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13. Abstract (Maximum 200 Words) <i>(abstract should contain no proprietary or confidential information)</i> We investigated the impact of combined exposure to the check-point abrogator (UCN-01) in conjunction with MEK1/2 inhibitors upon survival of mammary carcinoma cells. Treatment of cells with UCN-01 resulted in prolonged activation of the MAPK pathway. Inhibition of MEK1/2 caused modest reductions in basal MAPK activity and suppressed UCN-01-stimulated MAPK activity below that of MEK1/2 inhibitor alone. Significantly, combined, but not individual, exposure of cells to UCN-01 and MEK1/2 inhibitors enhanced BAX association with mitochondria and triggered release of cytochrome c into the cytosol, accompanied by activation of effector pro-caspases, resulting in a synergistic potentiation of apoptosis within 18-24h. Radiation exposure of drug treated cells did not further enhance apoptosis. Treatment of cells with both caspase 9 and caspase 8 inhibitors was required to completely inhibit apoptosis in carcinoma cells. Over-expression of Bcl-x _L blocked cytochrome c release and cell killing induced by the drug combination. Colony forming assays demonstrated that cells exposed to both agents exhibited a substantial reduction in clonogenic survival compared to either drug alone; moreover, radiation further reduced clonogenic survival despite failing to promote additional apoptosis.				
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INTRODUCTION FOR ANNUAL REPORT 2001/2002 OF DAMD17-99-1-9426.

UCN-01 (7-hydroxystaurosporine) is currently being evaluated as an anti-neoplastic agent in phase I clinical trials, both alone and in combination with chemotherapeutic agents and ionizing radiation [1,2]. UCN-01 has been shown to exert anti-proliferative activity both *in vitro* and *in vivo*, which may be related to inhibition of protein kinase C (PKC) isoforms. UCN-01 also enhances the cytotoxicity of chemotherapeutic agents by several postulated mechanisms, including inhibition of Chk1 [3]. Inhibition of Chk1 may directly promote activation of the protein phosphatase Cdc25C and can also interfere with Cdc25C elimination by blocking its binding to 14-3-3 proteins and subsequent degradation [3]. Down-regulation of Cdc25C results in enhanced phosphorylation and inactivation of cyclin-dependent kinases (Cdks) such as p34^{cdc2}, which are critically involved in G2/M cell cycle arrest following DNA damage [4]. Thus, UCN-01 can act as a checkpoint abrogator capable of enhancing the lethal actions of DNA-damaging agents, including ionizing radiation [5], cisplatin [6], mitomycin C [7], ara-C [8] and camptothecins [9]. It is thought that UCN-01 at physiologic concentrations promotes cell death *via* Cdk dephosphorylation rather than by inhibition of PKC enzymes [10].

The ability of the "classical" mitogen activated protein kinase (MAPK) pathway to regulate proliferation *versus* differentiation and survival appears to depend upon the amplitude and duration of MAPK activation. A short activation of the MAPK cascade by growth factors has been correlated with enhanced progression through the G1-S transition [11]. In contrast, prolonged elevation of MAPK activity has been demonstrated to inhibit DNA synthesis through super-induction of the Cdk inhibitor protein p21^{Cip-1/WAF1/mda6} (p21) [12]. In addition to a role for MAPK signaling in the G1/S phase transition, it has also been argued that MAPK signaling may be involved in the ability of cells to progress through G2/M, particularly following drug- or radiation-induced growth arrest [13,14].

Recent evidence has suggested that inhibition of the MAPK pathway sensitizes a broad range of tumor cells to the toxic effects of ionizing radiation and cytotoxic drugs [15-17]. In the former case, radiosensitization has been found to be dependent upon a prolongation of both radiation-induced G2/M phase arrest and Cdc2 tyrosine 15 phosphorylation [18,19]. On the other hand, in hematopoietic cells exposed to cytotoxic drugs, it has been argued that the ability of MAPK signaling to increase or maintain expression of p21 also plays an important cytoprotective role [1,20]. Inhibitors of the Raf/MEK/MAPK pathway are also in Phase I trials [21]. Thus, it is apparent that both inhibition of MAPK signaling as well as UCN-01-mediated check-point abrogation represent promising strategies for activation of the cell death pathway in neoplastic cells.

Despite the intense interest in UCN-01 as an anti-neoplastic agent, the mechanism(s) by which it induces cell death remain(s) incompletely understood particularly the relationship between UCN-01-mediated lethality and activation of signal transduction pathways. Recently, it was observed that the combination of UCN-01 and MEK1/2 inhibitors promoted cell death in a panel of p53-null human leukemia cell lines [22]. However, whether such interactions occur in tumors of epithelial origin, particularly those expressing mutant or wild-type p53, is presently unknown.

Body

Objective/Hypothesis.

Hypothesis 1. Combined inhibition of MAPK and UCN-01 treatment will enhance cell death in mammary carcinoma cells compared to either treatment alone, and that this will be synergistically augmented by radiation.

Hypothesis 2. The molecular mechanism(s) by which inhibition of MAPK and UCN-01 treatment, in the presence or absence of a subsequent radiation exposure, enhance mammary carcinoma cell death are identifiable.

Specific Aims.

Aim 1. We will determine the impact of combined inhibition of MAPK and UCN-01 treatment, with or without radiation exposure, on MCF-7 and T47D (estrogen-dependent), MDA-MB-231 (estrogen-independent) breast cancer cell survival with respect to apoptosis 24-96h after treatment and clonogenic survival 10-14 days after treatment.

Aim 2. We will elucidate the mechanisms by which inhibition of MAPK enhances UCN-01-induced apoptosis in carcinoma cells 24-96h after treatment. We will determine the molecular mechanisms by which exposure to ionizing radiation further enhances cell killing by MEK1/2 inhibition / UCN-01 treatment in these cells.

Specific Aim 1.

Significant portions of the studies in Aim 1 and Aim 2 are completed and published as McKinstry et al. (2002) Cancer Biology and Therapy 1, 243-253 (please also see appendix).

Task 1. Treat mammary carcinoma cells (MDA-MB-231, MCF7, T47D) with either the MEK1/2 inhibitor PD98059 (0-50 μ M), the Chk1 inhibitor UCN-01 (0-200 nM) or both drugs in combination. Cells will also be exposed to ionizing radiation (2 Gy) or mock irradiated. The percentage of apoptotic cells 12-96h after treatment/irradiation will be determined by nuclear morphology and fluorescent staining of double stranded DNA breaks. After 96h, cells will be re-plated in the absence of drugs to determine effects of various treatments on clonogenic survival.

Task completed. We re-plated cells at 48h, rather than 96h, for colony formation assays (see appendix paper).

Task 2. In parallel with Task 1, the MEK1/2 inhibitors U0126 (0-20 μ M) or PD184352 (0-20 μ M) will be used in lieu of PD98059. The percentage of apoptotic cells 12-96h after treatment/irradiation will be determined and after 96h, cells will be replated in the absence of drugs for clonogenic survival assays.

Task completed. Due to a lack of availability of PD184352, we only performed apoptosis assays with this drug. U0126 enhances apoptosis and reduces clonogenic survival in a similar manner to PD98059 when combined with UCN-01.

Task 3. Data from Tasks 1 and 2 will permit us to determine the maximum level of apoptosis and loss of clonogenic survival achievable in our three carcinoma cell lines. We will have determined the optimal concentrations of MEK1/2 inhibitors and UCN-01, which combined with radiation, result in the most profound cell killing. Task 3 will determine how long do cells need to be treated with MEK1/2 inhibitor and UCN-01 to obtain maximal apoptosis. For example, if we find that maximal apoptosis with the drugs and radiation is observed 60h after treatment, does the inhibitor need to be present for 24h or for the full 60h? Cells will be washed free of the drugs at 12-72h after treatment and the percentage apoptosis 12-96h after treatment/irradiation will be determined by nuclear morphology and fluorescent staining of double stranded DNA breaks. Furthermore, because apoptosis and loss of clonogenic survival do not always correlate, after removal of drug at each time point, portions of the cells will be re-plated in the absence of drugs for clonogenic survival assays.

Task completed. We determined that the maximum apoptotic response occurs 24-30h after exposure to UCN-01+MEK1/2 inhibitor. We determined that UCN-01+MEK1/2 inhibitor promote cytochrome c release within 6h of treatment and that this is the key commitment point in the death response. We determined that UCN-01 concentrations of less than 150 nM were marginally toxic to carcinoma cells. We determined that concentrations of the MEK1/2 inhibitor of less than 25 μ M were marginally toxic to carcinoma cells.

Specific Aim 2.

Task 4. Treat breast cancer cells with either the MEK1/2 inhibitor PD98059 (0-50 μ M), the Chk1 inhibitor UCN-01 (0-200 nM) or both drugs in combination. Cells will also be exposed to ionizing radiation (2 Gy) or mock irradiated. Based on data obtained in Tasks 1-3, we will know the concentrations of MEK1/2 inhibitor and UCN-01, in combination with radiation exposure, which generate peak levels of apoptosis and loss of colony formation ability. Using these optimal drug concentrations, we will determine via immunoblotting the expression and activity of pro-caspase 8, pro-caspase 9 and pro-caspase 3, 12-96h after exposure. In addition, activation/cleavage of BID, the mitochondrial membrane permeability transition ($\Delta\psi_m$), cytosolic cytochrome c levels and expression of Bcl-2, Bcl-XL, Bax and BAD will also be determined. Since BAD function is modulated by phosphorylation, and phospho-specific antibodies are commercially available, we will also determine BAD phosphorylation on S112 and S136.

Task completed. Both caspase 8 and caspase 9 inhibitors are required to inhibit apoptosis induced by UCN-01+MEK1/2 inhibitor. Bax translocates to the mitochondrion after UCN-01+MEK1/2 inhibitor treatment and BAD phosphorylation is reduced at both S112 and S136. Mammary carcinoma cells express significantly greater amounts of Bcl-2 and Bcl-XL compared to primary cultures of human mammary epithelial cells.

Task 5. We will generate cell lines expressing either dominant negative caspase 8 or dominant negative caspase 9. We will generate cell lines over-expressing either the inhibitor of DISC/caspase 8 processing c-FLIP-s or the caspase 3 inhibitor XIAP.

Task in progress. We have generated cells to over express c-FLIP-s. Preliminary studies indicate they have identical responses to UCN-01+MEK1/2 inhibitor as parental cells.

Task 6. We will generate recombinant adenoviruses to express Bcl-2, Bcl-X_L, p21^{Cip-1/WAF1} or an antisense mRNA to p21^{Cip-1/WAF1} to assess the role of these apoptotic regulatory proteins on cell death in breast cancer cells following exposure to MEK1/2 inhibitors in combination with UCN-01 ± IR.

Task completed. Over-expression of Bcl-X_L, but not Bcl-2, protected cells from UCN-01+MEK1/2 inhibitor treatment. This is abrogated by either irradiation or treatment with the death receptor ligand TRAIL.

Task 7. and Task 8. We will determine whether dominant-negative caspase 8 or the caspase 8 inhibitor IETD block the potentiation of apoptosis by drugs and radiation to define the role of the extrinsic apoptotic pathway on cancer cell death induced by these agents. We will determine whether dominant-negative caspase 9 or the caspase 9 inhibitor LEHD block the potentiation of apoptosis by drugs and radiation. These and the preceding studies will test the hypothesis that inhibition of both the death receptor / caspase 8 and the mitochondrial / caspase 9 pathways are necessary to block the potentiation of apoptosis in cancer cells by MEK1/2 inhibitors/UCN-01 ± IR.

Task 50% completed. Both caspase 8 and caspase 9 inhibitors are required to inhibit apoptosis induced by UCN-01+MEK1/2 inhibitor. Cell lines have been generated expressing dominant negative caspase 9 or dominant negative caspase 8, and we are currently investigating whether either molecule inhibits the apoptotic response.

Task 9. We will determine whether over-expression of Bcl-2 or Bcl-X_L alters the potentiation of apoptosis. We will also determine whether over-expression of Bcl-2 or Bcl-X_L impacts on the mitochondrial membrane permeability transition ($\Delta\psi_m$) and cytosolic cytochrome c levels after exposure to MEK1/2 inhibitors, UCN-01 and radiation. If combined MEK1/2 inhibition and UCN-01 treatment kill tumor cells via a mitochondria / cytochrome c / caspase 9 -dependent mechanism, overexpression of Bcl-2 and Bcl-X_L should inhibit the apoptotic response.

Task completed. Over-expression of Bcl-X_L, but not Bcl-2, protected cells from UCN-01+MEK1/2 inhibitor treatment.

Task 10. We will determine whether loss of the cyclin-dependent kinase inhibitor p21 expression alters the sensitivity of tumor cells to the combination of drugs and radiation exposure. Inhibition of MAPK signaling reduces radiation-induced expression of p21. In squamous carcinoma cells we observed that reduced basal expression of p21 radiosensitizes these cells. Furthermore, UCN-01 enhances expression of p21 in mammary carcinoma cells which is reduced below basal levels in cells treated with both UCN-01 and MEK1/2 inhibitor.

Task completed. Loss of p21 expression, by use of a p21 antisense construct, did not significantly enhance the apoptotic response of cells. Loss of p21 by itself enhanced basal apoptosis levels and enhanced cell killing by either UCN-01 or MEK1/2 inhibitor. Thus while the combination of UCN-01+MEK1/2 inhibitor reduces p21 protein levels, this appears to be a secondary effect in the death of drug treated cells.

Task 11. In parallel with task 10, we will determine whether over-expression of p21 inhibits the apoptotic response of breast cancer cells. If loss of p21 can enhance the apoptotic response, causality of a role for p21 could be proven by over-expressing p21 and protecting cells. It should be noted that the relative ability of a tumor cell to enhance p21 expression is less than that of a non-transformed primary cell. This finding may explain why CD34 + stem cells, peripheral lymphocytes and primary hepatocytes were relatively insensitive to the pro-apoptotic actions of combined MEK1/2 inhibition and UCN-01 treatment.

Task has not been initiated at present.

Key Research Accomplishments

(i) UCN-01 activates the Raf/MEK/MAPK pathway in multiple tumor cell types.

(ii) Inhibition of Raf/MEK/MAPK activation following UCN-01 treatment enhances apoptosis within 18-24h.

- (iii) Inhibition of Raf/MEK/MAPK activation following UCN-01 treatment reduces the clonogenic survival of cells: note, these are cells that are viable at the time of re-plating for survival, having survived the initial exposure to UCN-01 + MEK1/2 inhibitor.
- (iv) Exposure of UCN-01 + MEK1/2 inhibitor treated cells to low dose ionizing radiation enhances apoptosis in some, but not all, tumor cell types. However, this exposure causes a greater than additive reduction in the colony forming ability of tumor cells.
- (v) The UCN-01 + MEK1/2 inhibitor drug combination causes apoptosis by promoting the release of cytochrome c from the mitochondrion, that utilizes caspase 9, and a caspase 3-caspase 8 activation loop to promote high levels of effector caspase 3 activation, and apoptosis.
- (vi) Over-expression of Bcl-X_L to supra-physiological levels protects cells from UCN-01 + MEK1/2 inhibitor – induced apoptosis. The protective effect is either reduced by irradiation of cells or abrogated by treatment of cells with the clinically relevant death receptor ligand TRAIL.

Reportable Outcomes.

Manuscripts

- (i) Qiao, L., Yacoub, A., McKinstry, R., Park, J.S., Caron, R., Fisher, P.B., Hagan, M.P., Grant, S., and Dent, P. Pharmacologic Inhibitors of the Mitogen Activated Protein Kinase Cascade Interact With Ionizing Radiation Exposure to Induce Cell Death in Carcinoma Cells By Multiple Mechanisms *Cancer Biology and Therapy* (2002) **1**, 168-176.
- (ii) McKinstry, R., Qiao, L., Yacoub, A., Dai, Y., Decker, R., Holt, S., Hagan, M.P., Grant, S., and Dent, P. Inhibitors of MEK1/2 Interact with UCN-01 to Induce Apoptosis and Reduce Colony Formation in Mammary and Prostate Carcinoma Cells *Cancer Biology and Therapy* (2002) **1**, 243-256.
- (iii) Cartee L., Sankala H., Davis C., Smith R., Maggio S., Lin P.S., Dent P., and Grant S. 7-Hydroxystaurosporine (UCN-01) and ionizing radiation combine to inhibit the growth of Bcl-2-overexpressing U937 leukemia cells through a non-apoptotic mechanism. *Int J Oncol* (2002) **21**, 351-359.
- (iv) Dai, Y., Landowski, T.H., Rosen, S.T., Dent, P., and Grant, S. Combined treatment with the checkpoint abrogator UCN-01 and MEK1/2 inhibitors potently induces apoptosis in drug-sensitive and -resistant myeloma cells through an IL-6-independent mechanism *Blood First Edition Paper*, pre-published online July 5, 2002; DOI 10.1182/Blood-2002-03-0940.

Abstracts.

AACR 2001 meeting, San Francisco.

- (i) Poster Session - 3447 - Pharmacologic Inhibitors of the MEK/MAPK Pathway Dramatically Potentiate UCN-01-induced Apoptosis in Diverse Leukemia Cell Lines - Yun Dai, Chun-Rong Yu, Paul Dent, Steven Grant.
- (ii) Poster Session - 3616 - Inhibition of MAPK Signaling Combined with Chk1 Inhibition Increases Cell Killing in Human Carcinoma Cells. - Paul Dent, Robert McKinstry, Liang Qiao, Yun Dai, Steven Grant.
- (iii) Era of Hope Meeting, Orlando FL September 2002.

