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PRINCIPAL INVESTIGATOR: Rajagopalan Sridhar, Ph.D.

CONTRACTING ORGANIZATION: Howard University
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Rajagopalan Sridhar, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Howard University
Washington, DC 20059

E-Mail: sridhar_howard@hotmail.com

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13. ABSTRACT (Maximum 200 Words)

The wild type MCF7 human breast cancer cells and the multidrug resistant cell line designated as MCF7/ADR are reported to be genetically unrelated. Our attempts to produce stable multidrug resistant mutants from MCF7 were unsuccessful. However, we found reliable sources of wild type and related multidrug resistant breast cancer cell lines. This has further delayed our experiments. We are in the process of repeating our experiments with these genetically related cell lines. Both dipyridamole and raloxifene resembled tamoxifen in potentiating the cytotoxicity of doxorubicin towards MCF7/ADR cells, and inhibiting doxorubicin mediated microsomal lipid peroxidation. Raloxifene, dipyridamole and tamoxifen can inhibit protein kinase C, which is an attractive target for modulating multidrug resistance. Nicotine, which is cardiotoxic, can activate protein kinase C pathway. Raloxifene, tamoxifen and dipyridamole have basic amino groups in common, but unlike nicotine, they inhibit protein kinase C and sensitize multidrug resistant cells.

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Table of Contents

INTRODUCTION

The anti-cancer drug doxorubicin (adriamycin) causes severe adverse effects at high doses and loses its efficacy against multidrug resistant tumors including breast cancer. Potentially fatal cardiotoxicity can develop in patients when the cumulative dose of the drug exceeds 450 mg/m^2. For optimum use of doxorubicin it is necessary to overcome multidrug resistance of tumor with simultaneous protection against cardiotoxicity. Cardiotoxicity may result from the tendency of doxorubicin and its metabolites to accumulate in the heart and trigger free radical mediated injury (1-3). At present, R-verapamil, tamoxifen, raloxifene, toremifene and dipyridamole are being considered as chemosensitizers for reversing tumor resistance to doxorubicin. It is important to identify and avoid conditions under which they preferentially sensitize the tumor without subjecting the heart to the deleterious effects. Free radicals are known to induce apoptosis. If free radicals are the cause of cardiotoxicity, then R-verapamil, tamoxifen, toremifene, raloxifene and dipyridamole should protect the heart by virtue of their anti-peroxidant action. If apoptosis is the cause of cardiac injury then the alleged anti-apoptotic action of these chemicals should be beneficial to the heart. If cardiotoxicity is the result of altered kinetics and metabolism of doxorubicin to the more toxic doxorubicinol, then certain metabolic inhibitors would afford protection. Apoptosis in the tumor is desirable, whereas apoptosis in the heart is detrimental. Methods for decreasing the free radical burden in the heart and preventing the accumulation of doxorubicin and its metabolites in the heart may improve the clinical usefulness of doxorubicin. This may be achievable because the antitumor action of doxorubicin may occur through inhibition of DNA topoisomerase with minimal involvement of free radicals. The mechanisms involved in the actions of these pharmacologically diverse compounds may hold valuable clues for improved cancer therapy and protection against cardiotoxicity. The specific aims of the project are:

#1: To determine if R-verapamil, tamoxifen, toremifene, raloxifene and dipyridamole alter the metabolic and pharmacokinetic profiles of doxorubicin in athymic nude mice with multidrug resistant MCF-7 human tumor xenografts. High pressure liquid chromatography (HPLC) will be utilized to measure the concentration of doxorubicin, doxorubicinol and the chemomodulator in blood, tumor, heart, kidneys and liver of mice. The extent of lipid peroxidation and the concentration of reduced glutathione will be determined in heart and liver tissue by standard biochemical assays.

#2: To determine if R-verapamil, tamoxifen, toremifene, raloxifene and dipyridamole have differential effects on programmed cell death (apoptosis) in cardiac tissue compared to the tumor. The extent of apoptosis in heart tissue and tumor will be assayed *in situ* by histological methods and by DNA laddering assays in order to correlate cardiac damage with extent of apoptosis.

#3: To apply the electron spin resonance (ESR) technique of spin trapping to compare the influence of R- and S-verapamil on doxorubicin-induced free radical formation *in vitro* and *in vivo*. Alpha-(2,4,6-trimethoxyphenyl) N-tert-butylnitrone (TMeOPBN) will be administered to mice for *in vivo* studies and TMeOPBN and 5,5-dimethylpyrroline N-oxide (DMPO) will be used for *in vitro* experiments with cardiac microsomes and mitochondria isolated from animals in the different treatment groups. The long term goal is to find safer methods of treating multidrug resistant advanced breast cancer with doxorubicin while preventing cardiotoxic side effects of treatment.

BODY

There are several reports that the MCF-7/ADR cell line used at many research centers for investigating doxorubicin resistance in human breast tumor cells is not genetically related to its presumed parental cell line MCF-7 (4-6). Multidrug resistant breast cancer cell lines whose pedigree can be reliably traced to their parental cell lines have been obtained by researchers in other laboratories by transfection of the appropriate genes for drug resistance. Matched sensitive (wild type) and its multidrug resistant variant are now available to us from reliable sources such as Georgetown University and the National Cancer Institute. We have to repeat many of the experiments that were carried out earlier with MCF-7/ADR, using these matched pairs of breast cancer cell lines of varying drug sensitivities. Our attempts to produce multidrug resistant MCF-7 cells by serial exposures to graded concentrations of doxorubicin failed to yield stable drug resistant mutants. Therefore we have depended on the cell lines developed in Georgetown University by Dr. Robert Clarke (specifically MDA 435/LCC6 and its multidrug resistant derivative MDA 435/LCC6MR1) and by Dr. Michael Gottesman of the National Cancer Institute, National Institutes for Health (MCF-7 and MCF-7 MDR clone 10.3, which has been transduced with MDR1).

The in vivo studies associated with **task 3: statement of work** (pharmacokinetic analysis and apoptosis assays in animals) have been delayed. However, we have grown MCF-7/ADR cells as solid tumor xenografts in nude mice as part of another ongoing project dealing with *in vivo* phosphorus nuclear magnetic resonance spectroscopy. We will soon initiate tumor transplantation for pharmacokinetic studies and apoptosis assays using authentic multidrug resistant breast cancer cells. A prior assessment of the factors influencing apoptosis will be useful for interpreting the results of apoptosis assays on tissue and tumor samples from treated animals. Therefore the effects of different agents on doxorubicin-mediated apoptosis were studied.

Inhibitors of protein kinase C are known to play a regulatory role in cell proliferation and apoptosis. Protein kinases have been identified as therapeutic targets that are worth exploring (7). Tamoxifen, raloxifene and dipyridamole are inhibitors of protein kinase C, which is being recognized as an attractive target in cancer chemotherapy, especially for overcoming multidrug resistance (7-9). The importance of the sphingomyelinase - ceramide pathway in ceramide-mediated apoptosis and drug resistance is rapidly gaining recognition (10-17). The influence of oxidative stress, pH and calcium homeostasis in drug resistance is being actively studied in several laboratories (18-21).

The multidrug resistance reversing agents, tamoxifen, dipyridamole, verapamil and raloxifene are inhibitors of protein kinase C. Furthermore, these compounds possess some antiperoxidant activity and a tendency to perturb calcium homeostasis in cells. The basic amino groups in compounds such as nicotine, tamoxifen and dipyridamole, can alter intracellular pH and influence cellular uptake of anticancer drugs. The diverse pharmacological properties make in vivo study of these compounds exciting. In vitro experiments have been carried out as preparation for the projected in vivo studies on tumor bearing mice.

Necrosis was the major source of cell death in doxorubicin treated MCF-7 and MCF-7/ADR cells. The MCF-7/ADR cells are probably not of breast origin in spite of the nomenclature used. These cells have mutated p53, which may account for their resistance to apoptosis (4,22).

The protein kinase C inhibitors tamoxifen, raloxifene and dipyridamole partially sensitized MCF-7/ADR cells to doxorubicin, when cell viability was estimated using colony formation assays. These compounds also inhibited doxorubicin stimulated microsomal lipid peroxidation. All three compounds displayed non protein thiol sparing effect in microsomes treated with doxorubicin. This confirms the anti-peroxidant properties of these compounds.

Nicotine, tamoxifen, raloxifene and dipyridamole are amino compounds with some basic character. Nicotine activates protein kinase pathways in some cellular systems (23-27). This is in contrast to the other three compounds, which inhibit protein kinase C. Interestingly, nicotine decreased the cytotoxicity of doxorubicin towards MCF-7 cells (22).

Adenosine triphosphate (ATP) is needed for pathways leading to apoptosis, and ATP depletion promotes necrosis (28). Therefore it is important to understand the effects of different pharmacological agents on cellular energy production. In collaboration with Dr. Paul Wang of our University, we have utilized ^{31}P NMR spectroscopy to study ATP producing capacity of MCF-7/ADR cells (29). The technique should allow us to compare the effects of tamoxifen and doxorubicin on ATP production and glucose utilization by drug sensitive and resistant cells. We had noted differences in the glucose stimulated proliferation of MCF-7 and MCF-7/ADR cells (30). Since these cell lines are genetically unrelated, we will repeat the experiments with authentic genetically related pairs of drug sensitive and drug resistant breast cancer cell lines.

The roles of sphingomyelin and ceramide pathways in multidrug tumor cell sensitivity and resistance are being examined in several laboratories. We are teaming up with Dr. Xinbin Gu of our University, to investigate the effect of nitrone spin traps and nitroxyl spin labels on ceramide mediated apoptosis. Free radical initiated apoptosis can be triggered via sphingomyelinase pathway. Nitrones and nitroxyls exhibit antiperoxidant activity in systems where dipyridamole and tamoxifen also inhibit peroxidation. Therefore, we will compare the abilities of these compounds to perturb the sphingomyelinase pathway and apoptosis in drug sensitive and resistant breast cancer cells treated with and without doxorubicin.

Important differences were noted in the metabolic activation of doxorubicin and another anthracycline, mitoxantrone. Mitoxantrone yielded free radical intermediates during oxidative as well as reductive metabolism. Free radicals were detectable by electron spin resonance techniques only during reductive metabolism of doxorubicin (31).

We also observed that the neutral red assay for cell viability was unreliable when used with multidrug resistant cells, presumably due to lack of adequate retention of the dye by the drug resistant cells. This assay also overestimated the viability of heat inactivated cancer cells (32).

We have not submitted manuscripts on all our results (**task 4: statement of work**), but will do so after confirming the data obtained with authentic multidrug resistant human breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated necrosis to be the major cause of cell death in doxorubicin-treated MCF-7/ADR cells.

- Demonstrated the PKC inhibitors tamoxifen, raloxifene, and dipyridamole partially sensitized MCF-7/ADR cells to doxorubicin when cell viability was estimated using colony formation assays.

- Demonstrated that nicotine activates PKC pathways in some cellular systems in contrast to the other three compounds that inhibit PKC.

- Utilized ^{31}PNMR spectroscopy to distinguish drug sensitive and multidrug resistant cells.

- Nitrones and nitroxyls can perturb the sphingomyelinase pathway and apoptosis in drug-sensitive and –resistant cells treated with and without doxorubicin.

- Detected free radicals during reductive metabolism of doxorubicin.

- Observed that neutral red assay for cell viability was unreliable when used with MDR cells, and overestimated the viability of heat inactivated cancer cells.

REPORTABLE OUTCOMES:

Research

Manuscripts

- Sridhar R, Hanson-Painton O, and Cooper DR. 2000. Protein kinases as therapeutic targets. *Pharmaceutical Research* 17:1345-1353.

