maintained all of these lines in our laboratory.

(6)-1.2: Task 2 [Measurement of concentrations of GSH, GSSG, and total glutathione (GSH+GSSG) in E++, E+, E-, and T+ MCF-7 cells]

We have now measured the total glutathione content of the transfectants obtained from Dr. Thompson's laboratory. Accompanying the increase in Bcl-2 content afforded by the *bcl-2* transfection, there was a small but significant increase in glutathione content (see

Figure 2). In contrast, and in accordance with the demonstration of no change in Bcl-2 content, results from a single experiment recently completed demonstrate that, in native MCF-7 cells maintained in complete medium with added estradiol, there is a paradoxical small and not statistically significant decrease in the glutathione content with no change in the ratio of reduced to oxidized species. These studies suggest a relationship between Bcl-2 content and total glutathione content of MCF-7 cells.

(6)-1.3: Task 3 [Performance of concentration-response studies for E++, E+, E-, and T+ MCF-7 cells and MCF-7 transfectants exposed to neocarzinostatin]

Our previous studies have demonstrated the paradoxical increase in sensitivity of PC12 rat pheochromocytoma cells to NCS afforded by *bcl-2* transfection (11). This potentiation of NCS toxicity was abrogated by prior incubation of PC12 cells with BSO. To determine the generalizability of these findings to the overexpression of bcl-2 in different biological systems, similarly transfected MCF-7 human breast cancer cells were examined for the effects of *bcl-2* overexpression on sensitivity to NCS. Unlike PC12 cells, *bcl-2* overexpression protected MCF-7 cells from death induced by NCS (Figure 3). At each concentration, the survival of the *bcl-2* transfectants exceeds that of the mock transfectants.

Attempts to perform the same set of studies in cells exposed to E++, E+, E-, and T+ or E- and graded addition of E conditions were thwarted by the tendancy of estrogen-deprived cells to lift off of the culture surface over the final 5-10 days of the experimental exposure.

(6)-1.4: Task 4 (Preparation of a manuscript describing the findings relative to Tasks 1-4)

A manuscript to be submitted for publication is currently in draft form. This manuscript is entitled "Glutathione handling in *bcl-2*-transfected tumor cell lines: Implications for chemotherapy", and is co-authored by NF Schor, C Rudin, A-R Hartmann, CB Thompson, Y Tyurina, and VE Kagan. The support of the Department of the Army Breast Cancer Program has been acknowledged in this manuscript.

(7) CONCLUSIONS

The studies performed to date indicate that, at least in cultured breast cancer cell lines, increased sensitivity to NCS is not an inevitable consequence of altered Bcl-2 content. This is in contradistinction to the human pheochromocytoma cells on which we have previously reported (11). We have recently obtained preliminary results in connection with another project focussed on neuroblastoma and funded by the NIH, that indicate that this is because the relationship between Bcl-2 content and altered handling of GSH is cell line-dependent. We plan, in the next several months, to test this hypothesis in our human breast cancer model. This is in direct concert with the proposed studies for months 13-24 in our original IDEA Award application. These studies of the effects of Bcl-2 content on GSH handling and chemosensitivity may be of broad applicability, not only to breast cancer, but to the fundamental mechanism of action of Bcl-2 in the tumor cell.

Our studies also indicate that changes in Bcl-2 content require extreme changes in estrogen exposure (from estrogen deprivation to estrogen supplementation), and that these conditions may not be realistic reflections of the environment in which most breast cancers sit *in vivo*. The fact that serum in medium contains sufficient estrogen to maximally augment Bcl-2 content in MCF-7 cells is a reflection of this conclusion.

(8) REFERENCES

1. Joensuu, H, Pylkkanen, L, Toikkanen, S. Bcl-2 protein expression and long-term survival in breast cancer. Am J Pathol 145:1191-1198, 1994.

2. Troncone, G, Zeppa, P, Vetrani, A, D'Arcangelo, A, Fulciniti, F, DeDivitiis, B, Palombini, L. bcl-2 protein breast cancer cells obtained by fine needle aspiration (FNA): a preliminary report. Cytopathology 6:219-225, 1995.