Oral Presentation "PHARMACOLOGIC INHIBITORS OF THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) CASCADE INTERACT SYNERGISTICALLY WITH UCN-01 AND IONIZING RADIATION TO INDUCE APOPTOSIS AND REDUCE COLONY FORMATION IN MAMMARY AND PROSTATE CARCINOMA CELLS."

Invited Lecture Presentations.

- (i) Invited Speaker 4/2002 Massachusetts General Hospital / Harvard Medical School; Department of Radiation Oncology "Regulation of carcinoma and hepatocyte cell survival via the TGF alpha / EGFR / MAPK module".
- (ii) Invited Speaker 4/2002 Radiation Research Society 2002 annual meeting Reno NV. Refresher course "Regulation of signaling and survival by radiation and other stresses".
- (iii) Invited Speaker 11/2001 Virginia Polytechnic Institute; Blacksburg VA. Department of Biology "Regulation of carcinoma and hepatocyte cell survival via the TGF alpha / EGFR / MAPK module".
- (iv) Invited Speaker 11/2001 Virginia Commonwealth University; Department of Pharmacology and Toxicology "Regulation of carcinoma and hepatocyte cell survival via the TGF alpha / EGFR / MAPK

module”.

(v) Invited Speaker 10/2001 Mayo Clinic, Rochester MN Department of Pathology; “Regulation of carcinoma and hepatocyte cell survival via the TGF alpha / EGFR / MAPK module”.

(vi) Invited Speaker 10/2001 Virginia Commonwealth University; Department of Anatomy “Regulation of carcinoma and hepatocyte cell survival via the TGF alpha / EGFR / MAPK module”.

(vii) Invited Speaker 9/2001 Virginia Commonwealth University; Department of Cardiology “Regulation of carcinoma and hepatocyte cell survival via the TGF alpha / EGFR / MAPK module”.

Student training.

Robert McKinstry M.S. 2000-2001

Committees / Service.

(i) Permanent Member National Institutes of Health / National Cancer Institute Experimental Therapeutics 1 study section 2002.

(ii) Ad-Hoc member, National Institutes of Health / National Cancer Institute Radiation Research study section; February 2002.

(iii) Ad-Hoc member, and invited to become permanent member Veterans Administration Merit Review Committee for Oncology, 2002.

New Grant Applications.

Applied for Department of Defense Idea Award application (2002 cycle). “UCN-01 and MEK1/2 inhibitors synergize to reduce tumor growth in vivo.”

Conclusions

Main Points: The present studies have demonstrated that UCN-01 activates the MAPK pathway in multiple mammary carcinoma cell types, and that when pathway activation is inhibited using MEK1/2 inhibitors, apoptosis occurs. This also correlates with a reduction in clonogenic survival. Similar data have been obtained in leukemia, lymphoma, myeloma, prostate, pancreatic, hepatoma and glioma tumor cell types. When carcinoma cells treated with UCN-01 + MEK1/2 inhibitor are irradiated, and additional greater than additive loss in colony formation ability is observed. Supra-physiological over-expression of Bcl-XL can protect cells from the UCN-01 + MEK1/2 inhibitor drug combination, that is reduced by irradiation or by exposure of cells to a death receptor ligand under Phase I trial investigation, TRAIL. Of particular note, normal human mammary carcinoma cells, primary human and rodent hepatocytes, peripheral human lymphocytes and human CD 34+ stem cells are not killed by the drug combination.

So what?: The UCN-01 + MEK1/2 inhibitor drug combination selectively kills a broad range of tumor cells, but not non-transformed cells. Cell killing can be enhanced following irradiation. Collectively, these findings argue that if pre-clinic animal studies were to be performed showing UCN-01 + MEK1/2 inhibitor effects on tumor growth in vivo, it would be reasonable to propose Phase I clinic trials using this drug combination.

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Appendicies

- (i) Qiao, L., Yacoub, A., McKinstry, R., Park, J.S., Caron, R., Fisher, P.B., Hagan, M.P., Grant, S., and Dent, P. Pharmacologic Inhibitors of the Mitogen Activated Protein Kinase Cascade Interact With Ionizing Radiation Exposure to Induce Cell Death in Carcinoma Cells By Multiple Mechanisms *Cancer Biology and Therapy* (2002) **1**, 168-176.
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Pharmacologic Inhibitors of the Mitogen Activated Protein Kinase Cascade Interact With Ionizing Radiation Exposure to Induce Cell Death in Carcinoma Cells by Multiple Mechanisms

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Abstract

Recent studies have shown that inhibition of stress-induced signaling via the mitogen activated protein kinase (MAPK) pathway can potentiate the toxic effects of chemotherapeutic drugs and ionizing radiation. Because of these observations, we have further investigated the impact upon growth and survival of mammary (MDA-MB-231, MCF7, T47D), prostate (DU145, LNCaP, PC3) and squamous (A431) carcinoma cells following irradiation and combined long-term exposure to MEK1/2 inhibitors. Exposure of carcinoma cells to ionizing radiation resulted in MAPK pathway activation initially (0-4h) and modestly enhanced MAPK activity at later times (24h-96h). Inhibition of radiation-induced MAPK activation using MEK1/2 inhibitors potentiated radiation-induced apoptosis in two waves, at 21-30h and 96-144h after exposure. The potentiation of apoptosis was not observed in MCF7, LNCaP, or PC3 cells. At 24h, the potentiation of apoptosis was independent of radiation dose whereas at 108h, apoptosis correlated with increasing dose. Removal of the MEK1/2 inhibitor either 6h or 12h after exposure abolished the potentiation of apoptosis at 24h. At this time, the potentiation of apoptosis correlated with cleavage of pro-caspases -8, -9 and -3, and with release of cytochrome c into the cytosol. Inhibition of caspase function using a pan-caspase inhibitor ZVAD blocked the enhanced apoptotic response at 24h but did not block cytochrome c release. Selective inhibition of caspase 9 with LEHD or caspase 8 with IETD partially blunted the apoptotic response in MDA-MB-231, DU145 and A431 cells, whereas inhibition of both caspases reduced the response by > 90%. Removal of the MEK1/2 inhibitor either 24h or 48h after exposure abolished the potentiation of apoptosis at 108h. Incubation of cells with ZVAD for 108h also abolished the potentiation of apoptosis. In general agreement with the finding that prolonged inhibition of MEK1/2 was required to enhance radiation-induced apoptosis at 108h, omission of MEK1/2 inhibitor from the culture media during assessment of clonogenic survival resulted in either little or no significant alteration in radiosensitivity. Collectively, our data show that combined exposure to radiation and MEK1/2 inhibitors can reduce survival in some, but not all, tumor cell types. Prolonged blunting of MAPK pathway function following radiation exposure is required for MEK1/2 inhibitors to have any effect on carcinoma cell radiosensitivity.

Introduction

Mammary and prostate carcinomas are two of the leading causes of death for American women and men, respectively. A variety of modalities including surgery, chemotherapy and ionizing radiation have been used to treat these diseases with variable success at preventing re-occurrence. *In vitro*, carcinoma cells are often found to be relatively resistant to the toxic effects of ionizing radiation and thus further investigations designed to manipulate carcinoma cell survival are warranted.

The mechanisms by which radiation can either increase cell death or alter the proliferative rate of surviving cells are not fully understood. Recently, radiation has been shown to activate multiple signaling pathways within cells which can alter cell survival or proliferation depending upon the radiation dose, the cell type, and the culture conditions.¹⁻⁴ Several groups have shown that the epidermal growth factor receptor (EGFR) is activated in response to irradiation of carcinoma cells.^{1,5-8} Radiation exposure, via activation of the EGFR, can activate the Mitogen Activated Protein kinase (MAPK) pathway to a level similar to that observed by physiologic, growth stimulatory, EGF concentrations.^{5,6,9-11} The ability of the "classical" mitogen activated protein kinase (MAPK) pathway to regulate proliferation *versus* differentiation and survival appears to depend upon the cell type examined, as

well as upon the amplitude and duration of MAPK activation. A short activation of the MAPK cascade by growth factors has been correlated with increased proliferation, *via* both increased Cyclin D1 expression and an increased ability to progress through the G1-S transition.¹² In contrast, prolonged elevation of MAPK activity has been demonstrated to inhibit DNA synthesis, *via* super-induction of the cyclin dependent kinase inhibitor proteins p16 and p21.¹³ In addition to a role for MAPK signaling during G1/S phase, it has also been argued that MAPK signaling is involved in the ability of cells to progress through G2/M phase, particularly in cells following drug-induced growth arrest.^{14,15}

The proliferation of many carcinoma cells *in vitro* and *in vivo* is in part regulated by the synthesis and autocrine actions of ligands such as transforming growth factor α (TGF α).^{8,11} Irradiation of tumor cells can increase expression of TGF α , and this has been proposed to increase the proliferative rate of surviving cells.^{16,17} Increased proliferative rates and poor prognosis of carcinomas *in vivo* are also correlated with increased expression of the EGFR.¹⁸ MAPK signaling has also been linked to increased expression of other mitogens such as VEGF.¹⁹⁻²¹ Growth factors such as VEGF and TGF α , in addition to a growth promoting role *in vitro*, may also play an important role in the development of tumors *in vivo* due to their abilities in the promotion of angiogenesis. Collectively, these findings argue that radiation may have a self-limiting effect on its toxicity *via* increased autocrine growth factor function and the activity of downstream signaling modules, including the MAPK pathway.

We and others have recently demonstrated that increased signaling by the EGFR and the MAPK pathway can be cytoprotective versus ionizing radiation and various cytotoxic drugs in diverse cancer cell lines.^{1,8,10,11,22} Of note, several other groups have not observed MAPK signaling to be protective, which in part may be explained by both cell line differences and the fact that the ability of MAPK inhibition to sensitize DU145 cells in colony formation assays required a prolonged > 24h blunting of MAPK function.^{11,23-25} Despite the interest in MEK1/2 inhibitors as anti-neoplastic agents by promoting growth arrest, the mechanisms by which they can induce cell death after irradiation remain incompletely understood. The studies in this report were designed to determine the mechanisms by which MEK1/2 inhibitors enhance radiosensitivity in carcinoma cells.

Materials and Methods

Materials

Agarose conjugated anti-p42^{MAPK} antibody (sc-154-AC) was from Santa Cruz Biotechnology (Santa Cruz Biotechnologies, CA). Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (1:1000, rabbit polyclonal, NEB, Beverly, MA), anti-human Bcl-2 (1:2000, mouse monoclonal, Dako, Carpinteria, CA), Bax (N-20, 1:2000, rabbit polyclonal, Santa Cruz Biotechnology Inc.), Bcl-XS/L (S-18, 1:500, rabbit polyclonal, Santa Cruz Biotechnology Inc.), anti-human/mouse XIAP (1:500, rabbit polyclonal, R&D System, Minneapolis, MN), anti-cytochrome c (1:500, mouse monoclonal, Pharmingen), anti-caspase-3 (1:1000, rabbit polyclonal, Pharmingen), cleaved-caspase-3 (17kDa) antibody (1:1000, rabbit polyclonal, Cell Signaling Technology), anti-caspase-9 (1:1000, rabbit polyclonal, Pharmingen), anti-caspase-8 (1:1000, rabbit polyclonal, Pharmingen), anti-PARP (1:2500, mouse monoclonal, Calbiochem), and cleaved PARP (89kDa) antibody (1:1000, rabbit polyclonal, Cell Signaling Technology).

Radiolabelled [γ -³²P]-ATP was from NEN. Selective MEK1/2 inhibitors (PD184352, PD98059, and UO126) were supplied by Calbiochem (San Diego, CA) as powder, dissolved in sterile DMSO, and stored frozen under light-protected conditions at -20 °C. In all experiments, the final concentration of DMSO did not exceed 0.1%. The pan-caspase inhibitor (Z-VAD-FMK), the caspase 8 inhibitor (Z-IETD-FMK), and the caspase 9 inhibitor (Z-LEHD-FMK) were purchased from Enzyme System Products (Livermore, CA), dissolved in DMSO and stored at 4 °C. Western immunoblotting was performed using the Amersham Enhanced Chemi-Luminescence (E.C.L.) system. Other reagents were as in refs. 8 and 26.

Methods

Culture of Carcinoma Cells

Asynchronous cells (MCF7; MDA-MB-231; PC3; LNCaP; T47D; DU145; A431) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% (v/v) fetal calf serum at 37 °C in 95% (v/v) air / 5% (v/v) CO₂. Cells were plated at a density 3.2 x 10⁴ cells / cm² plate area and grown for 24-36h prior to further experimentation.

Exposure of Cells to Ionizing Radiation and Cell Homogenization

Cells were cultured in DMEM + 5% (v/v) fetal calf serum as above. UO126 / PD98059 / PD184352 treatment was from a 100 mM stock solution and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v). Cells were irradiated to a total of 2 Gy using a ⁶⁰Co source at dose rate of 2.1 Gy/min. Cells were maintained at 37 °C throughout the experiment except during the ~ 1 min irradiation itself. Zero time is designated as the time point at which exposure to radiation ceased. After radiation-treatment cells were incubated for specified times followed by aspiration of media and snap freezing at -70 °C on dry ice. Cells were homogenized in 1 ml ice cold buffer A [25 mM β -glycerophosphate, pH 7.4 at 4°C, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 1 mM phenylmethyl sulphonylfluoride, 1 mg/ml soybean trypsin inhibitor, 40 μ g/ml pepstatin A, 1 μ M Microcystin-LR, 0.5 mM sodium orthovanadate, 0.5 mM sodium pyrophosphate, 0.05 % (w/v) sodium deoxycholate, 1 % (v/v) Triton X100, 0.1 % (v/v) 2-mercaptoethanol], with trituration using a P1000 pipette to lyse the cells. Homogenates were stored on ice prior to clarification by centrifugation (4 °C).

Immunoprecipitations from Lysates

Fifty microliters of Protein A agarose (Ag) slurry (25 μ l bead volume) was washed twice with 1 ml PBS containing 0.1 % (v/v) Tween 20, and resuspended in 0.1 ml of the same buffer. Antibodies (2 μ g, 20 μ l), serum (20 μ l) were added to each tube and incubated (3h, 4°C). For pre-conjugated antibodies, 10 μ l of slurry (4 μ g antibody) was used. Clarified equal aliquots of lysates (0.25 ml, ~100 μ g total protein) were mixed with Ag-conjugated antibodies in duplicate using gentle agitation (2.5h, 4°C). Ag-antibody-antigen complexes were recovered by centrifugation, the supernatant discarded, and washed (10 min) sequentially with 0.5 ml buffer A (twice), PBS and buffer B [25 mM Hepes, pH 7.4, 0.1 mM Na₃VO₄].

Assay of p42^{MAPK} Activity

Immunoprecipitates were incubated (final volume 50 μ l) with 50 μ l of buffer B containing 0.2 mM [γ -³²P]ATP (5000 cpm/pmol), 1 μ M Microcystin-LR, 0.5 mg/ml myelin basic protein (MBP), which initiated reactions at time = 0. After 20 min, 40 μ l of the reaction mixtures were spotted onto a 2 cm circle of P81 paper (Whatman, Maidstone, England) and immediately placed into 180 mM phosphoric acid. Papers were washed four times (10 min each) with phosphoric acid, and once with acetone, and ³²P-incorporation into MBP was quantified by liquid scintillation spectroscopy.

SDS Poly-Acrylamide Gel Electrophoresis (SDS PAGE) and Western Blotting

Cells were irradiated and at specified time points / treatments media aspirated and the plates snap frozen. Cells were lysed with homogenization buffer and subjected to immunoprecipitation. Immunoprecipitates were solubilized with 100 μ l 5X SDS PAGE sample buffer (10% (w/v) SDS), diluted to 250 μ l with distilled water, and placed in a 100 °C dry bath for 15 min. One hundred microliter aliquots of each time point were subjected to SDS PAGE on 10% (w/v) acrylamide gels. Gels were transferred to nitrocellulose by the Method of Towbin and Western blotting using specific antibodies performed as indicated. Blots were developed using Enhanced Chemi-Luminescence (Amersham) using Fuji RX x-ray film. Blots were digitally scanned using Adobe Photoshop 5.5, their color removed, and Figures created in Microsoft PowerPoint.

Terminal Uridyl-Nucleotide End Labeling (TUNEL) for Apoptosis

Cells were treated with or without varying concentrations of U0126 / PD184352 / PD98059 / DMSO control 30 min prior to irradiation and irradiated (2 Gy). Cells were isolated 24h after irradiation by trypsinization followed by centrifugation onto glass slides (cytospin). Terminal Uridyl-Nucleotide End Labeling (TUNEL) was performed on these cells as described in refs 8, 26, 28. Randomly selected fields of fixed cells (~150 cells per field, n=5 per slide) were counted initially using propidium iodide counter stain, followed by examination and counting of TUNEL positive staining cells of the same field under FITC / fluorescence light.

Analysis of Cytosolic Cytochrome C

At various times after irradiation / MEK1/2 inhibitor treatment, 2×10^6 cells were washed in PBS and lysed by incubating for 30 seconds in lysis buffer (75mM NaCl, 8mM Na_2HPO_4 , 1mM NaH_2PO_4 , 1mM EDTA, and 350ug/ml digitonin). The lysates were centrifuged at 12,000g for 1 min, and supernatant was collected and added to equal volume of 2X sample buffer. The protein samples were quantified, separated by 15% SDS-PAGE, and subjected to immunoblot analysis as described above. Anti-cytochrome c (mouse monoclonal, Pharmingen) was used as primary antibody at a dilution of 1: 500.