Abstracts

- Zhou Y, Gu X, Dhingra S, et al. 2002. Nicotine inhibits doxorubicin-induced apoptosis of human breast cancer cells in culture. *Era of Hope, Department of Defense Breast Cancer Research Program Meeting Proceedings,* Volume I, Abstract P10-25, Orlando, Florida, 25-28, September.
- Sridhar R and Desai PB. 2001. Similarities and differences in mechanisms of free-radical formation from mitoxantrone and doxorubicin. Annual Meeting of the American Association for Cancer Research, *Proc. Amer. Assoc. Cancer Res.* 42: 935, New Orleans, Louisiana.
- Sridhar R, Balan KB, Shankar RA, et al. 2001. Observations on the application of neutral red assay for cell viability. *FASEB J.* 15 (4) : A238.
- Wang PC, Liu D, Agwu E, et al. 2000.Application of P31NMR spectroscopy to distinguish drug-sensitive and drug-resistant breast cancer. *Era of Hope, Department of Defense Breast Cancer Research Program Meeting Proceedings,* Volume I, Atlanta, Georgia, 8-11 June.
- Wang PC, Zhou J, Agwu CE et al. 2002 *Era of Hope, Department of Defense Breast Cancer Research Program Meeting Proceedings,* Volume III, Abstract P40-30, Orlando, Florida, 25-28, September.
- Zhou Y and Sridhar R. 2000. Interaction of tamoxifen with doxorubicin. *Era of Hope, Department of Defense Breast Cancer Research Program Meeting Proceedings,* Volume I, Atlanta, Georgia 8-11, June.

Presentations

- Sridhar R and Desai PB. 2001. Similarities and differences in mechanisms of free-radical formation from mitoxantrone and doxorubicin. Annual Meeting of the American Association for Cancer Research, *Proc. Amer. Assoc. Cancer Res.* 42: 935, New Orleans, Louisiana.
- Sridhar R, Balan KB, Shankar RA, et al. 2001. Observations on the application of neutral red assay for cell viability. *FASEB J.* 15 (4) : A238.

CONCLUSIONS

The MDR reversing agents, tamoxifen, dipyridamole and raloxifene partially inhibit doxorubicin mediated lipid peroxidation in tumor cells and in microsomal preparations. The results need to be confirmed using MCF-7, and MCF-7 MDR clone 10.3 cells developed by Dr. Michael Gottesman of the National Institutes for Health..

The MCF-7/ADR cells which was used in our studies has a mutated p53 and is not prone to undergo doxorubicin mediated apoptosis. Moreover this cell line may not even be a breast cancer. Therefore it is essential that we concentrate our efforts on MCF-7 and MCF-7 MDR clone 10.3. We also have MDA 435/LCC6 and its multidrug resistant derivative MDA/LCC6MR1 for comparison.

It is important to test the antiperoxidant and thiol sparing effects of tamoxifen, dipyridamole and raloxifene using authentic breast cancer cells differing in drug sensitivities. These results should be compared with those obtained using cardiac muscle cells. Electron spin resonance studies should be carried out to evaluate the thiol tone of muscle cells and tumor cells treated with doxorubicin alone and in combination with tamoxifen, raloxifene and dipyridamole.

The MCF-7 and MCF-7 MDR clone 10.3 will be grown as solid tumor xenografts in Balb/c nude mice for completing the pharmacokinetic studies as proposed . The results from these studies will help identify any preferential effects of doxorubicin and multidrug reversing agents on the tumor in comparison to the heart.

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APPENDICES (pages 13-39)

List of Appendices

Appendix 1.

Effect of nicotine on doxorubicin toxicity towards MCF-7 and KB3.1 cells

Introduction

In the United States approximately 7.5 % of cancer related deaths can be attributed to smoking. Tobacco use has been associated with carcinogenesis. Tobacco use is strongly discouraged in patients undergoing cancer therapy. Nicotine (nic) may decrease the effectiveness of cancer therapy and increase the risk of tumor recurrence.

The aim of cancer therapy is to eliminate clonogenic cells in tumor. Clonogenicity assays are useful for determining reproductive cell death, whereas assays for apoptosis are not always a measure of reproductive death in cells.Tumor recurrence depends on the presence of viable clonogenic cells that survive even after therapy.We tested the effect of nicotine on cytotoxicity of doxorubicin(dox) towards cancer cells in culture, using assays for apoptosis, necrosis and clonogenicity.

Methods and Materials

Cells: MCF-7 human breast cancer cells and KB-3.1 human nasopharyngeal carcinoma cells were cultured and maintained as exponential monolayers in a humidified 5% carbon dioxide air atmosphere in a 37°C incubator. RPMI 1640 medium fortified with 10% fetal bovine serum, glutamine (2mM), sodium pyruvate (1mM) and 100 units/ml each of penicillin and streptomycin was used for culturing MCF-7 cells. KB-3.1 cells were grown in Dulbecco's minimum essential medium that was fortified with fetal bovine serum, glutamine, pyruvate and antibiotics as indicated for RPMI 1640.

Apoptosis assays: Annexin V-EGFP apoptosis detection kit (MBL, Nagoya, Japan) was used for apoptosis assays using a flow cytometer. Apoposis was estimated by measuring caspase 3 and caspase 8 activities using a caspase-3/CPP32 fluorometric protease assay kit and a caspase-8/Flice fluorometric protease assay kit (MBL, Nagoya, Japan). Apoptosis was also detected on the basis of DNA Fragmentation analysis using 1.5% agarose gels, using ethidium bromide for staining.

Clonogenicity assay: Cells were seeded at a density of 300 and 1500 cells per 60mm diameter tissue culture dishesand the cells were allowed to attach overnight. The cultures were treated with nicotine (10 or 20 μM) for two hours. Control cultures were treated with same volume of medium without nicotine. After the two hour exposure to nicotine, the cultures were treated with different concentrations of doxorubicin (0.25 to 10 μM range) for 90 or 120 minutes. Appropriate controls without doxorubicin were also included. The medium was removed from each dish and the cells were washed with Dulbecco's phosphate buffered saline, and fresh drug free culture medium (5 ml) was added and the cultures returned to the incubator for colony formation to progress for ten days. Any colony containing more than 50 cells was considered to represent a viable clonogenic cell. The number of colonies in the different dishes were counted after staining with methylene blue. Survival was calculated relative to a 100% value for untreated controls. The experiment was performed at least four times for each cell line.

Results

Colony formation Assays: A 90 minute exposure of MCF-7 cells to doxorubicin (10 μM) in the absence of nicotine killed all cells. However, pretreatment with 10 and 20 μM nicotine afforded partial protection to MCF-7 cells treated with doxorubicin (10 μM) (**Fig. 1**).

In KB-3.1 cells the survival was 14.2% after a 90 minute treatment with 0.5 μM doxorubicin alone, but the survival increased to 27.2% when a two hour treatment with 10 μM nicotine preceded doxorubicin (0.5 μM) treatment (**Fig. 2**). Even this slight inhibition of doxorubicin cytotoxicity by nicotine will have a profound effect on tumor control.This is illustrated by simulating the effect of multiple treatments with doxorubicin alone and in the presence of nicotine. The graph (**Fig. 3**) depicts the number of clonogens remaining after multiple course of treatment of a hypothetical one gram tumor containing 10^9 clonogenic cells at the start of therapy. In this depiction, it is assumed that the tumor is homogeneous and that the effectiveness is the same for each cycle of therapy. Effects of tumor cell proliferation have also been ignored in this case.

Annexin V assay: Two color flow cytometry results indicated that a large proportion of cells exposed to doxorubicin were necrotic (as judged by propidium iodide staining) and the population of apoptotic cells was small. Pretreatment with nicotine (10 μM) decreased necrosis due to doxorubicin (10 μM) treatment of MCF-7 cells (**Fig. 4 and 5**). Nicotine treatment also protected KB 3.1 cells from necrosis induced by doxorubicin (1 μM) treatment for 90 and 120 minutes (**Fig. 6**).

Caspase 3 and caspase 8 activities and DNA fragmentation: Treatment with 10μM doxorubicin alone for 90 minutes increased caspase-3 activity to 142% and caspase-8 activity to 126% relative to 100% activity for each enzyme in untreated control cultures. However, a 2 hour pre-treatment with 20 μM nicotine reduced the caspase-3 and caspase-8 activities respectively, in cultures treated with doxorubicin to 114% and 101% of control (**Fig. 7**) . This corresponds to a 20% inhibition of doxorubicin-induced apoptosis by 20 μM nicotine in these cells. In a parallel set of experiments,+ the drug containing medium was replaced with drug free medium after the 90 minute exposure to doxorubicin and then incubated at 37°C for 24 hours. When these cells were examined, inhibition of doxorubicin-induced apoptosis by nicotine could be demonstrated on the basis of DNA fragmentation analysis (**Fig. 8**).

Our original intention was to compare MCF-7 and multidrug resistant MCF-7/ADR cells. Based on karyotype analysis, these cell lines were determined to be unrelated. However, we were able to obtain drug sensitive (KB-3.1) and related multidrug resistant (KB-V.1) nasopharyngeal carcinoma cells. We initiated experiments on these cells and used the results for comparison with the data for MCF-7 cells.

Conclusion

Nicotine decreases the cytotoxicity of doxorubicin as judged by assays for apoptosis, necrosis and clonogenicity. Necrosis and not apoptosis is the major cause of cell death in MCF-7 and KB-3.1 cells treated with doxorubicin under the conditions of our experiments. MCF-7 and KB-3.1 cells showed only a weak apoptotic respone to doxorubicin treatment.

Inhibition of apoptosis and reproductive cell death can affect tumor control. This statement must further be tempered by the fact that tumor response to therapy depends on tumor cell killing, cell proliferation, and cell loss factor. Chemotherapy is usually given in multiple doses or cycles of treatment. The slight inhibition of doxorubicin cytotoxicity by nicotine can be detrimental to the efficacy of multiple courses of doxorubicin treatment.

The U.S. Army Medical Research Material Command under DAMD 17-98-1-8109 supported this work.

Fig 1. Partial inhibition of doxorubicin cytotoxicity by nicotine as demonstrated by clonogenicity assays in MCF-7 cells

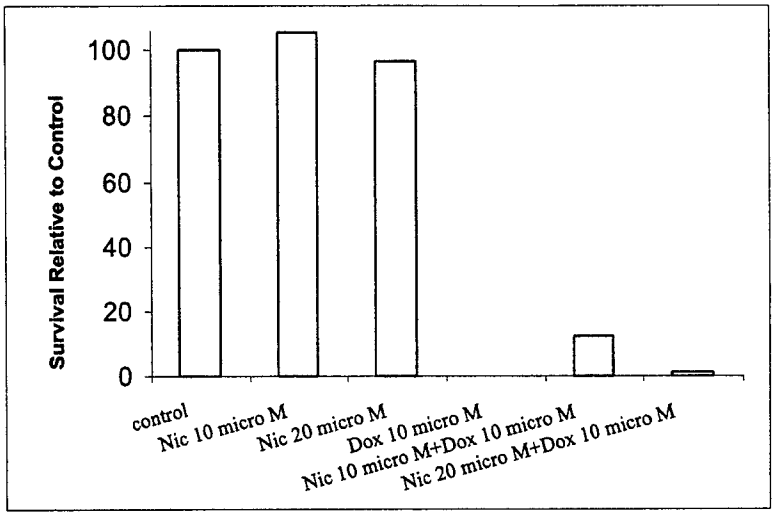


Fig 2. Partial inhibition of doxorubicin cytotoxicity by nicotine as demonstrated by clonogenicity assays in KB-3.1 cells