3. Teixeira, C, Reed, JC, Pratt, MA. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Research 55: 3902-3907, 1995.

4. Bonetti, A, Zaninelli, M, Pavanel, F, Sperotto, L, Molino, A, Pelosi, G, Cetto, GL, Biolo, S, Piubello, Q. Bcl-2 expression is associated with resistance to chemotherapy in advanced breast cancer. Proc Amer Assoc Cancer Research 37:192, 1996.

5. Kane, DJ, Sarafian, TA, Anton, R, Hahn, H, Gralla, EB, Valentine, JS, Ord, T, Bredesen, DE. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science 262:1274-1277, 1993.

6. Schor NF. Neocarzinostatin induces neuronal morphology of mouse neuroblastoma in culture. J Pharm Exp Therap 249:906-910, 1989.

7. Schor, NF. Targeted enhancement of the biological activity of the antineoplastic agent, neocarzinostatin: Studies in murine neuroblastoma cells. J Clin Invest 89:774-781, 1992.

8. Falcione, M, Milligan, KD, Schwartz, MC, Schor, NF. Prevention of neocarzinostatin-induced cell death and morphologic change in SK-N-SH human neuroblastoma cells by continuous exposure to nerve growth factor. Biochem Pharm 46:731-738, 1993.

9. Hartsell, TL, Yalowich, JC, Ritke, M, Martinez, AJ, Schor, NF. Induction of apoptosis in murine and human neuroblastoma cell lines by the enediyne natural product neocarzinostatin. J Pharm Exp Therap 275:479-485, 1995.

10. Hartsell, TL, Hinman, LM, Hamann, PR, Schor, NF. Determinants of the response of neuroblastoma cells to DNA damage: the roles of pre-treatment cell morphology and chemical nature of the damage. J Pharm Exp Therap 277:1158-1166, 1996.

11. Cortazzo, M and Schor, NF. Potentiation of enediyne-induced apoptosis and differentiation by Bcl-2. Cancer Research 56:1199-1203, 1996.

12. Cortazzo, MH, Kassis, ES, Sproul, KA, Schor, NF. p75/NGF-mediated protection of neuroblastoma cells from antimitotic agent-induced apoptosis. J. Neurosci. 16:3895-3899, 1996.

13. Kappen, LS, Goldberg, IH. Activation and inactivation of neocarzinostatin-induced cleavage of DNA. Nucleic Acids Research 5:2959-2967, 1978.

14. Beerman, TA, Poon, R, and Goldberg, IH. Single-strand nicking of DNA in vitro by neocarzinostatin and its possible relationship to the mechanism of drug action. Biochim. Biophys. Acta 475: 294-306, 1977.

15. DeGraff, WG, Mitchell, JB. Glutathione dependence of neocarzinostatin cytotoxicity and mutagenicity in Chinese hamster V-79 cells. Cancer Research 45:4760-4762, 1985.

16. Albrecht, H, Tschopp, J, and Jongeneel, CV. Bcl-2 protects from oxidative damage and apoptotic cell death without interfering with activation of NF-kappa B by TNF. FEBS Lett 351:45-48, 1994.

17. Johnston, SR, MacLennan, KA, Sacks, NP, Salter, J, Smith, IE, Dowsett, M. Modulation of Bcl-2 and Ki-67 expression in oestrogen receptor-positive human breast cancer by tamoxifen. Eur J Cancer 30A:1663-1669, 1994.

18. Doglioni, C, DeiTos, AP, Laurino, L, Chiarelli, C, Barbareschi, M, Viale, G. The prevanlence of bcl-2 immunoreactivity in breast carcinomas and its clinicopathological correlates, with particular reference to oestrogen receptor status. Virchows Arch 424:47-51, 1994.

19. Ratan, RR, Murphy, TH, and Baraban, JM. Macromolecular synthesis inhibitors prevent oxidative stress-induced apoptosis in embryonic cortical neurons by shunting cysteine from protein synthesis to glutathione. J. Neurosci. 14:4385-4392, 1994.

20. Sandstrom, PA, Mannie, MD, and Buttke, TM. Inhibition of activation-induced death in T cell hybridomas by thiol antioxidants: oxidative stress as a mediator of apoptosis. J. Leukoc. Biol. 55:221-226, 1994.