Analysis of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

At various times after irradiation / MEK1/2 inhibitor treatment, 2×10^5 cells were incubated *in situ* with 40nM 3,3'-dihexyloxacarbocynine (DiOC6, Molecular Probes Inc. Eugene, OR) in PBS at 37°C for 20 min, isolated by trypsinization, and then analyzed by flow cytometry as described in refs 26 and 28. The percentage of cells exhibiting low level of DiOC6 up-take, which reflects loss of mitochondrial membrane potential, was determined using a Becton-Dickinson FACScan analyzer.

Colony Forming (Clonogenic) Assay

Cells were pre-treated with MEK1/2 inhibitor as indicated, 2h prior to exposure. Cells were irradiated (2 Gy). After 48h, cells were isolated by tryptic digestion and re-plated as single cell suspensions plated on Linbro ® plates at densities of 500 cells / well and 1000 cells / well. Colony formation was defined as a colony of 50 cells or greater, 10-14 days after plating.

Data Analysis

Comparison of the effects of treatments was done using one way analysis of variance and a two tailed t-test. Differences with a *p*-value of < 0.05 were considered statistically significant. Experiments shown, except where indicated, are the means of multiple individual points from multiple separate experiments ($\pm$ SEM). Statistical analyses were made using SigmaPlot and SigmaStat.

Results

Radiation Induces Activation of the MAPK Pathway in Carcinoma Cells

The ability of ionizing radiation to modulate MAPK activity was investigated in carcinoma cells (Figures 1 and 2). In the representative studies shown, radiation caused immediate primary activation of the MAPK pathway (0-10 min) followed by a later secondary activation (Figure 1A). Incubation of cells with a relatively specific inhibitor of MEK1/2/5 (PD98059), blunted the ability of radiation to activate MAPK (Figure 1A). In some cell types, e.g. LNCaP, radiation appeared to cause a slight reduction in MAPK activity (data not shown). Of note, basal MAPK activity varied greatly between the cell lines used in our study (Figure 1B). Subsequent studies examined radiation-induced MAPK activity 24h-120h after exposure (Figure 2). Radiation enhanced MAPK activity ~ 1.3 to 1.4-fold above basal levels 24h following exposure, which declined to control levels by 72h and remained at or near control levels until 120h after exposure. In contrast, radiation appeared to reduce MAPK activity in cells treated with a MEK1/2 inhibitor beyond that of MEK1/2 inhibitor alone, 48-120h after exposure (Figures 2A and 2B).

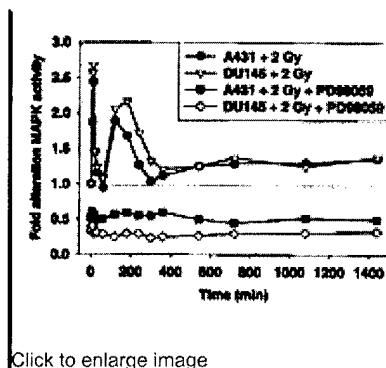


Figure 1A. Ionizing radiation activates the MAPK pathway in carcinoma cells, which is blocked by inhibitors of MEK1/2. Cells were cultured as described in Methods. A431 and DU145 cells.

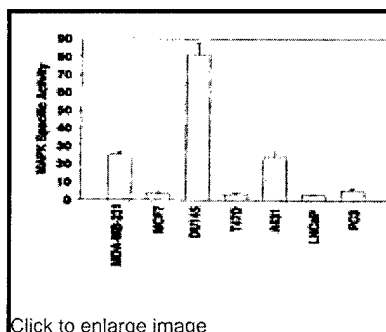


Figure 1B. Ionizing radiation activates the MAPK pathway in carcinoma cells, which is blocked by inhibitors of MEK1/2. Cells were cultured as described in Methods. Basal MAPK activity in MDA-MB-231, A431, DU145, MCF7, T47D, PC3 and LNCaP cells. Cells were pre-treated for 30 min with MEK1/2 inhibitor (PD98059; A431, 10 μ M; DU145 25 μ M) followed by irradiation (2 Gy) and MAPK activity determined over 0-1440 min as in Methods. Cells were lysed and portions (~100 μ g) from each plate used to immunoprecipitate MAPK followed by immune-complex kinase assays as in Methods. In Panel A, MAPK activity data are duplicate values from a single representative experiment and are shown as -fold increases in 32 P-incorporation into MBP substrate. In Panel B, data are shown as specific MAPK activity (fmol/min/mg) $\pm$ SEM (n=3).

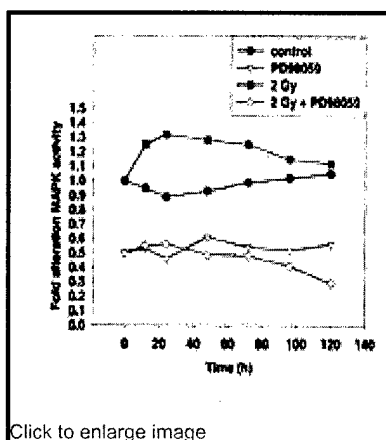


Figure 2A. MAPK pathway activity in carcinoma cells 24-120h after exposure to ionizing radiation. Cells were cultured as described in Methods. A431 cells.

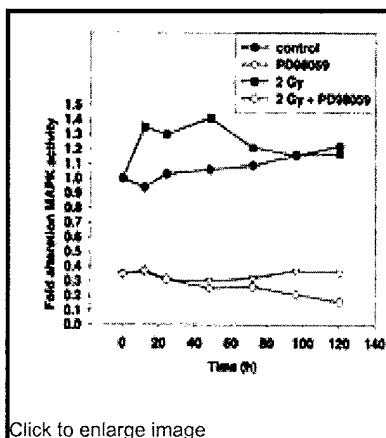
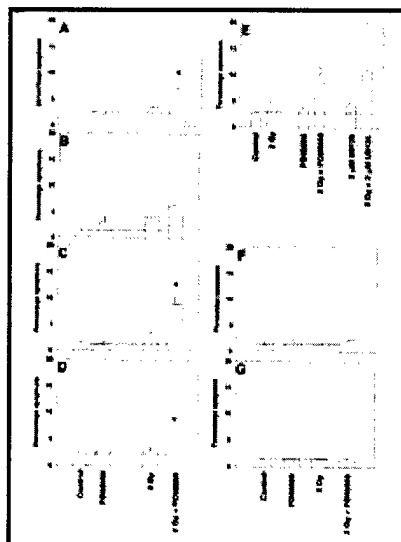


Figure 2B. MAPK pathway activity in carcinoma cells 24-120h after exposure to ionizing radiation. Cells were cultured as described in Methods. DU145 cells. Cells were pre-treated for 30 min with MEK1/2 inhibitor (PD98059, 10 μ M A431; 25 μ M DU145) followed by irradiation (2 Gy) and MAPK activity determined over 24h-120h as in Methods. Cells were lysed and portions (~100 μ g) from each plate used to immunoprecipitate MAPK followed by immune-complex kinase assays as in Methods. MAPK activity data are duplicate values from a single representative experiment (n=3) and are shown as -fold increases in 32 P-incorporation into MBP substrate.

Inhibition of Radiation-Induced MAPK Signaling Enhances Radiation-Induced Apoptosis at 24h

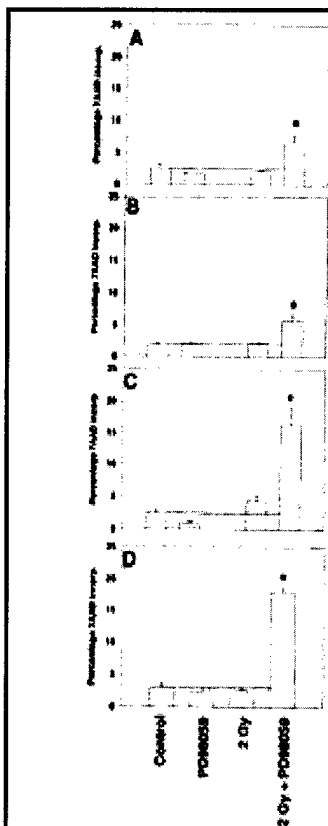
The impact of MEK1/2 inhibitors on radiation-induced apoptosis was initially determined 24h after treatment / exposure (Figures 3A-3G). Inhibition of radiation-induced MAPK signaling had a variable effect on the levels of apoptosis observed 24h after exposure: for example, MCF7 and PC3 cells exhibited no potentiation in apoptotic cell killing whereas A431 cells showed a greater sensitivity to MEK1/2 inhibitors alone as well as a larger significant enhancement in double stranded DNA breaks by

radiation. In the majority of cell lines examined, with the exception of T47D, the basal activity of ERK1/2 correlated with the ability of MEK1/2 inhibitors to enhance apoptosis (compare Figure 1B to Figure 3). Similar data were evident when plasma membrane integrity and staining of DNA was assessed using 7AAD (Figures 4A-4D). Removal of the MEK1/2 inhibitor either 6h or 12h after irradiation abolished the potentiation of apoptosis at 24h (Figure 5A), and increasing the radiation dose did not enhance the potentiation of apoptosis by MEK1/2 inhibitors at this time (Figure 5B).



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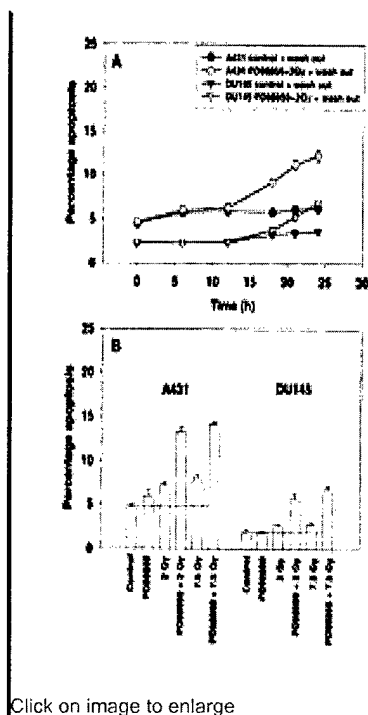
Figure 3. Combined exposure of carcinoma cells to ionizing radiation and MEK1/2 inhibitors causes apoptosis. Cells were incubated with matched vehicle control (DMSO) or with either 10 μ M PD98059 (A431) or 25 μ M PD98059 (MDA-MB-231, MCF7, T47D, DU145, LNCaP) alone. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. **Panel A.** MDA-MB-231 cells. **Panel B.** MCF7 cells. **Panel C.** DU145 cells. **Panel D.** T47D cells. **Panel E.** A431 cells. **Panel F.** LNCaP cells. **Panel G.** PC3 cells. Data shown are the mean number of staining cells from randomly selected fields of fixed cells ($n=5$ per slide, 3 parallel individual experiments $\pm$ SEM), which were examined and counted via fluorescent light microscopy. * $p < 0.05$ greater than corresponding value in unirradiated cells.



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Figure 4. Combined exposure of carcinoma cells to ionizing radiation and MEK1/2 inhibitors increases cell death as judged by 7AAD staining. Cells were incubated with matched vehicle control (DMSO) or with either 10 μ M PD98059 (A431) or 25 μ M PD98059 (MDA-MB-231, T47D, DU145) alone. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and incubated with 7AAD (10 mg/ml final) for 30 min at 37 $^{\circ}$ C as in Methods. Cells were subjected to flow cytometric analysis to determine the percentage of cells incorporating 7AAD. **Panel A.** MDA-MB-231 cells. **Panel B.** DU145 cells. **Panel C.** A431 cells. **Panel D.** T47D cells. Data shown are the mean percentage of staining cells (3 parallel individual experiments $\pm$ SEM). * $p < 0.05$ greater than corresponding value in unirradiated cells.

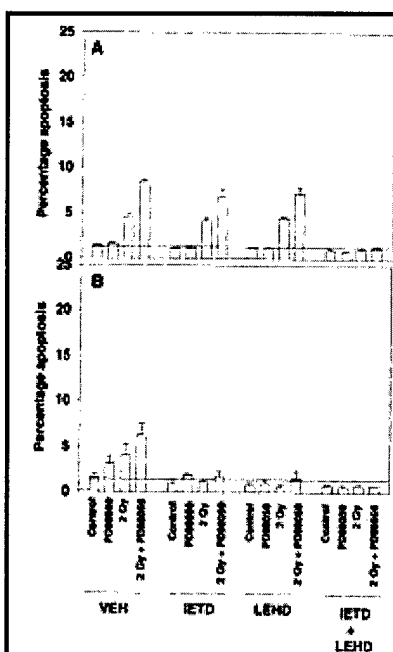
Figure 5. Combined exposure of A431 and DU145 carcinoma cells to ionizing radiation and MEK1/2 inhibitors causes apoptosis which requires >12h of



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incubation with MEK1/2 inhibitor. Cells were incubated with matched vehicle control (DMSO) or with either 10 μ M PD98059 (A431) or 25 μ M PD98059 (DU145) alone. Cells were either exposed to radiation (2 Gy, 7.5 Gy) or mock irradiated. Cells were either washed 6h, 12h, 18h and 21h following exposure with media containing either vehicle or PD98059. All cells were taken 24 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. **Panel A.** Cells with MEK1/2 inhibitor removed at 6h, 12h, 18h, and 21h after exposure. **Panel B.** Cells without MEK1/2 inhibitor removed irradiated with either 2 Gy or 7.5 Gy. Percentage apoptosis determined at 24h. Data shown are the mean number of staining cells from randomly selected fields of fixed cells ($n=5$ per slide, 3 parallel individual experiments $\pm$ SEM) which were examined and counted via fluorescent light microscopy. * $p < 0.05$ greater than irradiated value.

Simplistically, apoptosis is mediated via two inter-related cascades of proteases (caspases); the intrinsic mitochondrial (caspase 9) pathway and the extrinsic death receptor (caspase 8) pathway. Caspase 3 can also mediate additional activation of caspase 9 via a feed-forward amplification loop involving it in caspase 8 processing, leading to BID cleavage and further cytochrome c release from the mitochondrion. We found that the potentiation of radiation-induced apoptosis at 24h was blocked by the pan-caspase inhibitor ZVAD and in MDA-MB-231, DU145 and A431 cells partially blunted by individual additions of either the caspase 8 inhibitor IETD or the caspase 9 inhibitor LEHD (Figures 6A-6E). Combined use of both IETD and LEHD was required to completely prevent the apoptotic response in these cells. In contrast, IETD completely blocked the apoptotic response in T47D cells. In agreement with data arguing that both caspase 8 and caspase 9 played roles in the apoptotic process, enhanced apoptosis (Figures 4 and 6) correlated with cleavage and reduced expression of pro-caspase 8, pro-caspase 9 and pro-caspase 3 in A431 and DU145 cells (Figures 7A and 7B). Of note, in A431 cells, significant amounts of "active" p36 caspase 9 were observed in control cells. Combined exposure to radiation and MEK1/2 inhibitors also promoted release of cytochrome c from the mitochondria into the cytoplasm but surprisingly at this time did not alter the mitochondrial membrane potential (Figures 7C). Loss of the mitochondrial membrane potential was variably observed within the following 6-12h (data not shown).



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Figure 6A&B. The potentiation of apoptosis by combined irradiation and MEK1/2 inhibition requires both caspase 9 and caspase 8 functions. Cells were either treated with either vehicle, with IETD (20 μ M), with LEHD (20 μ M) or with both IETD and LEHD. In parallel, cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. **Panel A.** MDA-MB-231 cells. **Panel B.** MCF7 cells.

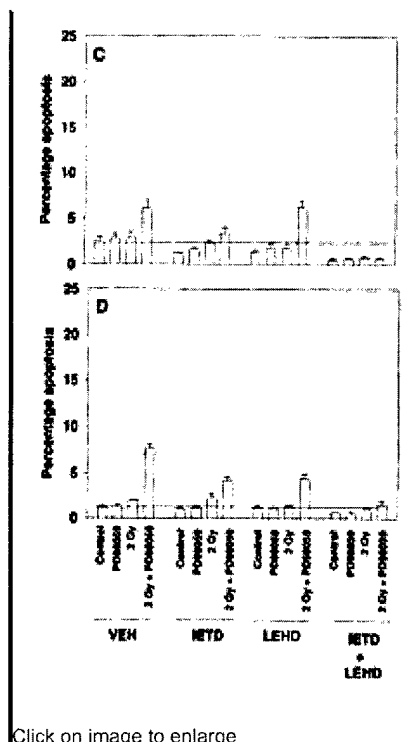


Figure 6C&D. The potentiation of apoptosis by combined irradiation and MEK1/2 inhibition requires both caspase 9 and caspase 8 functions. Cells were either treated with either vehicle, with IETD (20 μ M), with LEHD (20 μ M) or with both IETD and LEHD. In parallel, cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. T47D cells. **Panel D.** DU145 cells.

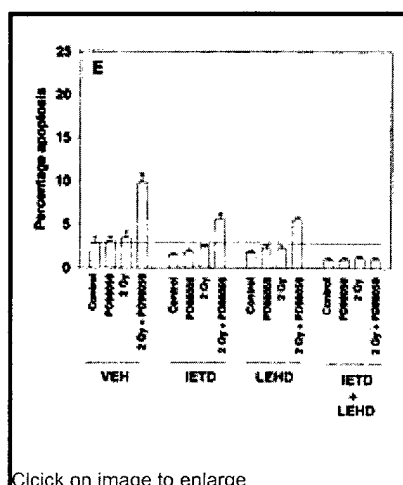


Figure 6E. The potentiation of apoptosis by combined irradiation and MEK1/2 inhibition requires both caspase 9 and caspase 8 functions. Cells were either treated with either vehicle, with IETD (20 μ M), with LEHD (20 μ M) or with both IETD and LEHD. In parallel, cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. T47D cells. A431 cells. Data shown are the mean number of staining cells from randomly selected fields of fixed cells (n=5 per slide, 3 parallel individual experiments $\pm$ SEM) which were examined and counted via fluorescent light microscopy. # $p < 0.05$ greater than corresponding value in unirradiated cells; * $p < 0.05$ greater than control value; % $p < 0.05$ less than corresponding value in cells not treated with caspase inhibitor. Cells treated with the pan-caspase inhibitor ZVAD (20 μ M) exhibited little or no apoptosis (data not shown).