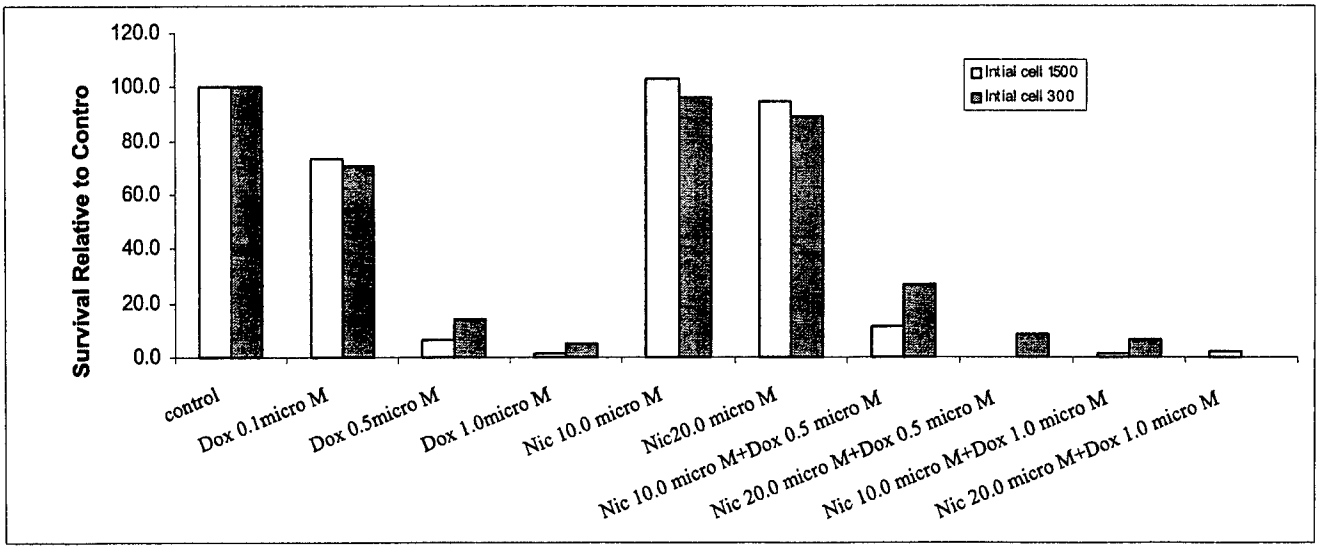


Fig 3. Theoretical curves for treatment efficacy

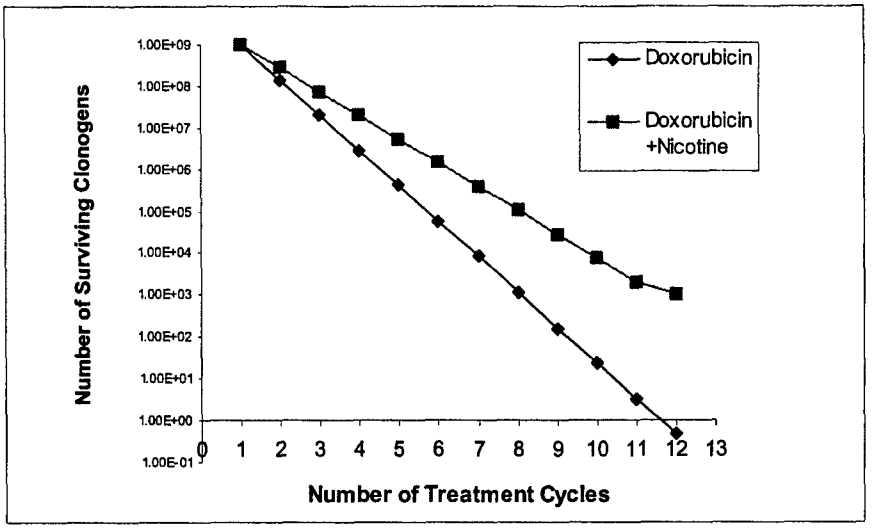


Fig 4. Flow cytometry assays for apoptosis and necrosis in MCF-7 cells

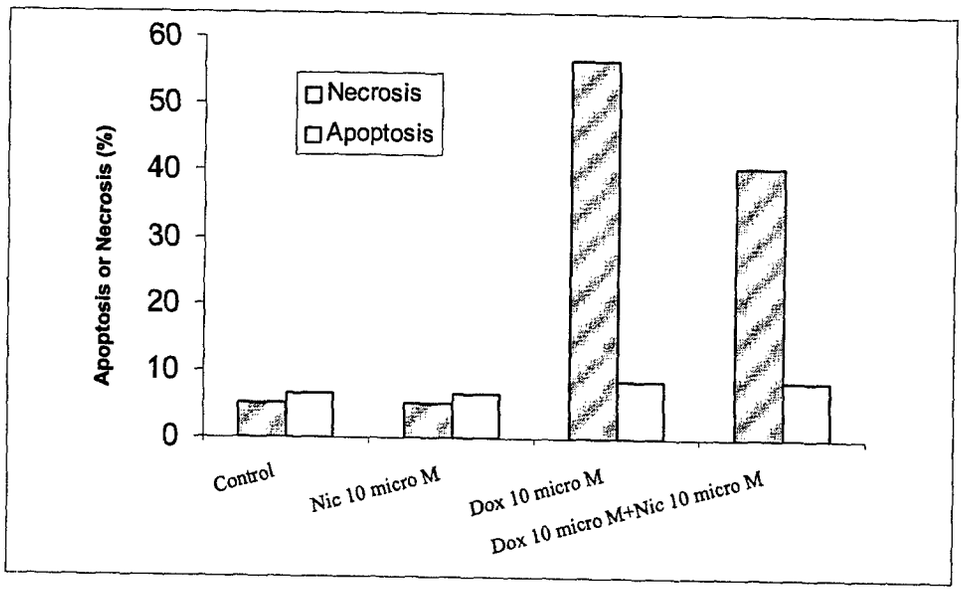
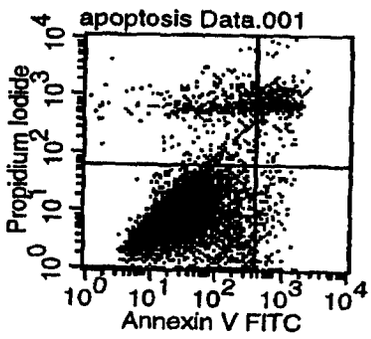
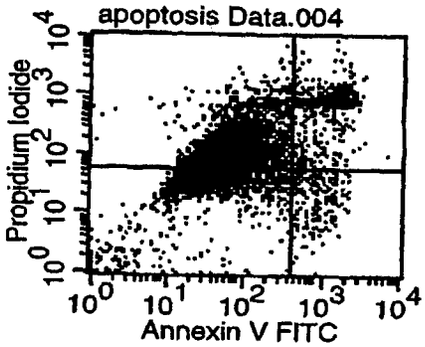


Fig 5. Partial inhibition of doxorubicin induced necrosis in MCF-7 cells by nicotine (Flow cytometry analysis)

A
Nic 20 μM



B
Dox 10 μM



C
Nic 10 μM +Dox 10 μM

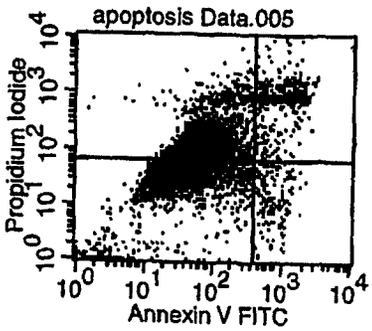


Fig 6. Flow cytometry assays for apoptosis and necrosis in KB-3.1 cells

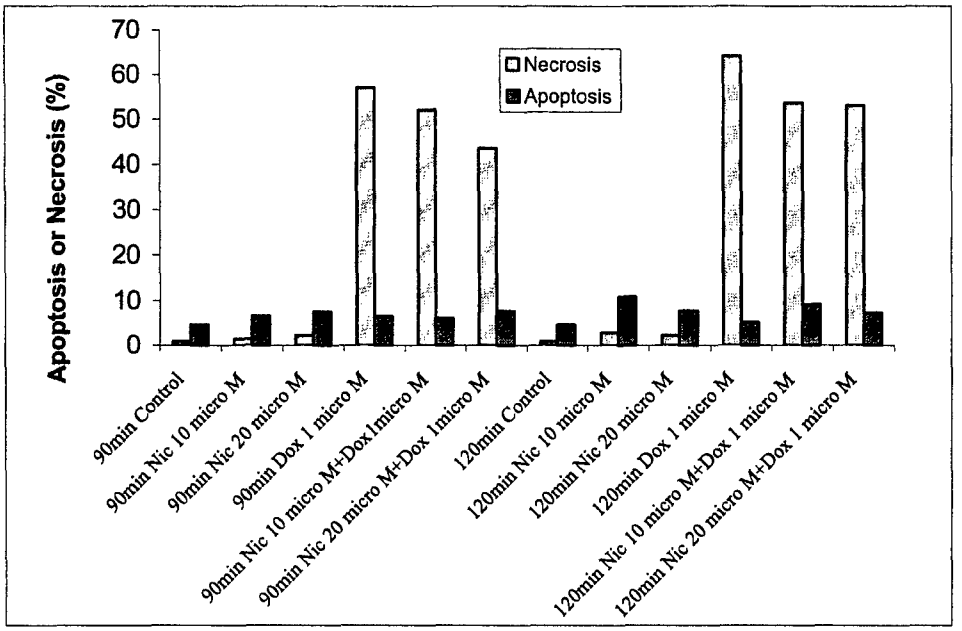


Fig 7. Caspase assays for apoptosis in MCF-7 cells

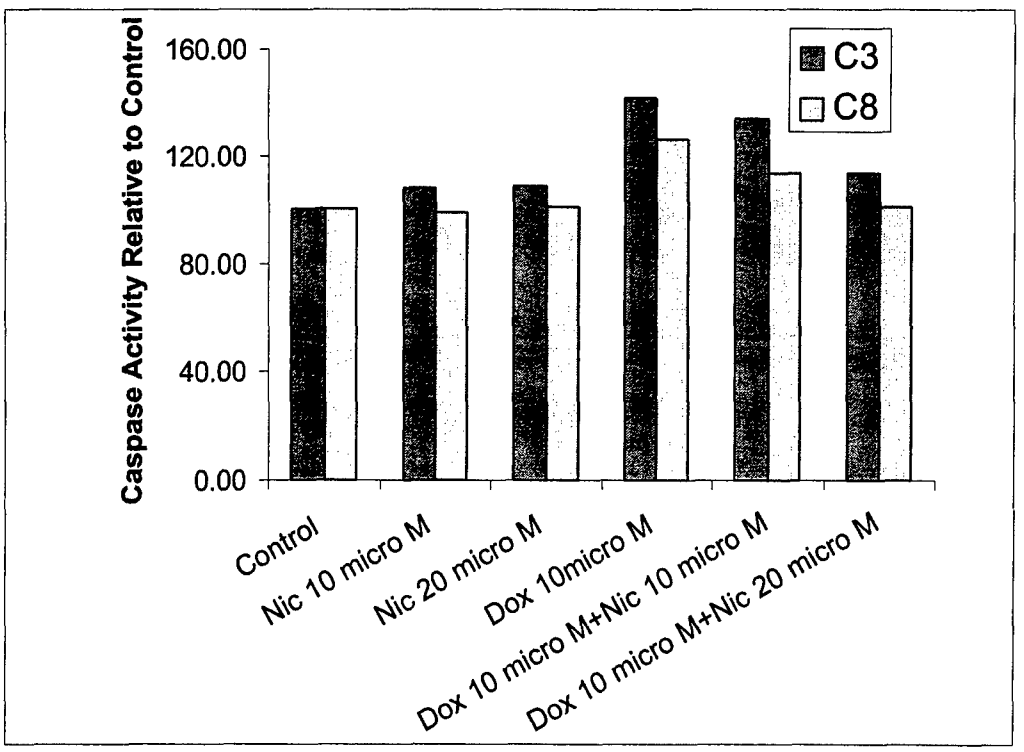
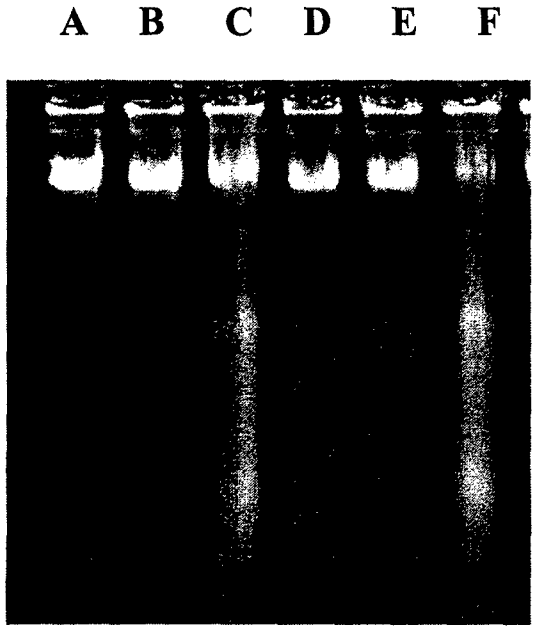


Fig 8. DNA fragmentation assays in MCF-7 Cells

A: Nicotine 10 μM, B: Nicotine 20 μM,
C: Nicotine 10 μM+Doxorubicin 10 μM, D: Nicotine 20 μM+Doxorubicin 10μM,
E: Control, F: Doxorubicin 10 μM.