21. Forrest, VJ, Kang, YH, McClain, DE, Robinson, DH, Ramakrishnan, N. Oxidative stress-induced apoptosis prevented by Trolox. Free Radic Biol Med 16: 675-684, 1994.

22. McKelvey, EM, Murphy, W, Zander, A, Bodey, GP. Neocarzinostatin: report of a phase II clinical trial. Cancer Treat Reports 65:699-701, 1981.

23. Nicolaou, KC, Dai, W-M, Tsay, S-C, Estevez, VA, Wrasidlo, W. Designed enediynes: a new class of DNA-cleaving molecules with potent and selective anticancer activity. Science 256:1172-1178, 1992.

24. Maeda, H, Ueda, M, Morinaga, T, Matsumoto, T. Conjugation of poly(styrene-co-maleic acid) derivatives to the antitumor protein of neocarzinostatin: pronounced improvements in pharmacological properties. J Med Chem 28:455-461, 1985.

25. Hinman, LM, Hamann, PR, Wallace, R, Menendez, AT, Durr, FE, Upslacis, J. Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. Cancer Research 53:3336-3342, 1993.

26. Meister, A. Methods for the selective modification of glutathione metabolism and study of glutathione transport. Meth Enzymol 113:571-585, 1985.

27. Schor NFT. Adjunctive use of Ethiofos (WR2721) with free radical-generating chemotherapeutic agents in mice: New caveats for therapy. Cancer Research 47:5411-5414, 1987.

28. Schor NF. Depletion of glutathione by the radioprotective agent, S-2-(3-aminopropylamino) ethyl phosphorothioic acid (WR2721). Biochem Pharm 37:562-563, 1988a.

29. Schor NF. Mechanisms of synergistic toxicity of the radioprotective agent, WR2721, and 6-hydroxydopamine. Biochem Pharm 37:1751-1762, 1988b.

30. Schor NF, Siuda JF, Lomis TJ, Cheng, B. Structural studies on the inactivation of γ–glutamylcysteine synthetase by the disulfide analogues of radioprotective cysteamine derivatives: Effects of aminoalkyl- and hydroxyalkyl- chain length and ß,ß-bis-dimethylation. Biochem J 267:291-296, 1990.

31. Schor, NF, Cheng, B, Siuda, JF. Inactivation of γ–glutamylcysteine synthetase by SAPH-3 disulfide: The role of the histaminyl moiety. J Pharm Sci 80:311-312, 1991.

32. Tyurina, YY, Tyurin, VA, Yalowich, JC, Quinn, PJ, Claycamp, HG, Schor, NF, Pitt, BR, Kagan, VE. Phenoxyl radicals of etoposide (VP-16) can directly oxidize intracellular thiols: Protective or damaging effects of phenolic antioxidants? Toxicol Appl Pharmacol 131: 277-288, 1995.

33. Tietze, F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. Analyt Biochem 27:502-522, 1969.

34. Seelig, GF, Meister, A. Glutathione biosynthesis; γ-glutamylcysteine synthetase from rat kidney. Meth Enzymol 113:379-389, 1985.

35. Carlburg, I, Mannervik, B. Glutathione reductase. Meth Enzymol 113:484-489, 1985.

36. Ritov, VB, Banni, S, Yalowich, JC, Day, BW, Claycamp, HG, Corongiu, FP, Kagan, VE. Non-random peroxidation of different classes of membrane phospholipids in live cells detected by metabolically integrated *cis*-parinaric acid. Biochim Biophys Acta 1283:127-140, 1996.

37. Stoyanovsky, DA, Goldman, R, Jonnalagadda, SS, Day, BW, Claycamp, HG, Kagan, VE. Detection and characterization of the electron paramagnetic resonance-silent glutathionyl-5,5-dimethyl-1-pyrroline *N*-oxide adduct derived from redox cycling of phenoxyl radicals in model systems and HL-60 cells. Arch Biochem Biophys 330:3-11, 1996.

38. Boise, LH, Gonzalez-Garcia, M, Postema, CE, Ding, L, Lindsten, T, Turka, LA, Mao, X, Nunez, G, and Thompson, CB. *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597-608, 1993.

(9) APPENDICES

Figure 1: Western blot for Bcl-2 of MCF-7 cells treated with varying concentrations of estradiol and PC12 cells transfected with the bcl-2 gene.