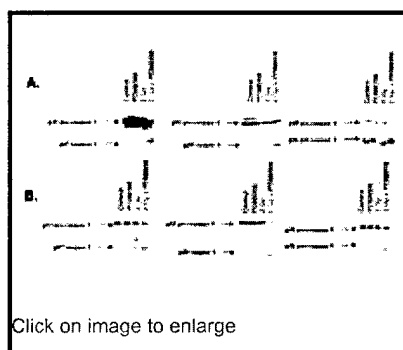
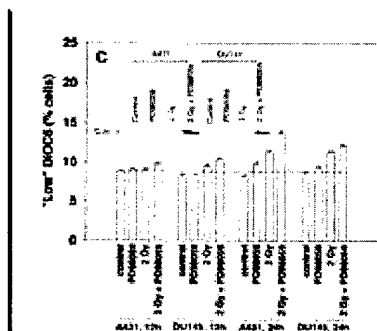


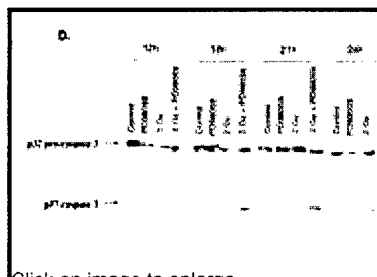
Figure 7A&B. Combined treatment of A431 and DU145 cells with ionizing radiation and MEK1/2 inhibitors enhances the cleavage of pro-caspase 3. Cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and lysed in SDS PAGE buffer containing bromophenol blue as described in Methods. Cells were subjected to SDS PAGE followed by immunoblotting to determine: **Panel A.** In A431 cells at 24h, the expression / integrity of pro-caspases -3, -8 and -9. **Panel B.** In DU145 cells at 24h, the expression / integrity of pro-caspases -3, -8 and -9. **Panel C.** Mitochondrial membrane potential in A431 and DU145 cells 24h after exposure: inset panel, cytosolic cytochrome c levels 24h after exposure. **Panel D.** A431 cells were irradiated and treated with 10 μ M PD98059, and the integrity of pro-caspase 3 determined after 12h, 18h, 21h and 24h after a 2 Gy exposure. Data shown are from a representative experiment (n=3).

Figure 7C. Combined treatment of A431 and DU145 cells with ionizing radiation and MEK1/2 inhibitors enhances the cleavage of pro-caspase 3. Cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells



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were taken 24 hours post irradiation and lysed in SDS PAGE buffer containing bromophenol blue as described in Methods. Cells were subjected to SDS PAGE followed by immunoblotting to determine: Mitochondrial membrane potential in A431 and DU145 cells 24h after exposure: inset panel, cytosolic cytochrome c levels 24h after exposure.



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Figure 7D. Combined treatment of A431 and DU145 cells with ionizing radiation and MEK1/2 inhibitors enhances the cleavage of pro-caspase 3.

Cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and lysed in SDS PAGE buffer containing bromophenol blue as described in Methods. Cells were subjected to SDS PAGE followed by immunoblotting to determine: A431 cells were irradiated and treated with 10 μ M PD98059, and the integrity of pro-caspase 3 determined after 12h, 18h, 21h and 24h after a 2 Gy exposure. Data shown are from a representative experiment (n=3).

Because removal of the MEK1/2 inhibitor at 12h abolished the potentiation of apoptosis at 24h (Figure 5), further studies were performed to examine the time course of pro-caspase cleavage. Reduced expression of p32 pro-caspase 3 and increased expression of p17 active caspase 3 was not observed 12h following combined radiation and MEK1/2 inhibitor exposure (Figure 7D), indicating that at this time cells were not undergoing apoptosis (in agreement with Figure 5A). However, at 18h, 21h and 24h after exposure loss of p32, and increased p17, expression was observed. The increase in p17 expression was greater in cells exposed to radiation and MEK1/2 inhibitor than to either radiation or MEK1/2 inhibitor alone. These findings suggest that MEK1/2 activity must be depressed for at least 18h to obtain a potentiation of radiation-induced apoptosis at 24h.

Inhibition of MAPK Signaling Enhances Radiation-Induced Apoptosis 96-144h After Exposure, Which is Dependent Upon Prolonged Inhibition of the MAPK Pathway

In a previous study, we had argued that inhibition of the MAPK pathway enhanced radiation-induced apoptosis in two waves, one at ~24h and a second ~96h after exposure. To further investigate these observations, we examined both the percentage apoptosis and the expression and integrity of pro-caspases in A431 and DU145 cells 24h-120h after irradiation (Figures 8 and 9). Sixty hours after exposure, apoptosis had returned to near basal levels under all conditions (data not shown). However, 108h after irradiation, the percentage of cells undergoing apoptosis in MEK1/2 inhibited and irradiated cells had again increased (Figure 8A). The potentiation of apoptosis at 108h appeared to correlate with increasing radiation dose (Figure 8B). Of note, removal of MEK1/2 inhibitor at either 24h or 48h after exposure abolished the increase in apoptosis at 108h (Figure 8A, data not shown). Removal at 72h, however, still permitted a limited apoptotic response from these cells (data not shown). These findings correlated with modulation of cellular radiosensitivity by MEK1/2 inhibitors in clonogenic survival assays (Table 1): in some cell types e.g. DU145 and A431, removal of the inhibitor 48h after exposure during re-plating abolished any impact of MEK1/2 inhibitors on clonogenic radiosensitivity. In contrast, other cell types e.g. MDA-MB-231 appeared to be radiosensitized even when the inhibitor was removed after 48h. In agreement with the finding that apoptosis had again increased at later times, prolonged inhibition of MEK1/2 enhanced the radiation-induced cleavage of pro-caspases 96-144h after exposure (Figures 9A and 9B).

Figure 8. Combined exposure of A431 and DU145 carcinoma cells to ionizing radiation and MEK1/2 inhibitors causes apoptosis 108h after exposure.

Cells were incubated with matched vehicle control (DMSO) or with PD98059 alone. Cells were either exposed to radiation or mock irradiated. Portions of cells were taken 24, 60 and 108 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. **Panel A.** A431 and DU145 cells irradiated with 2 Gy, either washed free of PD98059 at 48h or mock washed free at 48h. **Panel B.** A431 and DU145 cells at 108h irradiated with 7.5 Gy. Data shown are the mean number of staining

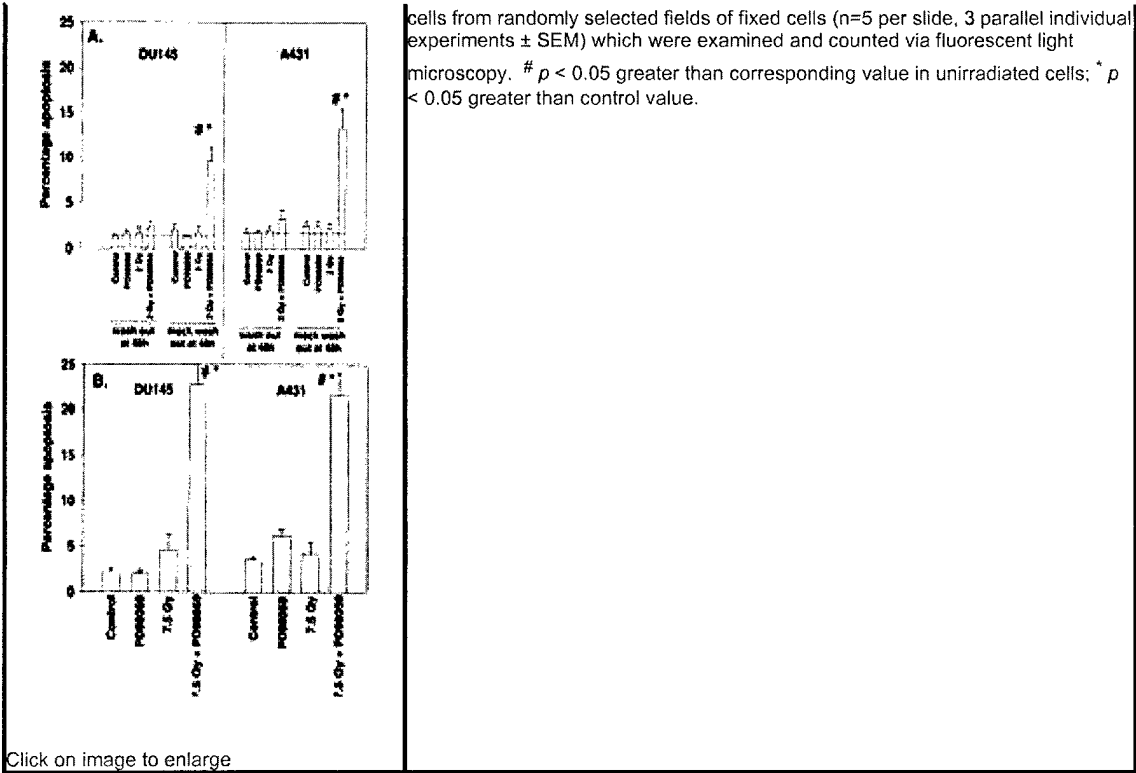


Table 1. Combined exposure of carcinoma cells to ionizing radiation and MEK1/2 inhibitors reduces clonogenic survival. Cells were incubated with matched vehicle control (DMSO), with 25 μ M PD98059 alone (DU145, MCF7, T47D, MDA-MB-231) or with 10 μ M PD98059 alone (A431). After 30 min, cells from each condition were either exposed to radiation (2 Gy) or mock irradiated. Identical portions of viable cells were taken 48 hours post irradiation and re-plated on Linbro plates at densities of 500 cells / well and 1000 cells / well. At this time cells were washed free, and re-plated in the absence of, MEK1/2 inhibitor. Cells were incubated for 10-14 days after which they were stained and colony number determined. A colony was defined as a cluster of 50 or more cells. MDA-MB-231 cells are referred to in the Table as MDA. Data are normalized to the plating efficiency of control-unirradiated cells, which is defined as 100% (Data shown are the number of staining colonies, 3-5 parallel individual experiments $\pm$ SEM), which were examined and counted via light microscopy. * p

Cell Type	Control	PD98059	2 Gy Control	2 Gy PD98059
DU 145	100 $\pm$ 1	99 $\pm$ 6	67 $\pm$ 3*	65 $\pm$ 2
MCF7	100 $\pm$ 1	91 $\pm$ 5	61 $\pm$ 4*	39 $\pm$ 3#
T47D	100 $\pm$ 4	97 $\pm$ 2	83 $\pm$ 3*	69 $\pm$ 5
A431	100 $\pm$ 2	93 $\pm$ 3	70 $\pm$ 2*	65 $\pm$ 3
MDA	100 $\pm$ 5	91 $\pm$ 1	63 $\pm$ 3*	44 $\pm$ 1#

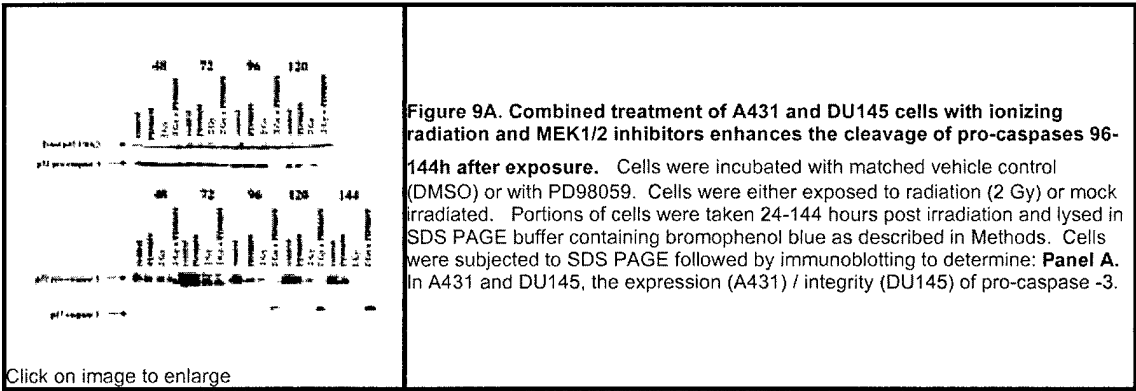


Figure 9A. Combined treatment of A431 and DU145 cells with ionizing radiation and MEK1/2 inhibitors enhances the cleavage of pro-caspases 96-144h after exposure. Cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24-144 hours post irradiation and lysed in SDS PAGE buffer containing bromophenol blue as described in Methods. Cells were subjected to SDS PAGE followed by immunoblotting to determine: **Panel A.** In A431 and DU145, the expression (A431) / integrity (DU145) of pro-caspase -3.

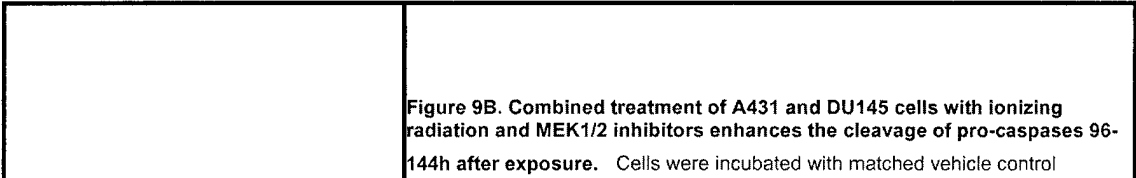
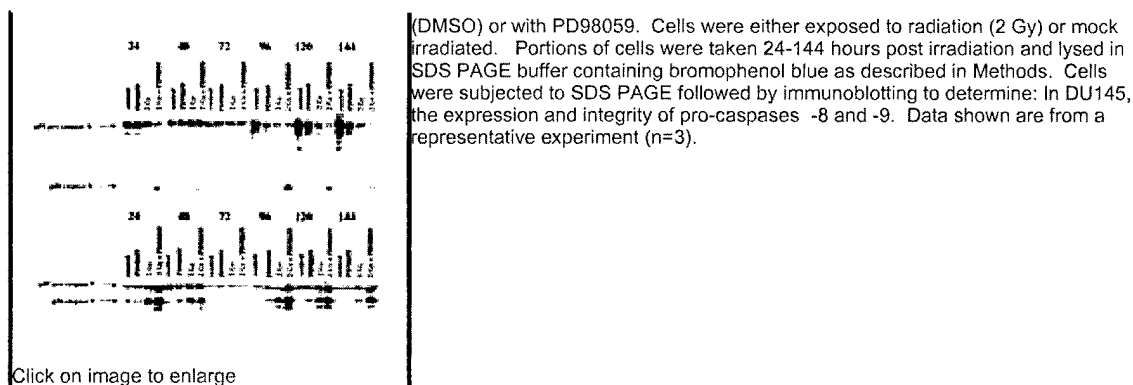
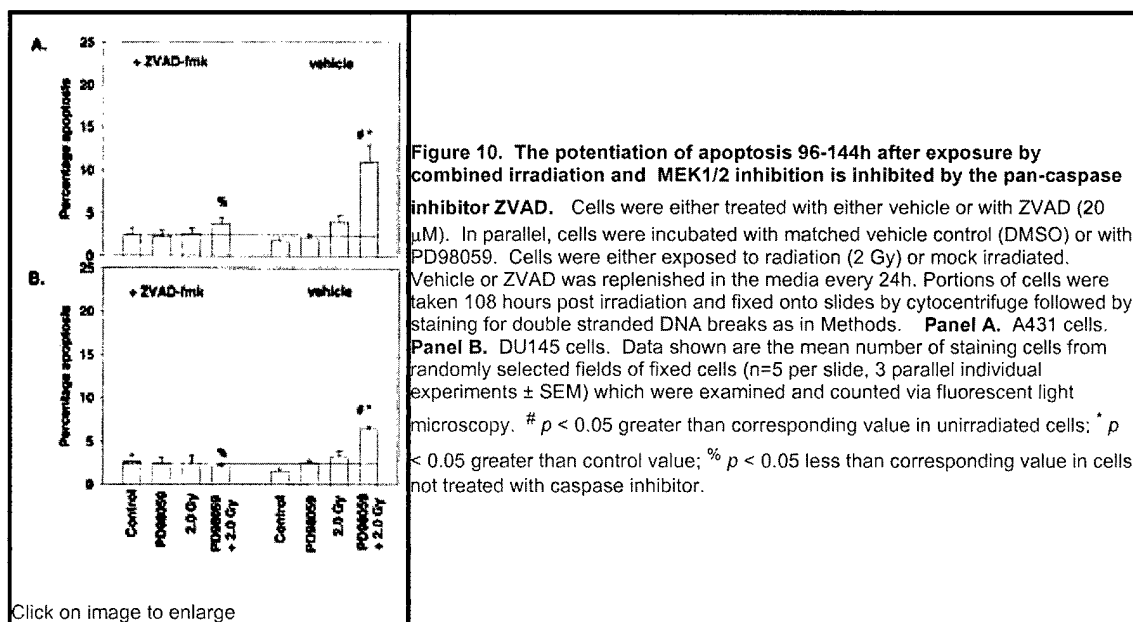


Figure 9B. Combined treatment of A431 and DU145 cells with ionizing radiation and MEK1/2 inhibitors enhances the cleavage of pro-caspases 96-144h after exposure. Cells were incubated with matched vehicle control



Because apoptosis and caspase cleavage were observed at later times, we investigated whether the pan-caspase inhibitor ZVAD could block this process and whether enhanced apoptosis played an important role in the previously reported enhancement in radiosensitivity caused by MAPK inhibition. Small molecule inhibitors of caspases are relatively unstable, and in all of the following experiments ZVAD was re-supplemented into the media every 24h. Prolonged exposure to ZVAD blocked the potentiation of apoptosis at later times (Figure 10) and also the cleavage of caspase 3 (data not shown). Collectively, these data argue that enhanced radiosensitivity caused by MEK1/2 inhibition is, in part, due to increased late phase apoptosis.