The results from DNA fragmentation assay indicated that doxorubicin induced most DNA fragments (Lane F) compared to other treatment groups. However, 20 uM nicotine inhibited this effect of doxorubicin (Lane D)



Appendix 2

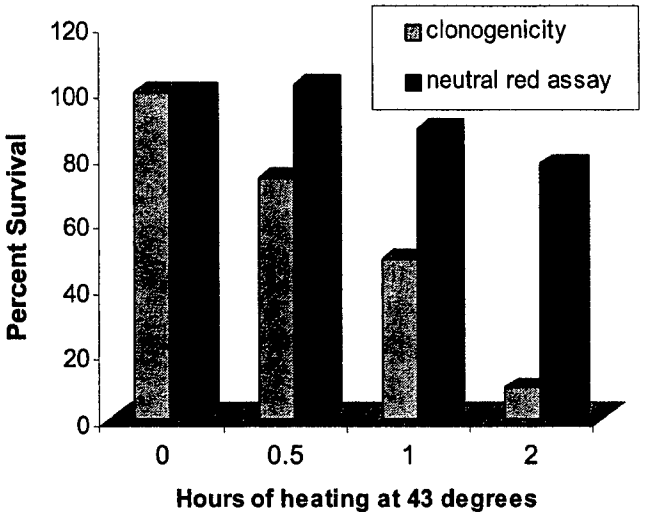


Fig 1.Comparison of Neutral Red and Clonogenicity Assays

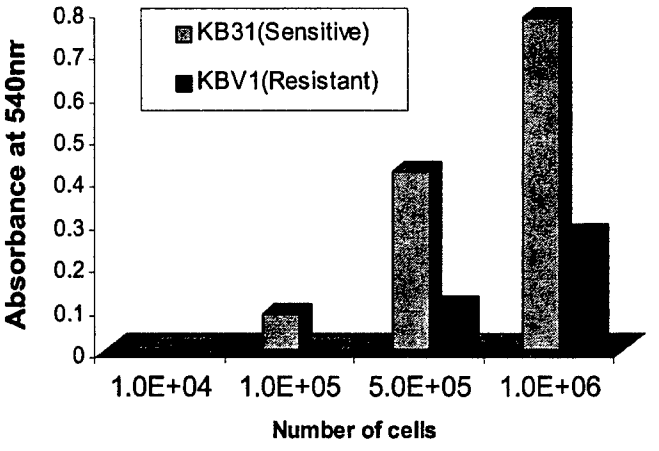
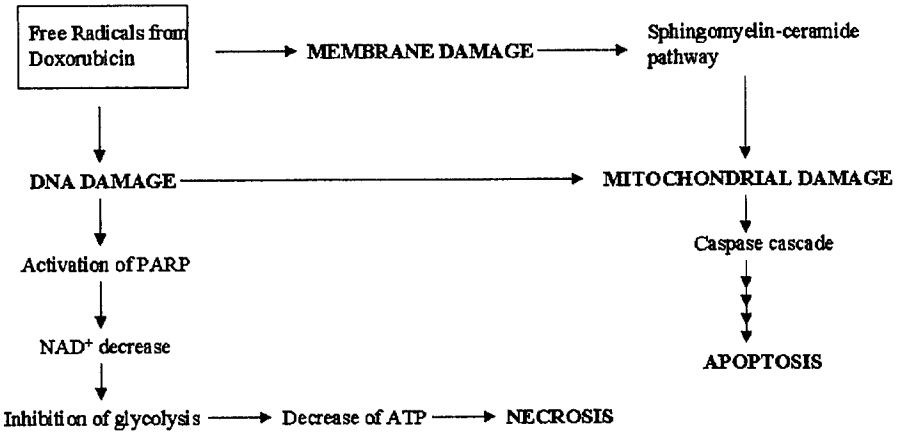


Fig 2. Comparison of Neutral Red Uptake By Drug Sensitive and Multidrug Resistant Cells

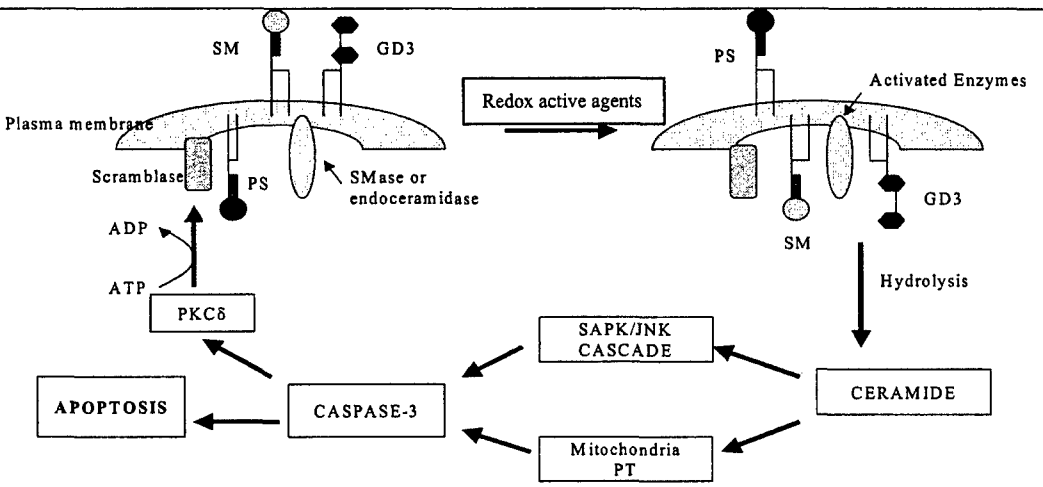
Appendix 3



Schematic Outline of Sequential Biochemical Pathways to Apoptosis Induced by Doxorubicin

Based on Martin D.S., Bertino, J.R. and Koutcher, J.A. Cancer Res. 60:6776-6783, 2001 (Ref.28).

Appendix 4



Redox active agents cause scrambling of plasma membrane phospholipids and active SMase/ endoceramidase. This initiates the ceramide-signaling pathway leading to apoptosis.

Appendix 5:

Abstract: Wang, P.C., Liu, D., Agwu, E. and Sridhar, R. Application of P31 NMR spectroscopy to distinguish drug sensitive and drug resistant breast cancer. Era of Hope. Department of Defense Breast Cancer Research Program Meeting. Proceedings Volume I, June 8-11, 2000, Atlanta, GA, pp 217.

APPLICATION OF P31 NMR SPECTROSCOPY TO DISTINGUISH DRUG SENSITIVE AND DRUG RESISTANT BREAST CANCER

Dr. Paul C. Wang, Dr. Dongsheng Liu, Mr. Emmanuel Agwu, and Dr. Rajagopalan Sridhar

Department of Radiology, Howard University
Washington, DC 20060

E-mail: pwang@howard.edu

Nuclear magnetic resonance (NMR) spectroscopy has emerged as one of the most promising techniques to improve specificity in the diagnosis and staging of breast cancer. It has been widely used in the study of tumor energy metabolism, vasculature and oxygenation, and response to drug treatment. The NMR technique has intrinsically weak signals, which limit the ultimate resolution and sensitivity. A new NMR probe was constructed using a high temperature superconductor, YBCO, to reduce electronic noise and improve detection sensitivity. To test the improvement of sensitivity, two experiments are conducted: an *in vitro* cell metabolism study on 9.4 T spectrometer and an *in vivo* tumor-bearing animal study on a 4.7 T scanner.

In the cell metabolism study, MCF7 human breast cancer cells and their variants are used to characterize the differences in levels of phosphate metabolites during growth phase as well as under Tamoxifen and Doxorubicin treatment. During the NMR study, the breast cancer cells (~10^7 cells) are restrained in agarose gel-thread matrices and continuously perfused with nutrients. Phosphorus metabolites identified include phosphocholine, inorganic phosphate, adenosine triphosphate, diphosphodiester, glycerophosphoethanolamine and glycerophosphocholine. The study of drug effect takes 10 to 12 hours, in which a series of NMR spectra are obtained at one hour intervals (4000 transients, 1.1 sec repetition time). The drug sensitive cells were dramatically affected by 2 μM Doxorubicin within two hours of perfusion but not responsive to Tamoxifen within 12 hours. In contrast, MCF7/ ADR multidrug resistant cells showed no effect by perfusion of 2 μM Doxorubicin.

In the animal study, healthy female athymic nude mice (20-25 g) are used. MCF7 wild type and drug resistant cells are grown as solid tumor xenografts in one hind leg. The other non-involved leg served as a control. The NMR spectroscopy study of tumor progression is done every two or three days during the tumor growth phase. There is a significant drop in high energy phosphate signals in the tumor sites compared with the control sites. In the course of tumor progression, the level of high-energy phosphates continuously dropped while that of inorganic phosphate increased. This study has demonstrated the importance of high quality NMR spectra in differentiating drug sensitive and drug resistant cells.

The U.S. Army Medical and Material Command under DAMD17-96-1-6289 supported this work.

Appendix 6:

Abstract: Wang, P.C., Zhou, J., Agwu, C.E., Li, E. and Sridhar, R. An improved perfusion system for NMR study of breast cancer cells. Era of Hope. Department of Defense Breast Cancer Research Program Meeting. Proceedings Volume III, Abstract P40-30, September 25-28, 2002.

AN IMPROVED PERFUSION SYSTEM FOR NMR STUDY OF BREAST CANCER CELLS

Paul C. Wang, Jianwei Zhou, Chikezirim E. Agwu, Ercheng Li, and Rajagopalan Sridhar

Department of Radiology, Howard University
Washington, DC 20060

pwang@howard.edu

Ex vivo nuclear magnetic resonance is useful for studying the metabolic activity and the response of cells to chemotherapy agents. Such studies involve data acquisition over extended periods up to several days. It is important to preserve the integrity of cells during such prolonged studies by providing adequate nutrients and oxygen to the cells by perfusing aerated culture medium maintained at the normal physiological temperature of 37°C. The presence of serum in the culture medium and temperature fluctuations can produce gas bubbles in the perfusion system. Air bubbles perturb magnetic field homogeneity in the NMR sample and causes line width broadening and loss of spectral resolution. We have utilized an improved perfusion system, which includes positive and negative pressure traps to prevent air bubble generation inside the NMR sample tube. This system has been used to prolong a typical 12 hour ex vivo study to longer than 10 days.

Wild type MCF-7 human breast cancer cells were grown to about 85% confluence in IMEM with 10% FBS. Approximately 1-2 x10^8 cells were harvested and mixed with low temperature gelling agarose. The agarose-cell mixture was extruded into a NMR tube through a 0.5 mm i.d. Teflon tubing. This resulted in thin strands of spaghetti like threads containing cells embedded in agarose. These threads in the NMR tube were perfused with IMEM (0.9 ml/min) using a peristaltic pump, with strategically located air bubble traps before and after the pump and a reservoir for waste collection was located to collect the effluent from the NMR tube. A 400 MHz instrument was used with a repetition time 2 sec, flip angle 45^0, spectral width of 5000 Hz and 1800 transients were acquired over an hour. The ex vivo studies were performed at 37° C and deuterium locked. Many phosphorus metabolites were identified on the basis of their chemical shifts and by spiking with the appropriate authentic compound, in particular phosphoethanolamine (PE), phosphocholine (PC), inorganic phosphate (Pi), glycerophospho-ethanolamine (GPE), glycerophospho-choline (GPC), phosphocreatine (PCr), γ-adenosine triphosphate (γ-ATP), α-adenosine triphosphate (α-ATP), diphosphodiester (DPDE), and β-adenosine triphosphate (β-ATP). The stability of the perfusion system was demonstrated over ten days by comparing a series of spectra obtained at one hour intervals.