Figure 2: Total glutathione +/- SEM of mock- and bcl-2-transfected MCF-7 cells.

Figure 3: NCS concentration-response curves for MCF-7 transfectants.

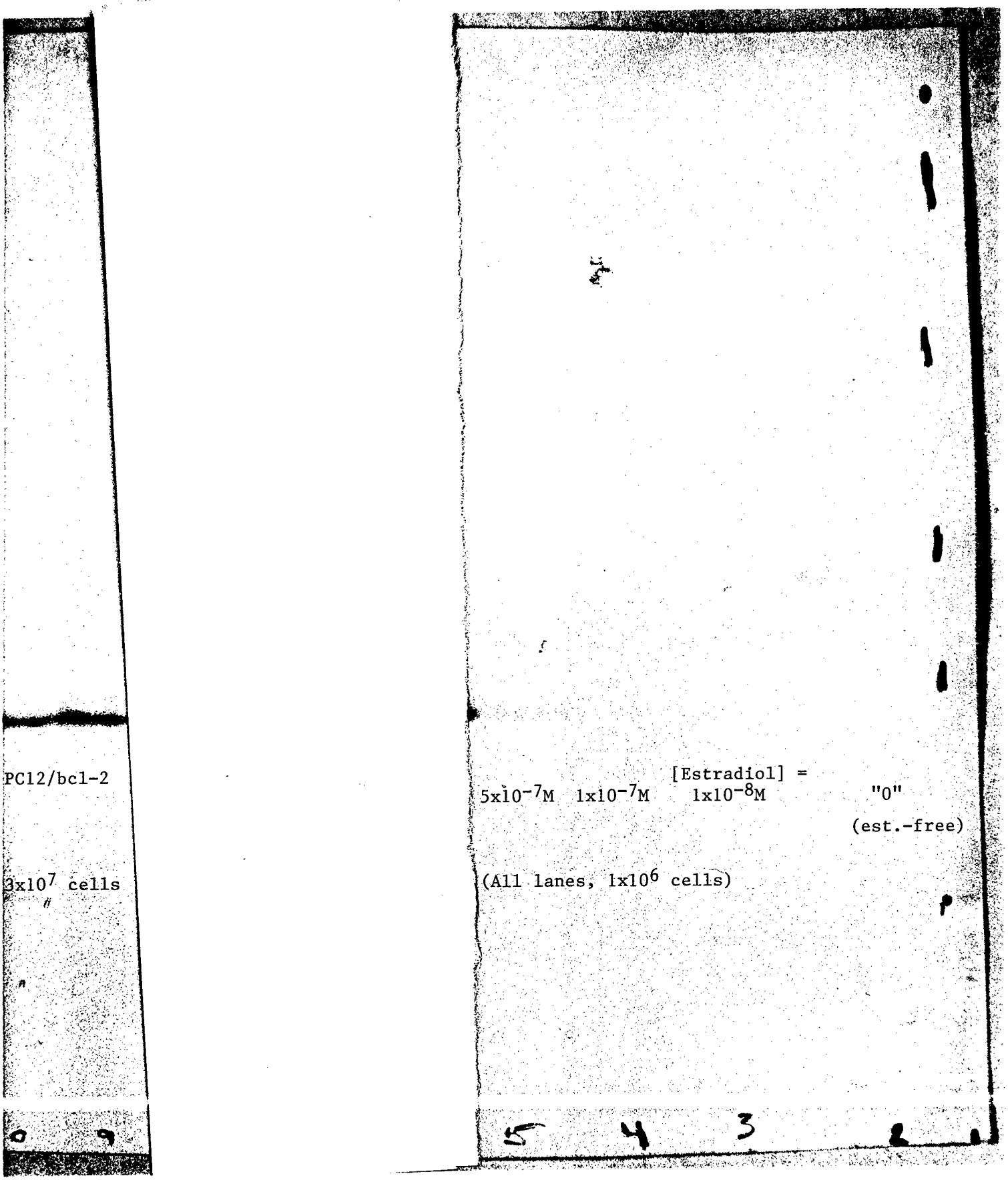

FIGURE 1: Western Blot for Bcl-2 of MCF-7 Cells Treated with Varying Concentrations of Estradiol (lanes 2-5) and PC12 Cells Transfected with the bcl-2 Gene (lane 9)