Discussion

Previous investigations by this group have demonstrated that low dose radiation exposure of carcinoma cells, in combination with inhibition of MEK1/2, causes a small but statistically significant increase in radiation-induced apoptosis within 24h. Prolonged inhibition of MEK1/2 caused a second wave of apoptosis 4-6 days after exposure in DU145 cells. Removal of the MEK1/2 inhibitor at 24h was found to abolish the second apoptosis wave and any impact upon long-term clonogenic survival. The studies described in this manuscript were designed to determine the mechanisms by which MEK1/2 inhibitors enhance apoptosis at both 24h and in the second wave at 108h.

We initially compared a variety of carcinoma cell types in their abilities to be killed by radiation in the presence or absence of a MEK1/2 inhibitor. Low dose radiation as a single agent was relatively ineffective at enhancing apoptosis in all of the cell types examined, including MCF7 and LNCaP that express wild type p53. In all cell types that had elevated basal MAPK activity greater than 10 fmol/min/mg (A431, MDA-MB-231, DU145), MEK1/2 inhibitors potentiated radiation-induced apoptosis to varying degrees. In cell types that had basal MAPK activity less than 10 fmol/min/mg (MCF7, PC3, LNCaP, T47D), with the exception of T47D, no potentiation of DNA strand breakage was observed using MEK1/2 inhibitors. Of note, serum starved cells also did not exhibit a potentiation of apoptosis (unpublished data). Within this group, it is also of note that LNCaP and PC3 cells do not express the inositol phospholipid phosphatase PTEN.²⁷ Loss of PTEN suggests these cells may utilize the PI 3-kinase signaling pathway, rather than the MAPK pathway, as a key growth and survival control signaling module. Collectively, these findings suggest that cells, which have elevated basal MAPK activity, are more responsive to MEK1/2 inhibitors as radiosensitizers than those with low basal MAPK activity.

Previously, we had discovered that removal of MEK1/2 inhibitor 6h following irradiation abolished the potentiation of apoptosis 24h afterwards. In the present study, we discovered that removal of the MEK1/2 inhibitor 12h-18h after exposure also abolished the potentiation of killing at 24h. Removal of the inhibitor at 18h had a partial blunting effect on the apoptotic response. These findings correlated with the cleavage of p32 pro-caspase 3: processing of pro-caspase 3 to p17 active caspase 3 was not observed until 18-21h after exposure. These findings suggest that prolonged blunting of MAPK signaling is required to enhance radiation-induced cell killing at 24h. The precise mechanisms that promote cell killing remain to be determined.

Two upstream pathways, the intrinsic and the extrinsic, mediate cleavage and activation of the effector pro-caspase 3. The intrinsic pathway utilizes the mitochondrion, cytochrome c and pro-caspase 9 and the extrinsic pathway that utilizes death receptors and pro-caspase 8. We found that combined exposure to radiation and MEK1/2 inhibitors caused release of cytochrome c from the mitochondria into the cytosol within 24h, but did not promote a large reduction in the mitochondrial membrane potential. While the apoptotic response was blocked by a pan-caspase inhibitor ZVAD, the release of cytochrome c in A431 and DU145 cells was independent of caspase function (Qiao, McKinstry and Dent, Unpublished results). These findings suggest that release of cytochrome c into the cytosol is a primary event in our apoptotic process. Induction of apoptosis, notably by chemotherapeutic drugs, has been linked to mitochondrial damage, including loss of the mitochondrial membrane potential or release of pro-apoptotic proteins from the mitochondria, such as cytochrome c. There is controversy regarding which of these events represents the central executioner of apoptosis, and the induction of apoptosis in both the presence or the absence of cytochrome c release has been described.²⁹ Release of cytochrome c is also often found to precede loss of the mitochondrial membrane potential, $\Delta\psi_m$.³⁰ The present findings suggest that cytochrome c release represents an important primary event in cells induced to undergo apoptosis when they are treated with MEK1/2 inhibitors and exposed to radiation.

The apoptotic responses of the various cell lines examined, surprisingly, differed with respect to inhibition of caspase 8 and caspase 9. In MDA-MB-231, A431 and DU145 cells, inhibitors of caspase 8 and caspase 9 individually had partial effects at blunting the apoptotic response. In MCF7 cells, that had little apoptotic response, either inhibitor abolished increases in cell death. In contrast, caspase 9 inhibitors had little impact on the apoptotic response of T47D mammary carcinoma cells whereas inhibitors of caspase 8 completely blocked apoptosis in these cells. These findings suggest that the mechanism by which MEK1/2 inhibitors potentiate cell killing, 24h after exposure, is cell-type specific. Caspase 9 inhibitors had no effect on the apoptotic response in T47D cells suggesting that MEK1/2 inhibitors are promoting death receptor signaling, as has previously been demonstrated by this group in primary hepatocytes treated with bile acids.²⁸

Previously, we have presented evidence that inhibition of MEK1/2 can radiosensitize cells when they are exposed repeatedly to low dose ionizing radiation. In these studies, cells were irradiated three times with 2 Gy per exposure and incubated with

MEK1/2 inhibitors for 96 hours prior to re-plating.⁸ In the present study we exposed cells to a single 2 Gy dose of radiation in the presence or absence of MEK1/2 inhibitor for 48h and examined apoptosis and clonogenic survival over the following week to fourteen days. Radiation, even several days after exposure, caused relatively little apoptosis in A431 and DU145 cells. However, the presence of MEK1/2 inhibitor, while by itself only marginally toxic, caused a significant enhancement in radiation-induced apoptosis. The pan-caspase inhibitor ZVAD blocked the increase in apoptosis. When the MEK1/2 inhibitor was removed 24h and 48h after irradiation, no enhancement in late phase apoptosis occurred, which correlated with negative data obtained when clonogenic survival was measured in the absence of this inhibitor. In contrast, when the MEK1/2 inhibitor was removed at 72h, a partial blunting effect on the potentiation of apoptosis at 108h became evident. Thus incubation of cells in the presence of a MEK1/2 inhibitor for ~72h, but not 48h, appears competent to produce a commitment for enhanced radiation-induced apoptosis at time periods distant to the initial exposure. These findings suggest that prolonged blunting of MAPK signaling is required to enhance radiation-induced cell killing at 108h. The precise mechanisms by which cell killing is promoted at this time point remain to be determined.

Surprisingly, in MDA-MB-231 and MCF7 cells, however, the MEK1/2 inhibitor PD98059 caused radio-sensitization in clonogenic survival assays, even though the drug was removed 48h after irradiation. The apoptotic responses of MCF7 cells at the 24h-48h time points were negligible, implying that in addition to potentiating radiation-induced apoptosis, MEK1/2 inhibition can also promote radiation-induced cell killing by other non-apoptotic mechanisms. Further studies will be required to determine: a) the mechanisms by which MEK1/2 inhibitors commit cells and then trigger an apoptotic response 18-24h after exposure, and b) the mechanisms by which MEK1/2 inhibitors commit cells and then trigger an apoptotic response 48-72h after irradiation.

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Research Article

Inhibitors of MEK1/2 Interact with UCN-01 to Induce Apoptosis and Reduce Colony Formation in Mammary and Prostate Carcinoma Cells

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KEY WORDS

UCN-01, MAPK, Apoptosis, Ionizing radiation, Clonogenic survival

ABBREVIATIONS

MAPK	mitogen activated protein kinase
UCN-01	7-hydroxy staurosporine

ABSTRACT

Recent studies have suggested that inhibition of the mitogen activated protein kinase (MAPK) pathway as well as abrogation of cell cycle check-point control can potentiate the lethal actions of chemotherapeutic drugs and radiation. We therefore investigated the impact of combined exposure to the check-point abrogator (UCN-01) in conjunction with MEK1/2 inhibitors upon survival of breast and prostate carcinoma cells. Treatment of cells with UCN-01 alone resulted in prolonged activation of the MAPK pathway. Inhibition of MEK1/2 caused modest reductions in basal MAPK activity and transiently suppressed UCN-01-stimulated MAPK activity below that of MEK1/2 inhibitor alone. Significantly, combined, but not individual, exposure of cells to UCN-01 and MEK1/2 inhibitors enhanced BAX association with mitochondria and triggered release of cytochrome c into the cytosol, accompanied by activation of effector pro-caspases, resulting in a greater than additive potentiation of apoptosis within 18-24h. Radiation exposure of drug treated cells did not further enhance apoptosis. Treatment of cells with both caspase 9 and caspase 8 inhibitors was required to completely inhibit apoptosis in carcinoma cells. Over-expression of Bcl-x_L blocked cytochrome c release and cell killing induced by the drug combination. Colony forming assays demonstrated that cells exposed to both agents exhibited a substantial reduction in clonogenic survival compared to either drug alone; moreover, radiation further reduced clonogenic survival despite failing to promote additional apoptosis. Collectively, these data demonstrate that combined exposure of carcinoma cells to UCN-01 and MEK1/2 inhibitors induces apoptosis and interacts with radiation to further reduce clonogenic survival.

INTRODUCTION

Mammary and prostate carcinomas represent two leading causes of death in The United States. A variety of therapeutic modalities, including surgery, chemotherapy, endocrine ablation, and ionizing radiation have been used in the treatment of these diseases with variable success. In vitro, breast and prostate carcinoma cells are often resistant to many commonly employed chemo- and radio-therapeutic strategies. In contrast to malignant hematopoietic cells, which are programmed to undergo cell death in response to multiple stimuli, the apoptotic machinery is often lacking or defective in epithelial carcinoma cells. In fact, it has been suggested that the relative resistance of epithelial carcinoma cells to apoptosis may account for or contribute to the poor response of such tumors to various therapeutic interventions.¹ Thus, further attempts enhance the susceptibility of epithelial carcinoma cells to apoptosis appear warranted.

UCN-01 (7-hydroxystaurosporine) is currently being evaluated as an anti-neoplastic agent in phase I clinical trials, both alone and in combination with chemotherapeutic agents and ionizing radiation. UCN-01 exerts anti-proliferative activity both in vitro and in vivo, an action which may be related to inhibition of protein kinase C (PKC) isoforms.² UCN-01 also enhances the cytotoxicity of chemotherapeutic agents by several postulated mechanisms, including inhibition of Chk1 catalytic activity.³ Inhibition of Chk1 may directly promote activation of the protein phosphatase Cdc25C.³ Down-regulation of Cdc25C activity in response to DNA damage results in enhanced phosphorylation and inactivation of cyclin-dependent kinases (Cdks) such as p34^{cdc2}, which are critically involved in G2/M cell cycle arrest.⁴ Thus, UCN-01 has been proposed to function as a G2/M checkpoint abrogator capable of enhancing the lethal actions of DNA-damaging agents, including ionizing radiation,⁵ cisplatin,⁶ mitomycin C,⁷ ara-C⁸ and camptothecins.⁹ It is thought that UCN-01, administered at pharmacologically achievable concentrations, promotes cell death via Cdk dephosphorylation rather than by inhibition of PKC enzymes.¹⁰

The ability of the "classical" mitogen activated protein kinase (MAPK) pathway to regulate proliferation versus differentiation and survival appears to depend upon the amplitude and duration of MAPK activation. A short activation of the MAPK cascade has been correlated with enhanced progression through the G1-S transition.¹¹ In contrast, prolonged elevation of MAPK activity can inhibit DNA synthesis through super-induction of the Cdk inhibitor protein p21^{Cip-1/WAF1/mda6} (p21).¹² In addition to a role for MAPK signaling in the G1/S phase transition, it has also been argued that MAPK signaling is involved in the ability of cells to progress through G2/M, particularly following drug- or radiation-induced growth arrest.^{13,14}

Recent evidence has suggested that inhibition of the MAPK pathway sensitizes a broad range of tumor cells to the toxic effects of ionizing radiation and cytotoxic drugs.¹⁵⁻¹⁷ In the former case, radiosensitization has been found to be dependent upon a prolongation of G2/M phase arrest.^{18,19} On the other hand, in hematopoietic cells exposed to cytotoxic drugs, it has been argued that MAPK activation plays an important cytoprotective role.^{1,20,21} Thus, both inhibition of MAPK signaling as well as UCN-01-mediated check-point abrogation may represent promising strategies for activation of the cell death pathway in neoplastic cells.

Despite the intense interest in UCN-01 as an anti-neoplastic agent, the mechanism(s) by which it induces cell death remain(s) incompletely understood, particularly the relationship between UCN-01-mediated lethality and activation of signal transduction pathways. To address these issues, we have examined the effects of a combined exposure to UCN-01 and pharmacologic MEK1/2 inhibitors in a variety of breast and prostate cancer cells. Our results indicate that when cells are exposed to UCN-01 in combination with MEK1/2 inhibitors, a striking increase in apoptosis occurs, accompanied by a greater than additive loss of clonogenic survival. In the majority of cells examined, exposure to ionizing radiation did not further stimulate apoptosis induced by the drug combination but did further reduce clonogenic survival.

MATERIALS AND METHODS

Materials. Agarose conjugated anti-p42^{MAPK} antibody (sc-154-AC) was from Santa Cruz Biotechnology (Santa Cruz Biotechnologies, CA). Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (1:1000, rabbit polyclonal, NEB, Beverly, MA), p44/42 MAP kinase antibody (1:1000, rabbit polyclonal, NEB), phospho-p38 MAP kinase (Thr180/Tyr182) antibody (1:1000, rabbit polyclonal, NEB), anti-phospho-CREB (1:1000, rabbit polyclonal, Upstate Biotechnology, Lake Placid, NY), phospho-cdc2 (Tyr15) antibody (1:1000, rabbit polyclonal, Cell Signaling Technology), anti-p21^{Cip/WAF1/mda6} (1:500, mouse monoclonal, Transduction Laboratories, Lexington, KY), anti-p27^{kip1} (1:500, mouse monoclonal, Pharmingen, San Diego, CA), anti-human Bcl-2 oncoprotein (1:2000, mouse monoclonal, Dako, Carpinteria, CA), Bax (N-20, 1:2000, rabbit polyclonal, Santa Cruz Biotechnology Inc.), Bcl-XS/L (S-18, 1:500, rabbit polyclonal, Santa Cruz Biotechnology Inc.), anti-human/mouse XIAP (1:500, rabbit polyclonal, R&D System, Minneapolis, MN), anti-cytochrome c (1:500, mouse monoclonal, Pharmingen), anti-caspase-3 (1:1000, rabbit polyclonal, Pharmingen), cleaved-caspase-3 (17kDa) antibody (1:1000, rabbit polyclonal, Cell Signaling Technology). Radiolabelled [γ -³²P]-ATP was from NEN. Selective MEK1/2 inhibitors (PD184352, PD98059, and U0126) were supplied by Calbiochem (San Diego, CA) as powder, dissolved in sterile DMSO, and stored frozen under light-protected conditions at -20°C. UCN-01 was kindly provided by Dr. Edward Sausville, Developmental Therapeutics Program/CTEP, NCI. It was dissolved in DMSO at a stock concentration of 1 mM, and stored at -20°C, and subsequently diluted with serum-free medium prior to use. In

all experiments, the final concentration of DMSO or chloroform did not exceed 0.1%. Caspase inhibitor (Z-VAD-FMK) and caspase 8 inhibitor (Z-IETD-FMK) were purchased from Enzyme System Products (Livermore, CA), dissolved in DMSO and stored at 4°C. Western immunoblotting was performed using the Amersham Enhanced Chemi-Luminescence (E.C.L.) system (Bucks, England). Other reagents were as in reference 18.

Methods

Generation of Primary Human Mammary Epithelial Cells. Primary human mammary epithelial cells were isolated from reduction mammoplasty and prepared as described.²²

Generation of Primary Human CD34⁺ Stem Cells. Cells were obtained from normal donor marrow. Cells were purified using an IsoplexTM 300i system utilizing anti-CD34⁺ antibody and Dynal beads as per manufacturers' instructions. For drug treatments, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% (v/v) fetal calf serum at 37°C in 95% (v/v) air / 5% (v/v) CO₂.

Culture of Primary Mammary Cells and Carcinoma Cells. Asynchronous carcinoma cells MCF7 (p53+,RB+,ER+); MDA-MB-231 (p53-,RB+,ER-); T47D (p53-,RB+,ER+); DU145 (p53-,RB-,AR-); LNCaP (p53+,RB+,AR+) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% (v/v) fetal calf serum at 37°C in 95% (v/v) air / 5% (v/v) CO₂. Cells were plated at a density 3.2 × 10⁴ cells/cm² plate area and grown for 36h prior to further experimentation.

Recombinant Adenoviral Vectors: Generation and Infection in Vitro. We generated recombinant adenoviruses to express Bcl-2 or Bcl-xl.²³ All cells, infected at an m.o.i. of 50 gave a >80% infection rate.

Exposure of Cells to Ionizing Radiation and Cell Homogenization. Cells were cultured in DMEM + 5% (v/v) fetal calf serum as above. U0126/PD98059/PD184352 treatment was from a 100 mM stock solution and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v). Cells were irradiated to a total of 2 Gy using a ⁶⁰Co source at dose rate of 2.1 Gy/min. Cells were maintained at 37°C throughout the experiment except during the ~1 min irradiation itself. Zero time is designated as the point at which irradiation ceased.