In order to ascertain that the spectra represented the metabolic activity of viable cells, iodoacetamide (0.1 mM), a respiratory poison was perfused through the cells and the change in metabolite profile observed. The cytotoxic effect of iodoacetamide caused a decline in β−ATP peak over the next ten hours. Furthermore, signal for extracellular Pi appeared immediately upon perfusion with iodoacetamide. The peak for extracellular peak increased with concomitant decrease in the intracellular peak for Pi, suggesting leakage of intracellular Pi into the extracellular space. The improved cell perfusion is stable for several days and is reliable for the study of cell metabolism in viable cells and for monitoring the effects of cytotoxic agents.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0291 supported this work.

Appendix 7:

Abstract: Zhou, Y. and Sridhar, R. Interaction of tamoxifen with doxorubicin. Era of Hope. Department of Defense Breast Cancer Research Program Meeting. Proceedings Volume II, June 8-11, 2000, Atlanta, GA. pp 684.

INTERACTION OF TAMOXIFEN WITH DOXORUBICIN

Yanfei Zhou and Rajagopalan Sridhar

Howard University Cancer Center and Department of Radiation Oncology, Washington, D.C. 20060

rsridhar@howard.edu

Doxorubicin (Adriamycin) is used alone or in combination with other drugs to treat metastatic breast cancer. Doxorubicin therapy is compromized by the development of a dose-limiting and potentially lethal cardiotoxicity. This may partly be due to free radical intermediates generated during metabolism of doxorubicin. The anti-estrogen tamoxifen protects biomembranes from free radical mediated oxidative damage. Tamoxifen has also shown some potential for reversing multidrug resistance in tumor cells. Ideally, the multidrug reversing agent should sensitize the tumor cells to the action of doxorubicin without any cardiotoxicity. Since cardiotoxicity may involve free radicals, it would be beneficial if the drug used to overcome drug resistance counteracted the free radical effects while potentiating the cytotoxicity of doxorubicin towards the tumor cells. Tamoxifen (10 μM) partially inhibited NADPH dependent lipid peroxidation in rat heart and rat liver microsomes in the presence and absence of doxorubicin. The multidrug resistant human breast cancer cell line MCF7/ADR is nearly 100 times more resistant than the parental MCF7 cell line as judged by clonogenic assays of cultures treated with graded concentrations of doxorubicin. In this study, tamoxifen (10 μM) treatment for 6 hours was toxic to both MCF7 and MCF7/ADR cells. Isobologram analysis revealed that combinations of tamoxifen and doxorubicin exhibited synergistic toxicity towards MCF7/ADR cultures, but exerted only additive effects against MCF7 cells. Both cell lines metabolized doxorubicin to the corresponding semiquinone free radical. Electron spin resonance (ESR) spectroscopy of cell suspensions treated with doxorubicin under anerobic conditions at 37°C, demonstrated the formation of semiquinone free radical interediate. Tamoxifen (10 μM) did not affect the formation of the semiquinone radical in the incubation mixtures. Inclusion of the spin trap 5,5-dimethylpyrroline-N-oxide (DMPO) in aerated incubation mixtures containing cells and doxorubicin gave the hydroxyl spin adduct of DMPO. Comparison of free radical yields from MCF7 and MCF7/ADR cells in these experiments was difficult because the proportions of viable cells and doxorubicin concentrations could not be kept the same for both cell lines. Optimization of electron spin resonance experiments are in progress.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8109 supported this work.

Appendix 8:

Abstract: Zhou, Y., Gu, X, Dhingra, S. and Sridhar, R. Nicotine inhibits doxorubicin–induced apoptosis of human breast cancer cells in culture. Department of Defense Breast Cancer Research Program Meeting. Proceedings Volume I, Abstract P10-25, September 25-28, 2002, Orlando, FL

NICOTINE INHIBITS DOXORUBICIN-INDUCED APOPTOSIS OF HUMAN BREAST CANCER CELLS IN CULTURE

Yanfei Zhou,[1] Xinbin Gu,[2] Sunny Dhingra,[2] and Rajagopalan Sridhar[1]

[1]Cancer Center and Department of Radiation Oncology, and
[2]College of Dentistry, Howard University,
Washington, DC 20060

sridhar_howard@hotmail.com

In the United States approximately 7.5 % of cancer related deaths can be attributed to smoking[1]. Use of tobacco after a cancer diagnosis decreases survival, promotes recurrence, field cancerization and decreases the efficacy of cancer therapy[2]. The chemotherapeutic drug doxorubicin induces apoptosis in cancer cells. The effect of nicotine on doxorubicin-induced apoptosis was evaluated *in vitro* using MCF-7 human breast cancer cell cultures. Exponentially growing monolayer cultures of MCF-7 cells were treated with 20μM nicotine for 120 minutes, followed by a co-exposure to 10μM doxorubicin for 90 minutes. Apoptosis was assayed immediately after exposure to doxorubicin. Twenty micromolar nicotine inhibited doxorubicin-induced apoptosis, based on DNA fragmentation analysis, annexin V binding, and assays for caspase-3 and caspase-8 activities. Treatment with 10μM doxorubicin alone for 90 minutes increased caspase-3 activity to 142% and caspase-8 activity to 126% relative to 100% activity for each enzyme in untreated control cultures. However, a 2 hour pre-treatment with 20μM nicotine reduced the caspase-3 and caspase-8 activities respectively, in cultures treated with doxorubicin to 114% and 101% of control. This corresponds to a 20% inhibition of doxorubicin-induced apoptosis by 20μM nicotine in these cells. In a parallel set of experiments the drug containing medium was replaced with drug free medium after the 90 minute exposure to doxorubicin and then incubated at 37°C for 24 hours. When these cells were examined, inhibition of doxorubicin-induced apoptosis by nicotine could be demonstrated clearly using DNA fragmentation pattern, and annexin V binding, but not on the basis of caspase-3 and caspase-8 activities. Nicotine by itself had no effect on apoptosis, although it inhibited doxorubicin-induced apoptosis. Clonogenicity assays for viability also confirmed the ability of nicotine (10 and 20μM) to protect MCF-7 cells against the cytotoxicity of a 90 minute treatment with doxorubicin (1 and 10μM). Nicotine (10 and 20μM) did not affect the viability of MCF-7/ADR multidrug resistant cells treated with 10μM doxorubicin for 90 minutes. Inhibition of apoptosis in cancer cells by nicotine can diminish the effectiveness of doxorubicin in cancer therapy. Apoptosis is a defense mechanism against carcinogenesis. If nicotine inhibits apoptosis in normal cells or cells progressing towards malignancy, then the risk of secondary malignancies may be higher in patients who use tobacco products during and after doxorubicin treatment.

References: (1) Mortality trends for selected smoking-related cancers and breast cancer. United States 1950-1990. MMWR 1993; 42:857-863. (2) Schnoll RA *et al.* Correlates of tobacco use among smokers and recent quitters diagnosed with cancer. Patient Educ Couns. 2002 Feb;46(2):137-45.

Original work supported by the U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8109.

Appendix 9:

Abstract: Sridhar, R., and Desai, P.B. Similarities and differences in mechanisms of free radical formation from mitoxantrone and doxorubicin. Abstract presented at the Annual Meeting of American Association for Cancer Research, New Orleans, LA. 2001. Proc. Amer. Assoc. Cancer Res. 42: 935,2001. (abstract #5017).

#5017 Similarities and Differences in Mechanisms of Free Radical Formation from Mitoxantrone and Doxorubicin. Rajagopalan Sridhar and Pankaj B. Desai. *College of Pharmacy, University of Cincinnati, Cincinnati, OH, and Howard University Hospital and Cancer Center, Washington, DC.*

Mitoxantrone is used for treating doxorubicin resistant breast cancer. Mitoxantrone is less cardiotoxic than doxorubicin. Since cardiotoxicity may involve free radical intermediates of drug metabolism, doxorubicin and mitoxantrone were evaluated for their tendencies to yield free radical species upon enzymatic reduction and oxidation. Doxorubicin, mitoxantrone and p-benzoquinone were each metabolized by rat liver microsomes and NADPH under anaerobic conditions to their corresponding semiquinone radicals as confirmed by their electron spin resonance (ESR) spectra. Interestingly, mitoxantrone (40μM) inhibited NADPH driven microsomal lipid peroxidation by 60%, whereas doxorubicin at the same concentration stimulated peroxidation nearly two fold. NADPH driven lipid peroxidation of rat liver microsomes, was estimated by the 2-thiobarbituric acid assay. Treatment of mitoxantrone (100μM) with horseradish peroxidase (HRP) and hydrogen peroxide (0.2 M potassium phosphate buffer, pH 7) yielded free radicals whose ESR spectrum was recorded. This matched the spectrum obtained for the product of HRP catalyzed attack of hydrogen peroxide on p-phenylenediamine. Spectrophotometric studies with this system showed the decay of characteristic peaks of mitoxantrone at 608 and 662 nm with concomitant build up of a peak at 586 nm. Treatment of doxorubicin with horseradish peroxidase and hydrogen peroxide caused rapid fading of the red color of the drug, but no free radical species could be detected by ESR spectroscopy. When tested at 10μM against MCF-7 cells in tissue culture, mitoxantrone was more toxic than doxorubicin. Mitoxantrone, in sharp contrast to doxorubicin, yielded a relatively stable free radical upon enzymatic oxidation. This could explain some of the differences in the pharmacodynamics of the two drugs. (Supported in part by the U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8109)

Appendix 10

Abstract: Sridhar, R., Balan, K.B., Shankar, R.A., Zhou, Y. and Goldson, A.L. Observations on the application of neutral red assay for cell viability. Abstract of presentation at EXPERIMENTAL BIOLOGY 2001, Orlando, FL, 2001. FASEB J. 15 (4): pp A238, 2001.

212.14

Observations On The Application Of Neutral Red Assay For Cell Viability

Rajagopalan Sridhar, Kannan V Balan, Yanfei Zhou, Ravi A Shankar, Alfred L Goldson: Howard University, 2041 Georgia Ave., NW, Washington, DC 20060

The vital dye, neutral red is taken up by the lysosomes of viable but not dead cells. This is the basis of widely used neutral red assay for rapid screening of cytotoxic drugs. During investigations on the effect of hyperthermia on a panel of tumor cells several limitations of this assay were evident. In particular, cells killed by exposure to 43 degrees C for one hour did not differ appreciably from viable cells maintained at 37 degrees C with respect to neutral red uptake measured immediately after heat treatment. The cytotoxicity of the 43 degrees C treatment was confirmed using clonogenicity assays. If the neutral red uptake was measured after allowing the cells to grow for 48 hours after heat treatment some degree of killing could be demonstrated by the neutral red assay. The viability was higher when assayed by neutral red assay compared to clonogenicity measurements. In attempts to evaluate the response of multidrug resistant MCF-7/ADR cells it was found that they did not accumulate neutral red to the same extent as other drug sensitive cells, perhaps due to the operation of the drug efflux action of p-glycoprotein (pgP170). This made neutral red assay ineffective for measuring the viability of drug resistant MCF-7/ADR cells. It was also observed that uptake of neutral red by cells depended on the composition of the medium. For instance, the uptake was enhanced in Dulbecco;"s minimal essential medium (DMEM) containing 4.5 g/L glucose compared to DMEM with 1 g/L glucose. The pH of the medium also affected dye uptake by cells. Although time consuming, clonogenicity assays are superior to neutral red assay for cytotoxicity studies. (Supported in part by the U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8109)

Pharmaceutical Research, Vol. 17, No. 11, 2000

Appendix 11

Review Article

Protein Kinases as Therapeutic Targets

Rajagopalan Sridhar,[1] Olivia Hanson-Painton,[2] and Denise R. Cooper[3,4]

Received May 19, 2000; accepted July 14, 2000

Protein kinases and phosphatases are likely targets for the development of therapeutic drugs since they are involved in specific signaling pathways which regulate cell functions such as metabolism, cell cycle progression, cell adhesion, vascular function and angiogenesis. Protein phosphorylation and dephosphorylation serve as molecular switches for modulating these processes and the level and duration of each is a highly regulated process in normal cells. Several compounds that inhibit the activity of tyrosine kinases are being evaluated as cancer therapeutic agents in clinical trials. Diabetes and complications of diabetes also involve deregulated levels of protein kinases. New approaches for regulating kinase gene expression include specific antisense oligonucleotides for inhibiting post-transcriptional processing of the messenger RNA, naturally occurring products and their chemical derivatives to inhibit kinase activity, and monoclonal antibodies to inhibit receptor linked kinases. Inhibition of phosphatases also serves to alter the duration of phosphorylation by kinases. Considerations for development of effective inhibitors include non-specific actions of compounds, cellular uptake, multiple intracellular targets that can dilute the effective cellular concentration of an agent, and tissue specificity. Kinase inhibitors may allow other therapeutic agents additional time to become effective and they may act synergistically with current treatments.