Figure 2: Total Glutathione +/- SEM of Mock- and bcl-2-transfected MCF-7 Cells

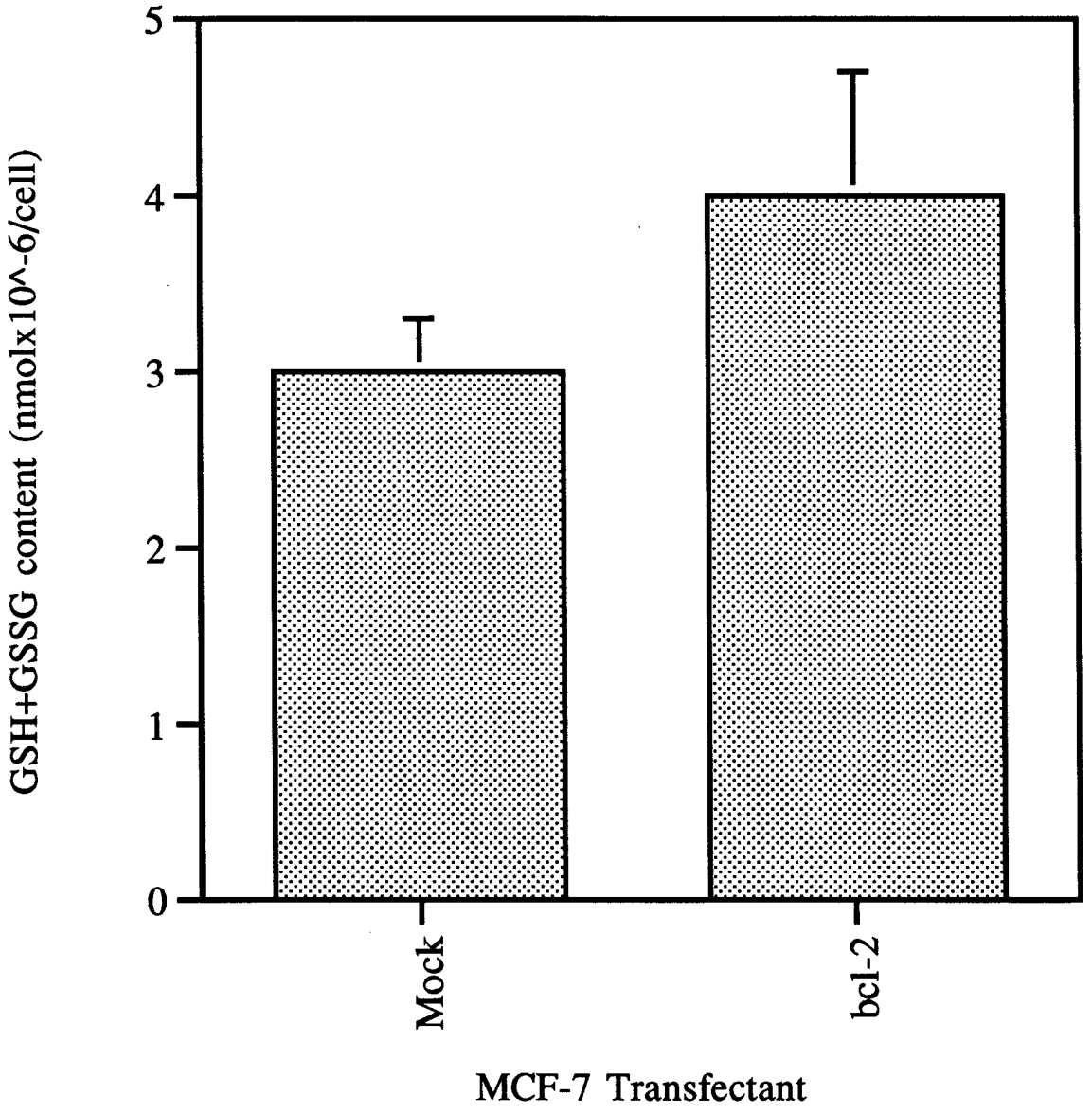

Figure 3: NCS Concentration-response Curves for MCF-7 Transfectants