After radiation-treatment cells were incubated for specified times followed by aspiration of media and snap freezing at -70°C on dry ice. Cells were homogenized in 1 ml ice cold buffer A [25 mM β -glycerophosphate, pH 7.4 at 4°C, 5 mM EDTA, 5 mM EGTA, 5 mM benzamide, 1 mM phenylmethyl sulphonylfluoride, 1 mg/ml soybean trypsin inhibitor, 40 μ g/ml pepstatin A, 1 μ M Microcystin-LR, 0.5 mM sodium orthovanadate, 0.5 mM sodium pyrophosphate, 0.05% (w/v) sodium deoxycholate, 1% (v/v) Triton X100, 0.1% (v/v) 2-mercaptoethanol], with trituration using a P1000 pipette to lyse the cells. Homogenates were stored on ice prior to clarification by centrifugation (4°C).

Immunoprecipitations from Lysates. Fifty microliters of Protein A agarose (Ag) slurry (25 μ l bead volume) was washed twice with 1 ml PBS containing 0.1 % (v/v) Tween 20, and resuspended in 0.1 ml of the same buffer. Antibodies (2 μ g, 20 μ l), serum (20 μ l) were added to each tube and incubated (3h, 4°C). For pre-conjugated antibodies, 10 μ l of slurry (4 μ g antibody) was used. Clarified equal aliquots of lysates (0.25 ml, ~100 μ g total protein) were mixed with Ag-conjugated antibodies in duplicate using gentle agitation (2.5h, 4°C). Ag-antibody-antigen complexes were recovered by centrifugation, the supernatant discarded, and washed (10 min) sequentially with 0.5 ml buffer A (twice), PBS and buffer B [25 mM Hepes, pH 7.4, 0.1 mM Na₃VO₄].

Assay of p42^{MAPK} Activity. Immunoprecipitates were incubated (final volume 50 μ l) with 50 μ l of buffer B containing 0.2 mM [γ -³²P]ATP (5000 cpm/pmol), 1 μ M Microcystin-LR, 0.5 mg/ml myelin basic protein (MBP), which initiated reactions at time = 0. After 20 min, 40 μ l of the reaction mixtures were spotted onto a 2 cm circle of P81 paper (Whatman, Maidstone, England) and immediately placed into 180 mM phosphoric acid. Papers were washed four times (10 min each) with phosphoric acid, and once with acetone, and ³²P-incorporation into MBP was quantified by liquid scintillation spectroscopy.

SDS Poly-Acrylamide Gel Electrophoresis (SDS PAGE) and Western Blotting. Cells were drug treated/irradiated and at specified time points/treatments media aspirated and the plates snap frozen. Cells were lysed with homogenization buffer and subjected to immunoprecipitation. Immunoprecipitates were solubilized with 100 μ l 5X SDS PAGE sample buffer (10% (w/v) SDS), diluted to 250 μ l with distilled water, and placed in a 100°C dry bath for 15 min. One hundred microliter aliquots of each time point were subjected to SDS PAGE on 10% (w/v) acrylamide gels. Gels were transferred to nitrocellulose by the Method of Towbin and Western blotting using specific antibodies performed as indicated. Blots were developed using enhanced Chemi-Luminescence (Amersham) using Fuji RX x-ray film. Blots were digitally scanned using Adobe Photoshop, their color removed, and Figures created in Microsoft PowerPoint.

Terminal Uridyl-Nucleotide End Labeling (TUNEL) for the Determination of the Percentage of Apoptotic Cells. Cells were grown as described, treated with or without varying concentrations of U0126/PD184352/PD98059/DMSO control 30 min prior to irradiation and irradiated (2 Gy). Cells were isolated 24h after irradiation by trypsinization followed by centrifugation onto glass slides (cytospin). Terminal Uridyl-Nucleotide End Labeling (TUNEL) that monitors double stranded DNA breaks was performed on these cells as described previously to determine the percentage of cells that were apoptotic (% apoptosis).^{17,18} Randomly selected fields of fixed cells (~150 cells per field, n=5 per slide) were counted initially using propidium iodide counter stain, followed by examination and counting of TUNEL positive staining cells of the same field under FITC/fluorescence light: this permitted the percentage of apoptotic cells to be determined.

Cell Cycle Analysis: Propidium Iodide Staining of Cells. Cells were isolated by tryptic digestion at the indicated times after various treatments and aliquots containing 1×10^6 cells were pelleted by centrifugation at 1500 rpm, 4°C for 5 min. and resuspended in 1.5 ml of PBS followed by the addition of 3 ml of 100% (v/v) ETOH (67% (v/v) ETOH Final) and incubated on ice at 4°C for 3h. Cells were pelleted by centrifugation as above, supernatant removed and resuspended in 1.0 ml of propidium iodide stain containing 3.8 mM sodium citrate, 0.5 mg/ml RNase A and 0.01 mg/ml propidium iodide and incubated on ice at 4°C overnight. Cells were pelleted by centrifugation as above, supernatant removed and resuspended in 1.0 ml of PBS. Cells were analyzed with a Becton-Dickinson FACScan flow cytometer and Verity Winlist software.

MTT Assay for Cell Growth. Cells were grown in 12 well plates and 36h after plating are pre-treated for 30 min with varying concentrations of MEK1/2 inhibitor/DMSO control before further drug treatment/irradiation. Cells were cultured for a further 48h. A 5 mg/ml stock solution of MTT reagent (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) was prepared in DMEM. For assay of mitochondrial dehydrogenase function, the MTT stock solution is diluted 1:10 in fresh media (DMEM + 10% fetal calf serum) and 1 ml of this solution is added to each aspirated well of a 12 well plate. Cells are incubated for a further 3h at 37°C. MTT is converted into an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes. After 3h, media is aspirated and cells lysed with 1 ml DMSO, releasing the purple product from the cells. Cells are incubated for a further 10 min at 37°C with gentle shaking. Absorbance readings at 540 nm are determined using a computer controlled micro-plate analyzer. The relationship between cell number and MTT absorbance/mitochondrial enzyme activity was linear over the range of 500-10,000 cells.

Analysis of Cytosolic Cytochrome C. 2×10^6 cells were washed in PBS and lysed by incubating for 30 seconds in lysis buffer (75mM NaCl, 8mM Na_2HPO_4 , 1mM NaH_2PO_4 , 1mM EDTA, and 350ug/ml digitonin). The lysates were centrifuged at 12,000g for 1 min, and supernatant was collected and added to equal volume of 2X sample buffer. The protein samples were quantified, separated by 15% SDS-PAGE, and subjected to immunoblot analysis as described above. Anti-cytochrome c (mouse monoclonal, Pharmingen) was used as primary antibody at a dilution of 1:100.

Analysis of Mitochondrial Membrane Potential $\Delta\psi_m$. 2×10^5 cells were incubated with 40nM 3,3-dihexyloxycarbocynine (DiOC6, Molecular

Probes Inc. Eugene, OR) in PBS at 37°C for 20 min, and then analyzed by flow cytometry as described previously.²⁴ The percentage of cells exhibiting "low" level of DiOC6 up-take, which reflects loss of mitochondrial membrane potential, was determined using a Becton-Dickinson FACScan analyzer.

Colony Forming (Clonogenic) Assay. Cells were plated 36h prior to experimentation. Cells were pre-treated with MEK1/2 inhibitor as indicated, 2h prior to exposure. Cells were irradiated (2 Gy). After a further 48h, cells were isolated by tryptic digestion and single cell suspensions plated on Linbro® plates at densities of 500 cells/well and 1000 cells/well. Colony formation was defined as a colony of 50 cells or greater, 10 days after plating.

Data Analysis. Comparison of the effects of treatments was done using one way analysis of variance and a two tailed t-test. Differences with a *p*-value of <0.05 were considered statistically significant. Experiments shown, except where indicated, are the means of multiple individual points from multiple separate experiments ($\pm$ SEM). Statistical analyses were made using SigmaPlot and SigmaStat.

RESULTS

Treatment of Carcinoma Cells with UCN-01 Activates the MAPK Pathway. UCN-01 is a known PKC and Chk1 inhibitor. Based on its capacity to inhibit PKC isoforms, we postulated that UCN-01 would suppress the activity of signaling pathways downstream of PKC, including the MAPK pathway. However, when MCF7, MDA-MB-231, T47D and DU145 cells were treated with a clinically achievable dose of UCN-01 (150 nM), activation of MAPK/ERK was observed in immune complex kinase assays that was variably prolonged for intervals of 12h (720 min) to 24h (1440 min) (Figs. 1A-1D). MAPK activation by UCN-01 was opposed by the MEK1/2 inhibitor PD98059 (PD, 25 μ M) (Figs. 1A-1D), and by other structurally unrelated MEK1/2 inhibitors PD184352 (10 μ M) and U0126 (5 μ M) (data not shown). Of note, the MEK1/2 inhibitor U0126, which is a more potent MEK1/2 inhibitor than PD98059, abolished both basal and UCN-01-stimulated MAPK activation in T47D cells (data not shown). In these studies, little modulation of JNK/SAPK or p38 MAPK pathway activity was observed following treatment with the drugs (data not shown).

MAPK activation correlated with enhanced phosphorylation of both MEK1/2 (S218/S222) and ERK1/2 (T183/Y185) as judged by immunoblotting of cell lysates (Fig. 1A, inset panel). Of note, while the decrease in ERK1/2 immune complex kinase activity in cells treated with UCN-01 and MEK1/2 inhibitor was reflected in a parallel loss of ERK1/2 phosphorylation, no additional loss of MEK1/2 phosphorylation or MEK1/2 activity was observed under these conditions (Fig. 1A, inset panel, data not shown). This finding argues that under conditions of the drug combination, either MEK1/2 activity is being suppressed independently of "Raf"-mediated S218/S222 phosphorylation or that a MAPK/ERK phosphatase is becoming activated.

Combined Treatment of Carcinoma Cells with UCN-01 and MEK1/2 Inhibitor Reduces Proliferative Potential and Increases Cell Killing within 24h. Because the activity of the MAPK pathway was reduced following drug treatment, we next investigated the impact of reduced pathway function on tumor cell proliferation. Individual treatment of cells with either MEK1/2 inhibitor or UCN-01 alone reduced the proliferative capacity of carcinoma cells, reflected by MTT assays, however, the proliferation of cells exposed to the combination of both drugs was significantly lower (>80%) than either treatment alone (data not shown).

Because exposure of cells to MEK1/2 inhibitor and UCN-01 reduced proliferative capacity, attempts were made to determine whether this effect reflected reduced cell viability. To assess the caspase-dependence of these events, cells were incubated with the pan-caspase inhibitor ZVAD. Furthermore, since both UCN-01 and MEK1/2 inhibitors are known as individual agents to be radio-sensitizers, studies were also performed in parallel using ionizing radiation. Cells were exposed to UCN-01 and MEK1/2 inhibitors ($\pm$ radiation; 2 Gy), after which cell viability was determined 24h following treatment by monitoring double stranded DNA breaks, as well as by 7AAD incorporation (7-amino actinomycin D) and nuclear morphology (data not shown).

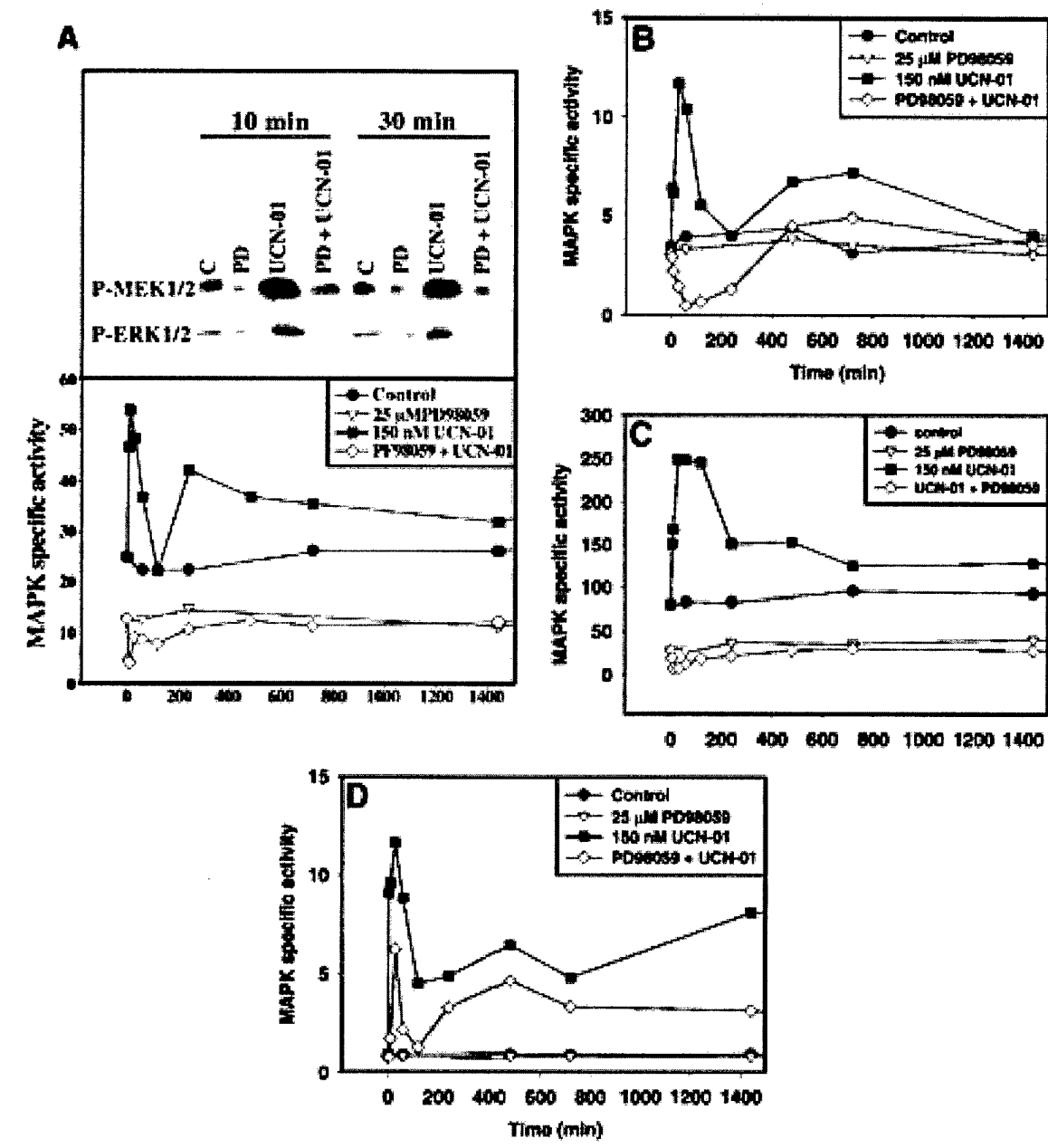


Figure 1. Prolonged activation of MAPK by UCN-01 in mammary and prostate carcinoma cells. Cells were cultured as described in Methods. Panel A. MDA-MB-231 cells. Panel B. MCF7 cells. Panel C. DU145 cells. Panel D. T47D cells. Cells were pre-treated for 30 min with MEK1/2 inhibitor (25 μM) followed at "time 0" by UCN-01 (150 nM) and MAPK activity determined over the next 0-1440 min (24h) as in Methods. Inset panel in Panel A.; MDA-MB-231 cells were pre-treated for 30 min with MEK1/2 inhibitor PD98059 (PD, 25 μM) followed at time 0 by UCN-01 (150 nM) and MEK1/2 phosphorylation and ERK1/2 phosphorylation determined 10 min and 30 min after drug addition. Cells were lysed and portions (~100 μg) from each plate used to immunoprecipitate MAPK followed by immune-complex kinase assays measuring increases in ³²P-incorporation into MBP substrate as in Methods. Phosphorylation status did not alter the ability of our antibody to immunoprecipitate MAPK (not shown). MAPK activity data are shown as specific activity (fmol/min/mg), and are from the means ± SEM of 3 independent experiments with MAPK activity values which differed by less than 20%.

In all cell types examined neither MEK1/2 inhibition, UCN-01 treatment, nor radiation exposure alone induced substantial reductions in cell viability within 24h. However, when cells were exposed to MEK1/2 inhibitor and UCN-01, a significant potentiation of apoptosis was observed after 24h that was abolished by the pan-caspase inhibitor ZVAD (Fig. 2). In all cell types examined, with the exception of DU145 cells which are null

for the pro-apoptotic protein BAX, radiation exposure did not further enhance the level of apoptosis in cells exposed to the combination of UCN-01 and MEK1/2 inhibitor (compare the apoptosis values of [PD98059 + UCN-01] ± 2 Gy in Figs. 2A, 2B, 2D and 2E to the data presented in Fig. 2C).