KEY WORDS: protein kinase; signal transduction; drug design; chemotherapeutic agents.

Eukaryotic protein kinases constitute a large family of homologous proteins that catalyze the transfer of the gamma phosphate group of ATP or GTP to the hydroxyl group of serine, threonine or tyrosine in a substrate protein. Protein kinases differ in structure, subcellular location, substrate specificity, and function. Cellular signaling cascades rely on the phosphorylation status of pathway proteins to alter their function. Some substrates transmit the signal, while the final protein targets are altered in activity. Phosphorylated serine, threonine, or tyrosine residues are substrates for specific protein phosphatases so that phosphorylation and dephosphorylation serve as molecular switches and each is highly regulated as to level and duration (Figure 1) (1–4).

Cellular functions such as gene expression, cytoskeletal integrity, cell adhesion, cell cycle progression, and differentiation are controlled by the complex interplay of protein kinases and phosphatases in specific signaling pathways (5–12). Malfunctions of cellular signaling have been associated with many diseases including cancer and diabetes. Regulation of signal transduction by cytokines and the association of signal molecules with protooncogenes and tumor suppressor genes have been subjects of intense research in the industrial setting as well as in academics. Many therapeutic strategies can now be developed through the synthesis of compounds which activate or inactivate protein kinases. In a multicellular organism, intercellular communication plays a crucial role under normal as well as pathological conditions. Coexistence of abnormal cells with normal cells provide the stroma and blood supply essential for maintaining growth and progression of tumors. Such codependence relies on a wide array of receptors and signal transduction pathways to the nucleus of either the host or cancer cell. Since aberrant expression/activation of protein kinase C appears to be involved in the development of certain types of cancer, diabetes and complications of diabetes, the search for selective PKC inhibitors is a major goal of many researchers. Mutant tyrosine kinases are also often associated with carcinogenesis in certain organs, making tyrosine kinase signaling pathways attractive targets for oncology research.

Cytokines, hormones and growth factors bind and activate specific receptors. The molecular mechanisms of signal transduction pathways were elucidated by identifying the specific protein kinase cascades along with their downstream targets, which include some specific transcription factors. Protein kinases act in concert with cytokines, cell cycle regulatory molecules, proteins of apoptotic machinery and transcription factors via pathways that regulate cell metabolism, differentiation, proliferation and death. Many therapeutic strategies are aimed at critical components in signal transduction pathways (9–13).

[1] Department of Radiation Oncology, Howard University Hospital and Cancer Center, Washington, D.C.

[2] Department of Chemistry, University of Central Oklahoma, Edmond, OK.

[3] J. A. Haley Veterans Hospital and Departments of Biochemistry and Molecular Biology and Medicine, University of South Florida College of Medicine, Tampa, FL.

[4] To whom correspondence should be addressed at J.A. Haley Veterans Hospital, 13000 Bruce B. Downs Blvd, Tampa, FL 33612. (e-mail: dcooper@com1.med.usf.edu)

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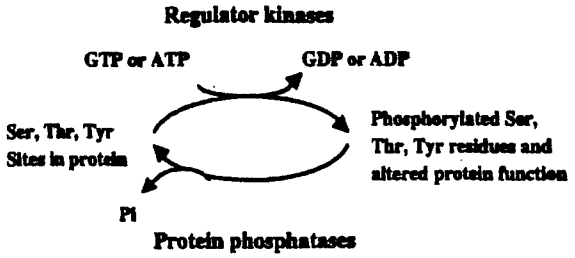

Fig. 1. Kinases and phosphatases provide molecular switches for altering protein function. Protein phosphorylation is a post-translational modification in cells that is reversible by the action of phosphatases. It is one of the most important mechanisms used by cells for signal transduction.

PROTEIN KINASES ARE GROUPED FUNCTIONALLY

Eukaryotic protein kinases typically encode a 250 amino acid catalytic domain that is commonly under the control of a separate regulatory domain or subunit. Hanks and Hunter have classified them on the basis of, their structural and functional properties (14). The protein kinase "phylogenetic tree" (Figure 2) was derived from aligning kinase domain amino acid sequences (14). There are five kinase categories: 1) the cyclic nucleotide-regulated and phospholipid-regulated kinases and ribosomal S6 kinases (AGC), 2) the Ca^{2+}/calmodulin kinases (CaMK), 3) the cyclin-dependent kinases (CMGC), 4) the protein tyrosine kinases (PTK), and 5) "other" kinases falling outside the four major groups. Members of groups share substrate preferences. For example, both the AGC and CaMK groups phosphorylate serine/threonine residues near arginine and/or lysine. Members of the serine/threonine kinase group, CMGC, phosphorylate serine/threonine in proline-rich domains. The CMGC kinases have larger catalytic domains than other kinases. The PTK group includes both receptor and non-receptor kinases that phosphorylate tyrosine residues. Other kinases can phosphorylate either serine/threonine and tyrosine residues and some are termed dual-specificity kinases. The cellular function of many kinases was elucidated initially by broad specificity inhibitors. With the rapid expansion of DNA sequence databases, it is likely that additional kinases will be discovered.

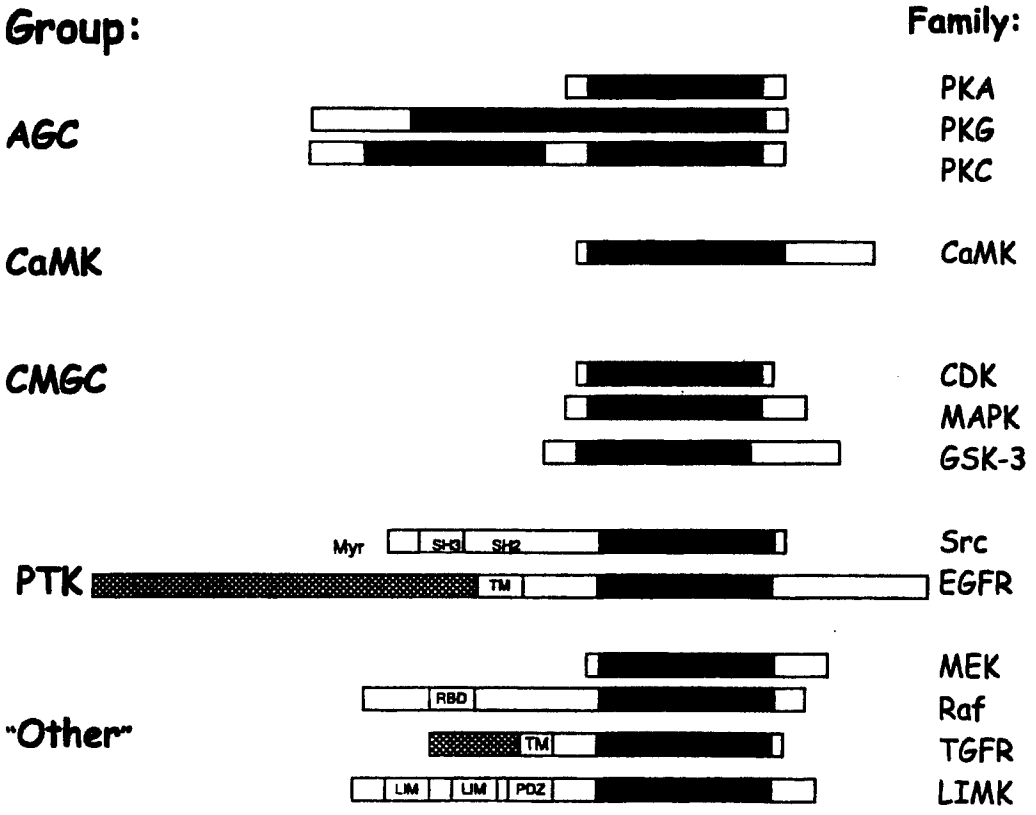

Fig. 2. Diagram of the major families of protein kinases. Kinase catalytic domains are shaded black. The AGC group includes cyclic nucleotide-dependent protein kinases (Protein kinase A (PKA) and Protein kinase G (PKG)) and lipid-dependent protein kinase C (PKC) families. The catalytic subunit of PKA is shown. PKG contains a cGMP binding domain which is shaded gray. The regulatory domain of PKC is also shaded gray. The CaMK group shows the shaded autoregulatory domain (site that binds Ca2+/calmodulin). The CMGC group includes the CDK, MAPK/ERK and GSK-3 families. The PTK group includes the Src family and the EGFR RTK family. The N-terminal myristoyl (Myr) modification allows for membrane attachment. The SH2 and SH3 domains are shown. RTKs contain transmembrane (TM) and extracellular binding domains for ligands (checked shading). The "other" group includes MEK, Raf, and the TGF-β receptor and LIM kinases. Raf kinases have a Ras binding domain (RBD). The TGF-β receptors have extracellular ligand binding domains (checked shading) and TM domains. The LIM and PDZ domains are noted in LIMK.

DEPHOSPHORYLATION BY PROTEIN PHOSPHATASES

Dephosphorylation by protein phosphatases plays an equally important role in regulating cellular processes. Protein phosphatases have specificities that are as distinct as those of the protein kinases, and a similar number of genes encode both family members (15,16). They are classified based on substrate specificity, dependence on metal ions, and sensitivity to inhibitory agents. Table 1 summarizes the distribution and known inhibitors of protein phosphatases which have been demonstrated to play a role in signal transduction. They possess a 230 amino acid catalytic domain and contain a number of regulatory subunits that govern subcellular localization and enzymatic activity (15,20). The activities of PP1 and PP2A are independent of metal ions (15,16). The catalytic subunit of PP1 binds to regulatory subunits that determine PP1 subcellular localization and activity (17) while PP2A is inactivated by transient phosphorylation of tyrosine residues on the molecule (18). PP2B, also known as calcineurin, consists of a catalytic subunit (A-subunit, 6kDa) and a regulatory subunit (B-subunit, 19kDa). It is dependent on the Ca^{2+}-calmodulin complex for complete activation (19). Over 40 protein tyrosine phosphatases (PTP) have been characterized. Specific activators of protein phosphatases are still being sought. The C2, C6 and C16 ceramides are reported activators of protein phosphatases (21–23).

THERAPEUTIC STRATEGIES FOR TREATING CANCER

Cancer treatment strategies include: (i) inhibiting tumor cell proliferation, (ii) inducing tumor cell death by necrosis or apoptosis, (iii) inhibiting tumor angiogenesis, (iv) facilitating host immune system, (v) inducing vegetative tumor cells to undergo terminal differentiation, (vi) inhibiting metastases by inhibiting tumor cell adhesion and invasiveness of normal tissues.

Table 1. Protein Phosphatases Involved in Signal Transduction

Protein phosphatase type	Subcellular distribution	Known inhibitors
PP1	Cytosol	Calyculin A
	Nucleus	Nodularin
	Myofibrils	Tautomycin
	Glycogen particles	
PP2A	Cytosol	*Calyculins*
	Nucleus	Microcystins
	Mitochondria	Nodularin
		Okadaic acid
PP2B (calcineurin)	Cytosol	Cyclosporin A
	Nucleus	FK506
	Plasma membrane	Immunophilin complexes
	Synaptosomes	Cypermethrin
		Deltamethrin
		Fenvalerate
PTP	Plasma membrane	bp V(phen)
	Nucleus	mpV(pic)
		Dephostatins
		Phenylarsine Oxide
		Sodium Orthovanadate

TYROSINE KINASES AS THERAPEUTIC TARGETS FOR CANCER CHEMOTHERAPY

Recent efforts in drug design have targeted specific kinases. Ras is one of the most frequently mutated oncogenes in human cancers (24,25), and Ras signaling is a downstream event of tyrosine kinase activation. Therefore modifiers of tyrosine kinases are actively being investigated as anti-cancer drugs. Cytoplasmic tyrosine kinases frequently contain SH2 and SH3 domains (*src* Homology 2 and 3 domains) which mediate intra- and interprotein interactions (Figure 2). SH2 domains bind to phosphotyrosine sites with flanking amino acids that are specific for the particular SH2 sequence, and SH3 domains latch on to proline-rich regions (10).

Receptor tyrosine kinases (RTKs), many of which are growth factor receptors, are transmembrane glycoproteins with a membrane spanning domain and a conserved cytoplasmic tyrosine kinase domain. The RTK superfamily consists of 18 families in vertebrates and includes 56 different receptors including insulin, fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), and many other receptors.

There is considerable structural similarity among each RTK subfamily. Related receptors usually bind related ligands such as the HER family of receptors (EGF receptor, HER2, HER3 and HER 4) and their ligands (TFG-α), amphiregulin, heparin binding EGF (HB-EGF), betacellulin, and heregulin. Each of these receptors contains two domains that share the conserved sequence pattern of nearly 50 cysteine residues; additionally, each of the ligands contains a conserved motif of cysteine sextet present in the prototypical EGF (10).

Binding of a ligand to an RTK leads to receptor dimerization and activation of the intracellular catalytic (kinase) domain. In the dimer, the catalytic unit of one receptor subunit phosphorylates specific tyrosines in the other subunit. The phosphorylated receptors then phosphorylate or interact with other adapter and signaling molecules through phosphotyrosines, triggering a cascade of further phosphorylations and/or dephosphorylations. After a series of downstream events involving several proteins, the signal reaches the nucleus in the form of a molecule which can alter the activity of the genetic machinery to control cell proliferation, differentiation, cell metabolism, and even programmed cell death (apoptosis) (10,26,27).

Insulin-like growth factor 1 (IGF-1) and related receptors exist as preformed dimers of α and β chains. Following activation, the ligand-induced phosphorylation is similar to the RTK pathway. The phosphotyrosines of the receptor can bind to an adapter molecule or substrate such as phosphatidyl inositol (PI) 3-kinase. The association of PI-3-kinase with the intracellular domain of phosphorylated RTK enhances PI-3 kinase activity via allosteric activation of the catalytic subunit (10,28). In contrast to substrates of RTKs such as PI-3-kinase, the adapter molecules contain no intrinsic catalytic activity. An example of an adapter molecule in this signaling is Grb2 in the MAP kinase pathway (29).

There is strong evidence for the involvement of RTKs in human cancer making these a target for inhibition (10,30). Examples of these are *erbB (EGF receptor)*, *neu* (HER2), *kit*

(stem-cell factor receptor), *fms* (CSF1 receptor), *met* (HGF receptor), *trk* (neurotrophin receptor), *sea, ros, ret, eyk,* and *axl* (10). A number of cytoplasmic tyrosine kinases including *src* and *abl* behave as oncogenes when mutated or inappropriately expressed (10). Nearly 30% of human breast and ovarian cancers show amplified expression of the receptor tyrosine kinase HER2 (31). Amplification of HER2 gene also correlates with decreased patient survival and a shorter time for recurrence of disease (32).

Blocking of the receptor/ligand interaction is also an effective therapeutic target. Herceptin (Genentech, San Francisco, CA) is a humanized monoclonal antibody against HER2. The success of Herceptin in cancer treatment supports the hypothesis that blocking certain RTKs can curtail cancer progression. Alteration or overexpression of RTKs such as PDGF and EGF receptors has also been associated with certain cancers. Inhibitors of RTKs may inhibit the growth and proliferation of such cancers, since RTKs stimulate tumor cell proliferation.

Inhibitors of RTKs are useful in preventing tumor angiogenesis and can eliminate support from the host tissue by targeting RTKs located on vascular cells (e.g., blood vessel endothelial cells and stromal fibroblasts (FGF receptor)). Another example of restricting blood supply to a tumor could be through vascular endothelial growth factor (VEGF) and its receptor. Several splice variants of VEGF are known (e.g., $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) which vary in the number of amino acids in the peptide (33). VEGF stimulates endothelial cell growth during angiogenesis, and increases the permeability of tumor vasculature so that proteins and other growth factors become accessible to the tumor (10). Broad-spectrum antitumor efficacy of an oral dosage form of an inhibitor of VEGF signaling has been reported (33). Thus, inhibition of VEGF receptor signaling presents an important therapeutic target. An extracellular receptor can also be a target for inhibition. For example, the EGF receptor family and its ligands are overexpressed and exist as an autocrine loop in many tumor types. One EGF-related peptide, amphiregulin, is coexpressed in pancreatic and ovarian cancer (10). HB-EGF is expressed as an autocrine loop in gastric cancer. EGF receptor is found in over half of breast tumors unresponsive to hormone (10). EGF is found in many tumors, and EGF may be required for tumor cell growth. Antibody to EGF blocked the growth of tumor xenografts in mice (35). An antisense oligonucleotide for amphiregulin inhibited growth of a pancreatic cancer cell line (36). A variety of inhibitors of RTKs are listed in Table 2, some of which are already in clinical trials.

OTHER TARGET POSSIBILITIES

Many tyrosine kinase inhibitors are derived from natural products including flavopiridol, genistein, erbstatin, lavendustin A, staurosporine, and UCN-01. Inhibitors directed at the ATP binding site are also available (11,37). Signals from RTKs can also be inhibited at other target sites such as: nuclear tyrosine kinases, membrane anchors (inhibition of farnesylation) and transcription factors.

DEFINING THE TARGET

Targeting the signaling potential of growth promoting tyrosine kinases such as EGFR, HER2, PDGFR, *src,* and *abl,* will block tumor growth while blocking IGF-1 and TRK will interfere with tumor cell survival. Inhibiting these kinases will lead to tumor shrinkage and apoptosis. Flk-1/KDR and *src* are kinases necessary for neovascularization (angiogenesis) of tumors and inhibition of these will slow tumor growth thereby decreasing metastases (38). *Met* promotes cell migration, and inhibiting this kinase should also decrease metastases (39).

The usual criteria applicable for evaluating conventional chemotherapy drugs may fail to detect the efficacy of drugs targeted against RTKs. Inhibitors of RTKs stabilize the tumor in terms of cell proliferation, normal cell loss via apoptosis, and prevent cell migration, invasion and metastases. These drugs are likely to increase the time required for tumor progression, and may inhibit or attenuate the aggressiveness of the disease but may not initially result in measurable tumor regression. Therefore, specially designed trials are needed to evaluate the usefulness of drugs designed for RTK inhibi-

Table 2. Inhibitors of Receptor Tyrosine Kinases

Inhibitor	Tyrosine kinase target	Clinical trial	Source
Genistein	EGF-R, v-src		
Herbimycin A	EGF-R		
HNMPA-(AM)3	IR-K		
Lavendustin A	IR-K		
Tyrphostin 23	IR-K, EFG-R		
PP1	v-src		Pfizer
PP2	v-src		Pfizer
ZD 1839 (Iressa™)	EGF-R	Phase I (NSCLC, breast, colorectal, ovarian, renal, esophageal, pancreatic, and mesothelioma solid tumors)	Zeneca
CP-358774	EGF-R		Pfizer
CGP 59326	EGF-R		Novartis
PD 166285	EGF-R, PDGF-R, v-src		Parke-Davis
CGP 57148	PDGF-R		Novartis
SU101 (Leflunomide)	PDGF-R	Phase III (glioblastoma multiform) and Phase II (prostate, lung and ovarian cancer)	Sugen
SU 5416	Flk-1/KDR	Phase II (AIDS-related Kaposi sarcoma)	Sugen
STI 571	abl	Phase I (Philadelphia-chromosome-positive leukemia)	Novartis
ZD4190	VEGF-R		Astra Zeneca

tion. Perhaps drugs will act synergistically with the currently utilized chemotherapeutic agents. Inhibitors of RTKs are less likely to have adverse systemic toxicity since they are cytostatic and not cytocidal. They are likely to delay tumor progression by inhibiting cell cycle transit allowing other therapeutic agents additional time to cause tumor regression.

An example of cancer arising from a defective tyrosine kinase is a class of ALK positive lymphomas referred to as "ALKomas" which display inappropriate expression of a neural-specific tyrosine kinase, anaplastic lymphoma kinase (ALK) (40). Many solid tumors overexpress epidermal growth factor receptor (41). Iressa (ZD1839) is an orally active selective EGF-R inhibitor. This compound disrupts signaling involved in cancer cell proliferation, cell survival and tumor growth support by the host (42). The clinical efficacy of this agent shows that it is well tolerated by patients undergoing Phase I/II clinical trials (43,44). The compound has shown promising cytotoxicity towards several cancer cell lines (43).

Many growth factors and cytokines regulate cellular functions via the Janus kinase (JAK) signal transducers and activators of transcription (STAT). Membrane-associated JAK tyrosine kinases are activated upon ligand binding to an RTK. This preferentially recruits dormant cytoplasmic transcription factors (STATs) which are subsequently activated by phosphorylation. The phosphorylated STATS migrate to the nucleus and activate transcription of the target gene (45,46). Cells derived from rat and human cancers have constitutively activated Stat3, and the malignant potential of cancer has been associated with Stat3 activation (47,48). The JAK inhibitor AG940 prevents Stat3 activation and suppresses the growth of human prostate cancer cells (48).

DNA DEPENDENT PROTEIN KINASES

DNA-dependent protein kinase (DNA-PK) is involved in the repair of double-strand breaks in mammalian cells. This enzyme requires ends of double stranded DNA or transitions from single stranded to double stranded DNA in order to act as a serine/threonine kinase (49–54). Cells with defective or deficient DNA-PK activity are unable to repair radiation induced DNA double strand breaks and consequently very sensitive to the lethal effects of ionizing radiation (50–53). DNA-PK dependent repair of DNA double strand break involves DNA ligase IV and XRCC4 (53–55). Inhibition of DNA-PK has the potential to increase the efficacy of antitumor treatment with radiation or chemotherapeutic agents.

CELL CYCLE REGULATION BY CYCLIN DEPENDENT KINASES

Progression through the cell cycle is controlled in part by a series of regulatory molecules called cyclins and the cyclin-dependent kinases (CDK) which they activate. In addition to the cyclins, CDK activity is also regulated by phosphorylation and dephosphorylation. Cellular inhibitors of CDKs also play a major role in cell cycle progression (56). Alterations in the expression, function, and structure of cyclin and CDK are encountered in the cancer phenotype. Therefore CDKs may be important targets for new cancer therapeutic agents. Cell cycle perturbations occur in tumors and tumor cells treated with ionizing radiation and or chemotherapeutic agents. Whether or not the DNA damage caused in cells leads to cell death depends on normal cell cycle control mechanisms that are in place. Often chemotherapy resistant cells tend to escape apoptosis. Under certain circumstances, inappropriate CDK activation may even promote apoptosis by encouraging the progression of the cell cycle under unfavorable conditions, *i.e.*, attempting mitosis while DNA damage is largely unrepaired.