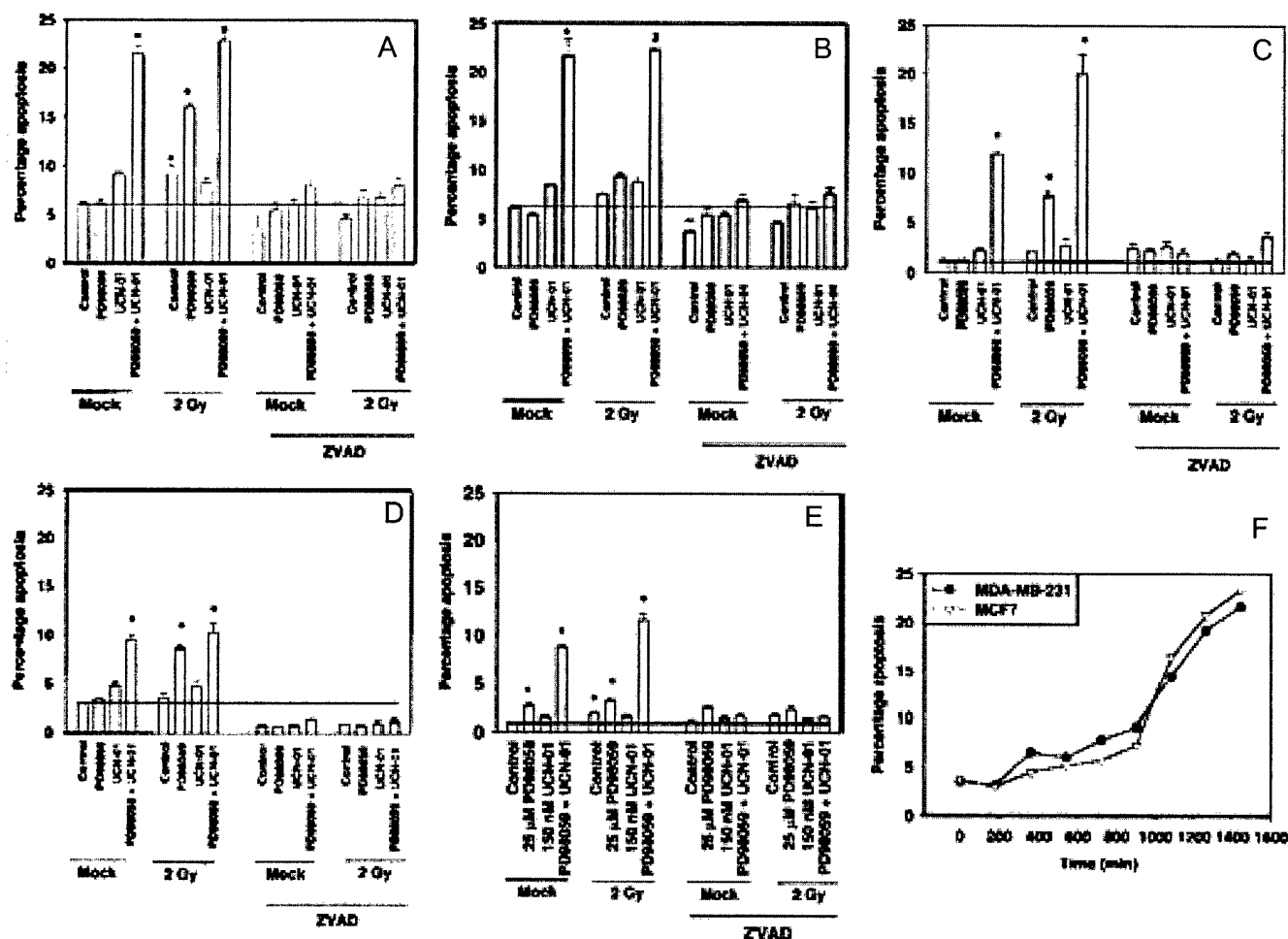


Figure 2. Combined exposure of mammary and prostate carcinoma cells to UCN-01 and MEK1/2 inhibitors enhances apoptosis. Cells were either treated with vehicle or with ZVAD (20 μ M). In parallel, cells were incubated with matched vehicle control (DMSO), with 25 μ M PD98059 alone, with 150 nM UCN-01 alone, or with 25 μ M PD98059 and 150 nM UCN-01. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours (1440 min) post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. Panel A. MDA-MB-231 cells; Panel B. MCF7 cells; Panel C. DU145 cells; Panel D. T47D cells; Panel E. LNCaP cells; Panel F. Time course of apoptosis for MDA-MB-231 and MCF7 cells treated with 25 μ M PD98059 and 150 nM UCN-01 (cf Panels A and B). Data shown are the mean number of staining cells from randomly selected fields of fixed cells ($n=5$ per slide, 3 parallel individual experiments $\pm$ SEM) which were examined and counted via fluorescent light microscopy. * $p < 0.05$ greater than control value.

In addition to monitoring apoptosis 24h after exposure, studies were also performed to characterize the ability of UCN-01 and MEK1/2 inhibitor treatment to potentiate apoptosis over a 24h time course. Combined inhibitor treatment weakly enhanced apoptosis in MDA-MB-231 and MCF7 cells 6h after exposure, with only a doubling in the basal percentage of dead cells, from ~3% to ~6% (Fig. 2F). However, it was only after an exposure interval of 18-24h that approximately 15-25% of the cells were classified as apoptotic based upon evidence of TUNEL staining/DNA strand breakage (Figs. 2A and 2B cf Fig. 2F). The potentiation of apoptosis was not limited to the MEK1/2 inhibitor PD98059, as we found that the chemically dissimilar MEK1/2 inhibitors PD184352 and U0126 also enhanced apoptosis in combination with UCN-01 (data not shown).

In contrast to these malignant tumor cells, primary human mammary epithelial cells, primary mouse hepatocytes, normal human peripheral blood mononuclear cells, or a purified population of human CD34⁺ stem cells

were relatively refractory, and did not exhibit an enhancement in apoptosis following exposure to UCN-01 and MEK1/2 inhibitor (Fig. 3, data not shown). Also, notably, the PKC inhibitor bisindolylmaleimide (1 μ M) did not mimic the actions of UCN-01, consistent with a PKC-independent mechanism of action for UCN-01 (data not shown). Collectively, these findings argue that the reduction in proliferation of cells exposed to UCN-01 and MEK1/2 inhibitors reflects, at least in part, potentiation of caspase-dependent apoptosis.

Combined Treatment of Carcinoma Cells with UCN-01 and MEK1/2 Inhibitor Increases Release of Cytochrome c into the Cytosol and Correlates with Reduced Cdc2 Y15 Phosphorylation. In view of evidence that combined exposure to UCN-01 and MEK1/2 inhibitors promoted cell death in epithelial carcinoma cells, the effects of co-treatment with these agents was examined in relation to various events implicated in the apoptotic process, including mitochondrial damage, pro-caspase cleavage/activation,

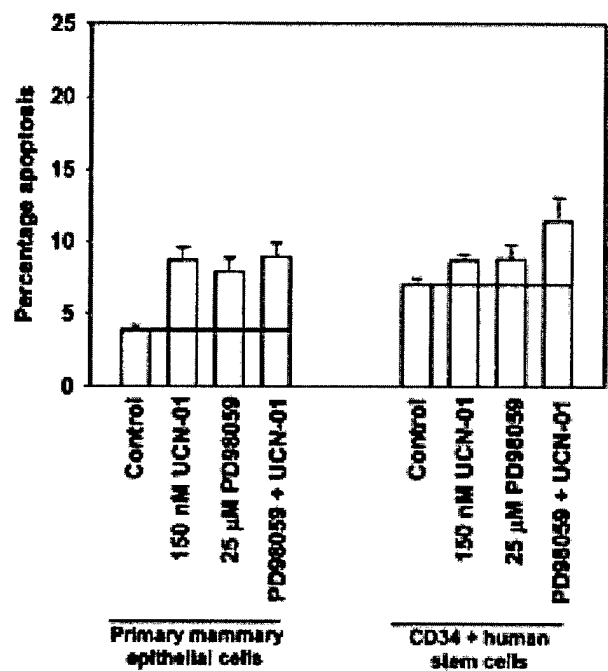


Figure 3. MEK1/2 inhibitor and UCN-01 cause weak additive increases in apoptosis in normal mammary epithelial cells and in a purified population of human CD34⁺ stem cells. Cells were incubated with matched vehicle control (DMSO), with PD98059 (25 μ M) alone, with 150 nM UCN-01 alone, or with PD98059 (25 μ M) and 150 nM UCN-01. Portions of cells were taken 24 hours (1440 min) post treatment and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. Panel A. Normal mammary epithelial cells; Panel B. CD34⁺ stem cells. Data shown are the mean number of staining cells from randomly selected fields of fixed cells (n=5 per slide, 3 parallel individual experiments $\pm$ SEM) which were examined and counted via fluorescent light microscopy. * $p < 0.05$ greater than control value.

and perturbations in cell cycle regulatory proteins. Combined exposure of MDA-MB-231 carcinoma cells to a MEK1/2 inhibitor and UCN-01, but not individual treatment, induced cleavage of pro-caspases -8, -9 and -3 within 24h (Fig. 4A). Notably, reduced expression (cleavage) of pro-caspase 9, and to a lesser extent pro-caspase 3, occurred within 3-6h of combined drug treatment and prior to any significant cleavage of pro-caspase 8. Significantly, this cleavage did not correlate with enhanced apoptosis at this time (Fig. 2F). For unknown reasons, p32 pro-caspase 3, but not p49 pro-caspase 9, levels in combined drug treated cells seemed to recover somewhat 9-12h after drug exposure but the intensity of these bands were still less than that observed in control samples.

UCN-01 is known in some cell types to cause activation of Cdk enzymes.^{1,3,4} However, as an individual agent in MDA-MB-231 cells, UCN-01 weakly modulated neither Cdc2 tyrosine 15 phosphorylation nor Cdc2 catalytic activity (Figs. 4B and 4C). In addition, combined exposure of MDA-MB-231 cells to a MEK1/2 inhibitor and UCN-01 also did not significantly alter Cdc2 Y15 phosphorylation 3-9h after drug addition (Fig. 4B). In contrast, however, cleavage of the pro-apoptotic protein BID, a profound reduction in Cdc2 Y15 phosphorylation, enhanced Cdc2 activity and reduced 14-3-3 protein association with Cdc25C correlated temporally with the delayed 18-24h cleavage of pro-caspase 8 and pro-caspase 3 (Figs. 4B and 4C, data not shown) and with the large increase in apoptosis observed at 18-24h post-treatment (Fig. 2F). Similar data for the time course of pro-caspase cleavage were obtained in DU145 cells (data not shown).

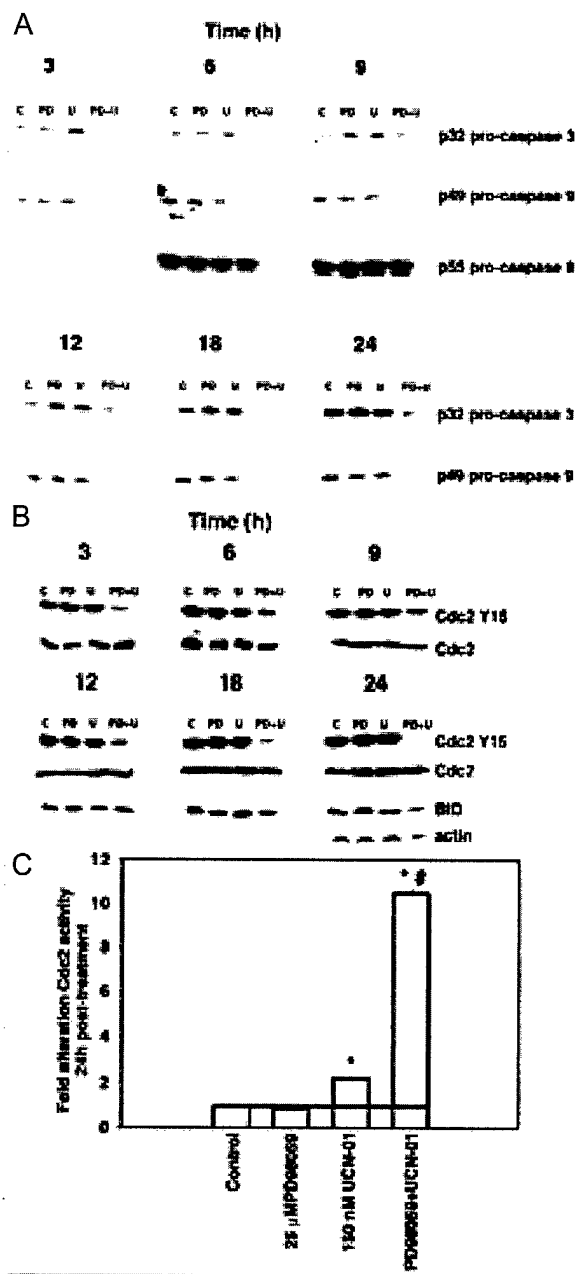


Figure 4. Combined treatment of MDA-MB-231 cells with UCN-01 and MEK1/2 inhibitors reduces the expression of pro-caspases. Cells were incubated with matched vehicle control (DMSO), with 25 μ M PD98059 alone, with 150 nM UCN-01 alone, or with 25 μ M PD98059 and 150 nM UCN-01. Portions of cells were taken 0-24 hours post treatment and lysed in SDS PAGE buffer containing bromophenol blue as described in Methods. Cells were subjected to SDS PAGE followed by immunoblotting to determine: Panel A. The expression / integrity of pro-caspases -3, -8 and -9 as judged by the intensity of the immunoblotted pro-caspase band; Panel B. The expression and integrity of PhosphoY15 and total Cdc2, and BID as judged by the intensity of the immuno-reactive band. Data shown are from a representative experiment (n=3); Panel C. The activity of Cdc2 24 hours after drug treatment. Data shown are the mean activity from 3 parallel individual experiments ($\pm$ SEM). * $p < 0.05$ greater than control value; # $p < 0.05$ greater than UCN-01 alone value. Identical parallel data were obtained in immune complex assays for the G1/S cyclin dependent kinase, Cdk2 (not shown).

Cotreatment of MDA-MB-231 cells with UCN-01 and MEK1/2 inhibitor also resulted in a marked increase in the number of cells exhibiting loss of, or "low" mitochondrial membrane potential, 24h, but not 6h, after exposure to the drugs (i.e., $\Delta\psi_m$; Fig. 5). The ordinate axis shows the percentage difference between conditions in the "low" mitochondrial membrane potential. This differential was obtained by determining and then plotting the percentage "low" mitochondrial membrane potential value for cells treated with combined PD98059 and UCN-01 minus the values of cells treated individually with either PD98059 or UCN-01.

In contrast to our observations examining the mitochondrial membrane potential, release of cytochrome c into the cytosol was observed at both 6h and 24h after drug exposure (Fig. 5, inset panel). Similar data were also obtained in DU145 cells (data not shown). This is in general agreement with the cleavage of pro-caspase 9 observed at this time (Fig. 4A). Treatment of cells with a pan-caspase inhibitor ZVAD failed to reduce cytochrome c release in UCN-01 and MEK1/2 inhibitor-treated cells 6h after exposure but prevented loss of $\Delta\psi_m$ at 24h. These findings are compatible with the concept that cytochrome c release represents an early event in the apoptotic response of these cells to UCN-01 and MEK1/2 inhibitor treatment, whereas the loss of $\Delta\psi_m$ is a secondary process likely stemming from subsequent activation of pro-caspases, including pro-caspase 3, pro-caspase 8, and the cleavage of facilitator proteins, i.e., BID.

Attempts were then made to determine whether peptide inhibitors specific for caspase 9 (LEHD-fmk) and for caspase 8 (IETD-fmk) could block the apoptotic response following combined treatment with MEK1/2 inhibitor and UCN-01. Incubation of MDA-MB-231 cells individually with either LEHD-fmk or IETD-fmk partially attenuated the potentiation of apoptosis following treatment of carcinoma cells with MEK1/2 inhibitor and UCN-01 (Fig. 6A). Of note, however, treatment with both inhibitors was required to abolish the drug-induced apoptotic response. Similar data were obtained for MCF7, T47D and LNCaP cells (data not shown).

Because radiation potentiated cell killing by the drug combination only in DU145 cells, the impact of the caspase inhibitors was also examined following combined drug treatment and irradiation (Fig. 6B). Incubation of DU145 cells with LEHD blunted the overall apoptotic response of MEK1/2 inhibitor and UCN-01 treated cells, but still permitted radiation to enhance the apoptotic response of these drug treated cells. In contrast, incubation of these cells with IETD had a weaker inhibitory effect on the overall apoptotic response of MEK1/2 inhibitor and UCN-01 treated cells, but abolished the further enhancement of cell killing following irradiation; this suggests the potentiation of apoptosis by radiation requires activation of pro-caspase 8.