INHIBITION OF CDKs TO INDUCE APOPTOSIS IN CANCER CELLS

Purines and purine analogs act as CDK inhibitors. Flavopiridol (L86-8,275) is a flavonoid that causes 50% growth inhibition of tumor cells at 60 nM (57). It also inhibits EGFR and protein kinase A (IC_{50} about 100 μM) (57). Flavopiridel induces apoptosis and inhibits lymphoid, myeloid, colon, and prostate cancer cells grown *in vivo* as tumor xenografts in nude mice. At the molecular level, flavopiridol affects CDK function and arrests cells in the G_2/M and G_1/S border. Both cycling and non-cycling cells are killed by flavopiridol. At concentrations above 1 micromolar, flavopiridol loses its selectivity and starts inhibiting other kinases (e.g., 6 μM is the IC_{50} for protein kinase C) (57). Staurosporine and its derivative, UCN-01, in addition to inhibiting protein kinase C, inhibit cyclin B/CDK (IC_{50} 3 to 6 nM). Staurosporine is toxic, but its derivative 7-hydroxystaurosporine (UCN-01) has antitumor properties and is in clinical trials (58). UCN-01 affects the phosphorylation of CDKs and alters the cell cycle checkpoint functioning. These compounds illustrate that multiple intracellular targets may be affected as the concentration of an inhibitor is increased within cells.

TISSUE SPECIFICITY AS A COMPONENT OF IDENTIFYING THE THERAPEUTIC TARGET

Tamoxifen, a protein kinase C inhibitor with antiestrogen activity, is currently a standard treatment for hormone-dependent breast cancer. The use of this compound may increase the risk of developing cancer in other tissues such as the endometrium (59). Raloxifene, a related compound, has been shown to protect against osteoporosis (59). The tissue specificity of inhibitors must be considered when identifying therapeutic targets.

MITOGEN ACTIVATED KINASE (MAP KINASES) IN CARCINOGENESIS

Signal transduction to the nucleus in response to extracellular stimulus by a growth factor involves the mitogen activated protein (MAP) kinases. MAP kinases are a family of protein serine threonine kinases which mediate signal transduction from extracellular receptors or heat shock, or UV radiation (some receptors are tyrosine kinase receptors) (60,61). These kinases, in concert with other signal transduction pathways can network to differentially alter the phosphorylation of transcription factors. Cell proliferation and differentiation in normal cells are under the regulation and control of multiple MAP kinase cascades. Aberrant and deregulated functioning of MAP kinases can initiate and support carcinogenesis (62,63). Insulin and IGF-1 also activate a mitogenic MAP kinase pathway that may be important in

acquired insulin resistance occuring in type 2 diabetes (64).

PHOSPHATIDYLINOSITOL 3-KINASE, PKB/AKT AND CELL SURVIVAL

Many cancers become refractory to chemotherapy by developing a survival strategy involving the constitutive activation of the phosphatidylinositol 3-kinase-protein kinase B/Akt signaling cascade. This survival signaling pathway thus becomes an important target for the development of specific inhibitors that would block its function (65–68). PI-3 kinase/Akt signaling is equally important in diabetes (69). The pathway activated by RTKs subsequently regulates glycogen synthase kinase 3 (GSK3) and glucose uptake. Since Akt has decreased activity in type 2 diabetes, it provides a therapeutic target (69).

KINASE INHIBITORS AS TOOLS FOR STUDYING CELLULAR SIGNALING

Protein kinase inhibitors provide much of our knowledge about regulation and coordination of physiological functions. Endogenous peptide inhibitors occur *in vivo* (70). A pseudosubstrate sequence within PKC acts to inhibit the kinase in the absence of its lipid activator (71). A PKC inhibitor such as chelerythrine acts on the catalytic domain to block substrate interaction, while calphostin C acts on the regulatory domain to mimic the pseudosubstrate sequence and block ATPase activity, or by inhibiting cofactor binding. The ability to inhibit specific PKC isozymes is limited. The most specific inhibitors appear to be directed toward the conventional PKCs (regulated by phospholipids, calcium, and diacylglycerol) with at least two inhibitors of PKCβII identified (72,73). Most PKC inhibitors, including those for PKCβII, inhibit insulin-induced glucose uptake (73,78). The importance of PKC activation for insulin action has been the topic of numerous studies. Multiple PKC isozymes appear to be involved in regulating glucose uptake (78) and insulin resistance mediated by the insulin receptor (79).

The caveat for evaluating the specific function of a kinase using inhibitors lies in the non-specific actions of some compounds and their ability to inhibit a number of different protein kinases or, at higher concentrations, similar isozymes. The cellular uptake, half-life, diffusion, or multiple intracellular receptors are also considerations when interpreting inhibitor effects on metabolic and mitogenic function.

Activated kinases can have multiple substrates that are then trafficked to subcellular locations via phosphorylation/dephosphorylation signals. Nuclear targets of activated kinases are thought to be transcriptional activation factors. Another mechanism of activating transcription factors that are dormant in the cytoplasm is their translocation into the nucleus upon phosphorylation. This mechanism of signal transduction is observed in the case of NF-κB proteins (74). NF-κB complexes are inactive due to complexing with IκB inhibitors, but upon phosphorylation of the regulatory IκB by PKC and PKA, free NF-κB complexes are dissociated and are then translocated to the nucleus (74).

OTHER MODES OF REGULATING PROTEIN KINASES

Although some protein kinases have, to date, no known system of physiological regulation, many are activated or in-

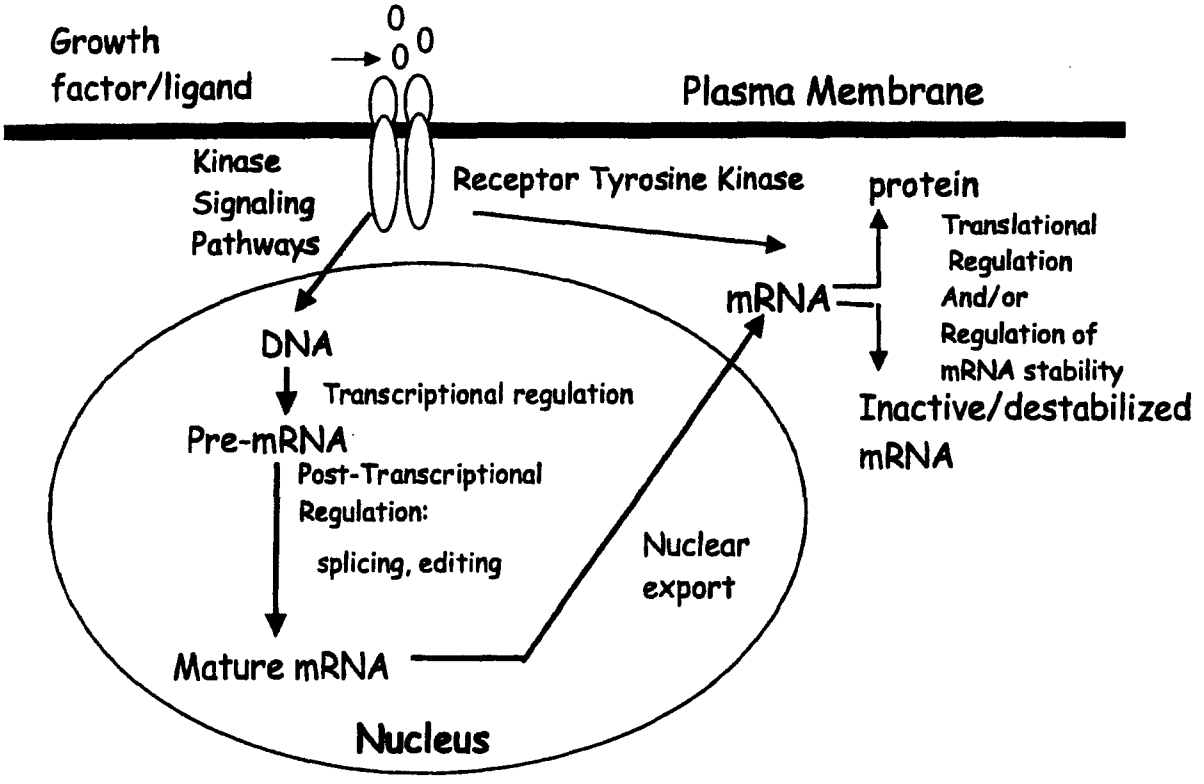

Fig. 3. Regulatory control points in gene expression by growth factors. Protein kinase signaling pathways activated by protein tyrosine kinases are known to regulate RNA transcription, post-transcriptional processing of pre-mRNA, mRNA stability, and protein phosphorylation.

activated by autophosphorylation or phosphorylation by upstream protein kinases. The regulation of protein kinases also occurs transcriptionally, post-transcriptionally, and post-translationally (Figure 3). The mechanism of post-transcriptional regulation is alternative splicing of precursor mRNA (75). Protein kinase C-βI and -βII are two isoforms of a single PKCβ gene derived from differences in the splicing of the exon encoding the C-terminal 50–52 amino acids. Splicing can be regulated by a kinase cascade in response to peptide hormones such as insulin and IGF-1 (75). PKCβI and βII have different specificities for phosphorylating members of the mitogen activated protein (MAP) kinase family, for glycogen synthase 3β, for nuclear transcription factors such as TLS/Fus, and for other nuclear kinases (76–78). By inhibiting the post-transcriptional alternative splicing of PKCβII mRNA, PKCβII-dependent processes are inhibited.

The stability of mRNA encoding the PKC isozymes is also apparently regulated by kinase cascades. Destabilization of PKCδ mRNA by phorbol esters is one example (80). The destabilization of PKCβII mRNA by glucose is another case in which stability is modulated by protein kinases (81). Thus, regulation of PKCβII expression by insulin via alternative splicing of pre-mRNA and glucose via destabilization of mRNA, suggests that post-transcriptional processing may be a likely target for altering kinase levels. The development of antisense oligonucleotides to inhibit the expression of various protein kinases has been successful. Antisense oligonucleotides are short lengths of synthetically manufactured, chemically modified DNA or RNA designed to specifically interact with mRNA transcripts encoding target proteins. The interaction of the antisense moiety with mRNA inhibits protein translation and, in some cases, post-transcriptional processing (e.g., alternative splicing and stability) of mRNA. Antisense oligonuclotides have been developed to alter alternative splicing of BclX long to short mRNA forms and for inhibiting the translation of PKCα and PKC ζ (82).

PROTEIN KINASE INHIBITORS IN CARDIOVASCULAR DISEASE AND VASCULAR COMPLICATIONS IN DIABETES MELLITUS

Protein kinase C isoforms have been implicated in cellular changes observed in the vascular complications of diabetes. Hyperglycemia is associated with increased levels of PKCα and β isoforms in renal glomeruli of diabetic rats (72). Oral administration of a PKCβ inhibitor prevented the increased mRNA expression of TGF-βI and extracellular matrix component genes (72). Administration of the specific PKCβ inhibitor (LY333531) also normalized levels of cytokines, caldesmon and hemodynamics of retinal and renal blood flow (72). Overexpression of the PKCβ isoform in the myocardium resulted in cardiac hypertrophy and failure (72). The use of LY333531 to prevent adverse effects of cardiac PKCβ overexpression in diabetic subjects is under investigation (72). The compound is also in Phase II/III clinical trials for diabetic retinopathy and diabetic macular edema indicating that it may be pharmacodynamically active (83).

CONCLUSIONS

Our original understanding of kinases and their roles in cellular metabolism are based on work by Krebs, Graves and Fisher (84), more recently, information provided from investigations on such diverse species as *D. melanogaster, S. cerevisiae, D. discoideum, and C. elegans* has identified new kinase genes and allowed cloning of their mammalian counterparts (1,3). The further use of peptide libraries, protein-RNA, protein-DNA, and protein-protein interaction systems will advance our understanding of kinase specificity and how kinases are regulated by protein interaction, and will provide additional molecular possibilities for drug intervention.

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