Overexpression of Bcl-X_L Blocks the Potentiation of Apoptosis Caused by Combined Treatment with MEK1/2 Inhibitor and UCN-01. In view of evidence that activation of pro-caspase-9 was involved in the enhanced apoptosis observed in cells exposed to UCN-01 and MEK1/2 inhibitors, an effort was made to determine whether over-expression of the mitochondrial anti-apoptotic proteins Bcl-X_L and Bcl-2 protected carcinoma cells from drug-induced apoptosis. Cells were infected with recombinant adenoviruses to express an empty vector, Bcl-2, or Bcl-X_L. Increased expression of Bcl-X_L in MDA-MB-231 cells abolished the potentiation of apoptosis induced by combined treatment with PD98059 and UCN-01 (Fig. 7), as well as in DU145 cells (data not shown). Over-expression of Bcl-X_L abolished the release of cytochrome c into the cytosol but did not alter the drug-stimulated increase in mitochondrial BAX levels (data not shown). Contrary to expectations, but consistent with the results of previous studies suggesting a differential cytoprotective effect of these anti-apoptotic proteins, over-expression of Bcl-2 did not protect cells from increased cell killing.^{35,39} Of note, in primary hepatocytes, we recently demonstrated that the adenoviruses to express Bcl-2 and Bcl-X_L were both capable of blocking cytochrome c-dependent bile acid-induced apoptosis, arguing that our observations in carcinoma cells were not due to a lack of functional Bcl-2 expression.⁴⁴

Potentiation of Apoptosis Correlates with Reduced Cell Numbers in S Phase and G2/M Phase. The interactions between PD98059 and UCN-01 were next examined in relation to cell cycle events (Fig. 8). Administration

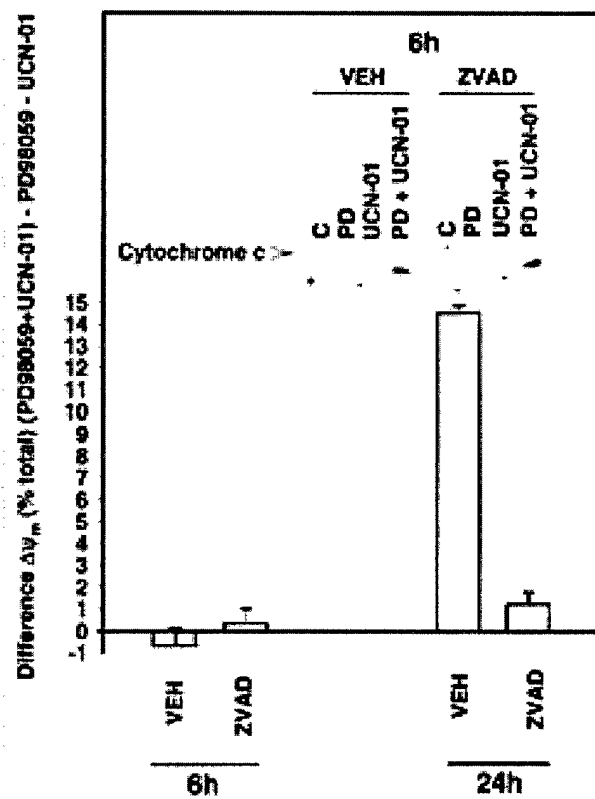


Figure 5. Combined treatment of MDA-MB-231 cells with UCN-01 and MEK1/2 inhibitors causes a loss of the mitochondrial membrane permeability transition and release of cytochrome c into the cytosol. Cells were either treated with vehicle or with ZVAD (20 μ M). In parallel, cells were incubated with matched vehicle control (DMSO), with 25 μ M PD98059 alone, with 150 nM UCN-01 alone, or with 25 μ M PD98059 and 150 nM UCN-01. Portions of cells were taken 6 and 24 hours post treatment and the mitochondrial membrane potential determined. The ordinate axis shows the percentage difference between conditions in the "low" mitochondrial membrane potential. This differential was obtained by plotting the value for cells treated with combined PD98059 and UCN-01 minus the values of cells treated individually with either PD98059 or UCN-01. Data are the means of 3 parallel individual experiments $\pm$ SEM. * $p < 0.05$ greater than control value. Inset panel; Increased release of cytochrome c into the cytosol, which is potentiated by MAPK inhibition. Cells were treated as above and the release of cytochrome c into the cytosol determined 6h after addition. Data are representative of 3 experiments.

of either a MEK1/2 inhibitor or UCN-01 individually for 24hr enhanced cell numbers in G1 phase and primarily depleted the S phase population. When the agents were combined, a substantial reduction in the G2/M fraction occurred, an event accompanied by a significant increase in the S-phase population and corresponding increase in the sub-diploid apoptotic fraction. Of note, only exposure to the combination of UCN-01 and PD98059, but not the drugs individually, exhibited a pronounced reduction in phosphorylation of Cdc2 on tyrosine 15 (Fig. 4B). Together, these findings raise the possibility that interactions between PD98059 and UCN-01 may interfere with checkpoint function and lead to unscheduled Cdc2 activation.

Combined Exposure to MEK1/2 Inhibitor and UCN-01 for 48h Diminishes Clonogenic Survival of Carcinoma Cells which is Reduced Further by Radiation Exposure. Cells were treated with UCN-01, PD98059 or radiation in various combinations. Cells were re-plated 48h

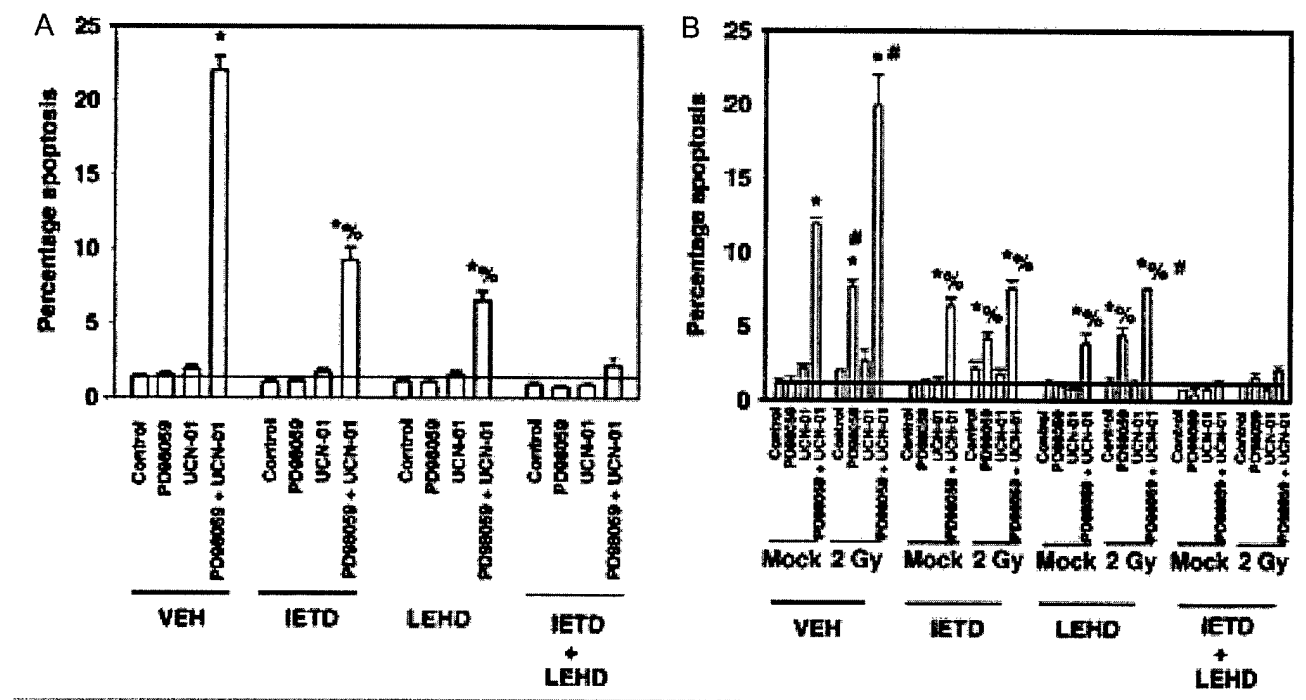


Figure 6. The potentiation of apoptosis by combined UCN-01 treatment / MEK1/2 inhibition requires both caspase 9 and caspase 8 functions. Cells were either treated with either vehicle, with IETD (20 μ M), with LEHD (20 μ M) or with both IETD and LEHD. In parallel, cells were incubated with matched vehicle control (DMSO), with 25 μ M PD98059 alone, with 150 nM UCN-01 alone, or with 25 μ M PD98059 and 150 nM UCN-01. In Panel B, cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. Panel A. MDA-MB-231 cells. Panel B. DU145 cells. Data shown are the mean number of staining cells from randomly selected fields of fixed cells ($n=5$ per slide, 3 parallel individual experiments $\pm$ SEM) which were examined and counted via fluorescent light microscopy. # $p < 0.05$ greater than corresponding value in unirradiated cells; * $p < 0.05$ greater than control value; % $p < 0.05$ less than corresponding value in cells not treated with caspase inhibitor.

after treatment in the absence of drugs and the impact of combined treatment of carcinoma cells with UCN-01 and PD98059 was examined in relation to effects on clonogenic survival (Table 1). Treatment with either UCN-01 or PD98059 by themselves generally had very modest effects on subsequent colony formation. However, combined treatment of cells with both agents resulted in a substantial reduction in clonogenicity (Table 1). These findings indicate that enhanced apoptosis in cells exposed to UCN-01 in combination with a MEK1/2 inhibitor is accompanied by a subsequent significant reduction in carcinoma cell viability and self-renewal

capacity. Of note, while UCN-01/MEK1/2 inhibitor-induced apoptosis was not further enhanced following radiation exposure as noted above, irradiation of drug-treated cells resulted in a further reduction in clonogenic survival. This effect was quite pronounced in DU145, T47D and MDA-MB-231 cells. MCF7 cells, expressing wild type p53, were very sensitive to the drug combination, and this effect was increased in an additive fashion by radiation. These data suggest that radiation enhances cell killing in the presence of the drug combination through a non-apoptotic mechanism.

TABLE 1 COMBINED EXPOSURE OF CARCINOMA CELLS TO UCN-01 AND MEK1/2 INHIBITORS REDUCES CLONOGENIC SURVIVAL AND IS ENHANCED BY RADIATION EXPOSURE.

Cell Type	control	PD98059	UCN-01	PD + U	2 Gy control	2 Gy PD98059	2 Gy UCN-01	2 Gy PD + U
DU145	100 $\pm$ 1	99 $\pm$ 6	82 $\pm$ 4	55 $\pm$ 1*	67 $\pm$ 3	65 $\pm$ 2	68 $\pm$ 2	21 $\pm$ 1* %
MCF7	100 $\pm$ 1	91 $\pm$ 5	69 $\pm$ 6	25 $\pm$ 2*	61 $\pm$ 4	39 $\pm$ 3#	44 $\pm$ 3	16 $\pm$ 4* %
T47D	100 $\pm$ 4	97 $\pm$ 2	103 $\pm$ 8	62 $\pm$ 1*	83 $\pm$ 3	69 $\pm$ 5	43 $\pm$ 4	42 $\pm$ 4 %
MDA	100 $\pm$ 5	91 $\pm$ 1	97 $\pm$ 3	43 $\pm$ 1*	63 $\pm$ 3	44 $\pm$ 1#	55 $\pm$ 2	10 $\pm$ 1* %

Cells were incubated with matched vehicle control (DMSO), with 25 μ M PD98059 alone, with 150 nM UCN-01 alone, or with 25 μ M PD98059 and 150 nM UCN-01. After 30 min, cells were either exposed to radiation (2 Gy) or mock irradiated. Identical portions of viable cells were taken 48 hours post irradiation and re-plated in the absence of drugs on Linbro plates at densities of 500 cells / well and 1000 cells / well. Cells were incubated for 10-14 days after which they were stained and colony number determined. A colony was defined as a cluster of 50 or more cells. MDA-MB-231 cells are referred to in the Table as MDA. Data are normalized to the plating efficiency of control unirradiated cells which is defined as 100% (Data shown are the number of staining colonies, 3-5 parallel individual experiments $\pm$ SEM) which were examined and counted via light microscopy. * $p < 0.05$ less than control value; # $p < 0.05$ less than irradiated value without MAPK inhibition; % $p < 0.05$ less than corresponding unirradiated value.

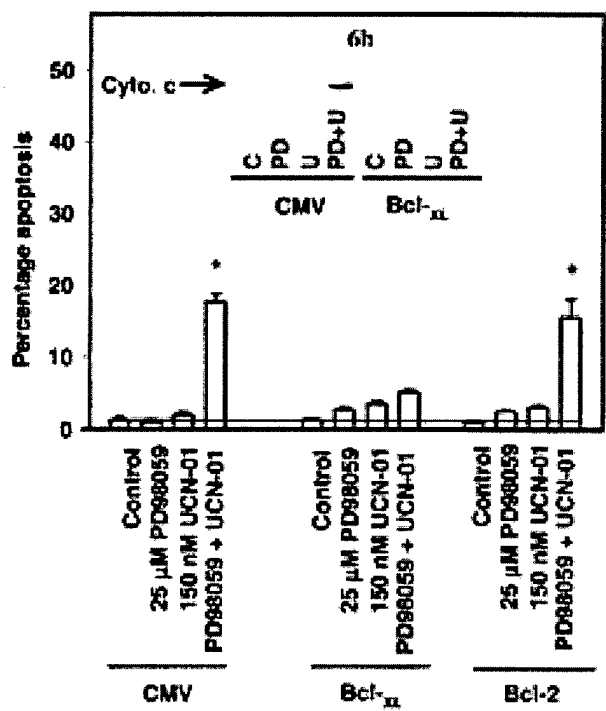


Figure 7. Over-expression of Bcl-XL protects carcinoma cells from the toxic effects of combined UCN-01 and MEK1/2 inhibitor treatment. MDA-MB-231 cells were infected with recombinant adenoviruses to express either null (CMV), Bcl-2 or Bcl-XL. Twenty four h after infection, cells were incubated with matched vehicle control (DMSO), with 25 μ M PD98059 alone, with 150 nM UCN-01 alone, or with 25 μ M PD98059 and 150 nM UCN-01. Portions of cells were taken 24 hours post treatment and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. Data shown are the mean number of staining cells from randomly selected fields of fixed cells ($n=5$ per slide, 3 parallel individual experiments $\pm$ SEM) which were examined and counted via fluorescent light microscopy. # $p < 0.05$ greater than corresponding value in unirradiated cells; * $p < 0.05$ greater than control value. Inset panel. Six hours after treatment, cells over-expressing Bcl-XL were prepared to determine cytochrome c release into the cytosol. Data shown are from a representative experiment ($n=3$).

DISCUSSION

The present investigations demonstrate that relatively non-toxic concentrations of the PKC / Chk1 inhibitor, UCN-01, cause activation of the MAPK pathway in carcinoma cells. Furthermore, co-administration of MEK1/2 inhibitors interfere with this process, leading to BAX association with the mitochondria and release of cytochrome c into the cytosol, followed by activation of caspases and apoptosis within 24h. This process does not appear to require p53 function or dependence on endocrine hormones in that cells expressing wild type/mutant p53 or estrogen/androgen receptor were sensitive to the drug combination.

The present data with epithelial carcinoma cells, which are generally regarded as highly resistant to apoptosis,^{1,34,35} are similar, although not identical, with contemporaneous findings by our group involving malignant hematopoietic cells,²¹ which are in most cases highly vulnerable to programmed cell death. However, and in contrast to malignant hematopoietic cells, the mechanism(s) of cell

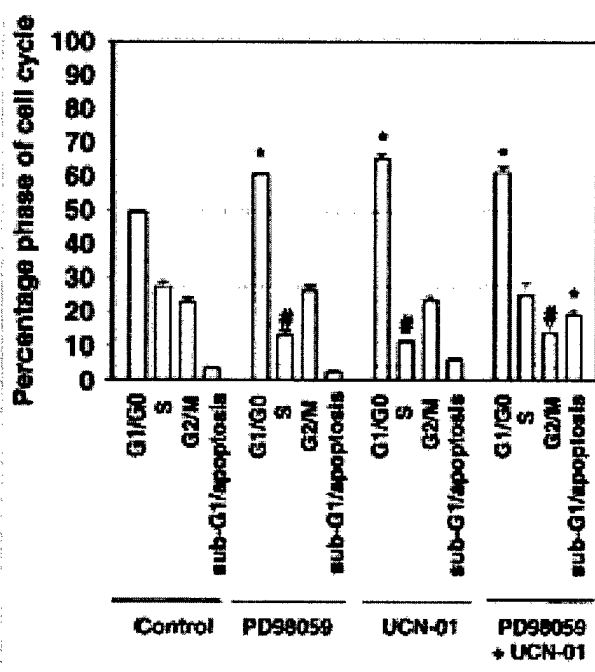


Figure 8. Combined treatment of carcinoma cells with UCN-01 and MEK1/2 inhibitors depletes cell numbers in G2/M phase and increases sub-G1 DNA fragmentation. Cells were incubated with matched vehicle control (DMSO), with 25 μ M PD98059 alone, with 150 nM UCN-01 alone, or with 25 μ M PD98059 and 150 nM UCN-01. Portions of cells were taken 24 hours post irradiation, fixed, digested with RNAase, stained with propidium iodide and flow cytometric analysis performed to assess cell cycle progression of MDA-MB-231 cells. Data shown are the means of duplicate determinations ($n=3 \pm$ SEM). * $p < 0.05$ greater than control value; % $p < 0.05$ less than corresponding value in cells.

killing in epithelial tumor cells appeared to be a more complex and protracted process that was independent of Bcl-2 expression, requiring activation of both caspase 9 and an amplification loop with caspase 3 and caspase 8, BID, as well as being independent of increased p38 MAPK signaling.

That UCN-01 activated the MAPK pathway was surprising, based on its ability to inhibit PKC enzymes. Upstream activators of the MAPK pathway, such as Raf-1 are known to be regulated both in a positive and a negative fashion by phosphorylation.^{15,18,26,27} Thus, it is possible that UCN-01 enhanced MAPK pathway activity by reducing phosphorylation on a negative regulatory site on Raf-1. Similar findings for negative regulatory phosphorylation have also been reported for MEK1, which was shown to be phosphorylated and inhibited by Cdk enzymes.²⁸ Thus in theory UCN-01, by activating Cdk enzymes, could blunt ERK1/2 activity. However, in our cells, 150 nM UCN-01 by itself only weakly enhanced the in vitro measured activities of Cdc2 or Cdk2 approximately 2-fold (Fig. 4C, our unpublished results). The combination of UCN-01 with a MEK1/2 inhibitor induced a rapid further decrease in ERK1/2 activity and phosphorylation beyond that of MEK1/2 inhibitor alone, in 4 of the 5 cell lines examined. The further decline in ERK1/2 phosphorylation did not correlate with an additional reduction in MEK1/2 S218/S222 phosphorylation, suggesting that MEK1/2 activity was being reduced by another mechanism, potentially Cdk-mediated inactivation. Alternatively, the drug

combination could be acting by increasing the activity of an ERK1/2 phosphatase. Further studies will be required to define the multiple mechanisms by which UCN-01 modulates MAPK pathway signaling.

That MEK1/2 inhibitors, which exhibit different mechanisms of action, interact in a greater than additive fashion with UCN-01 to induce apoptosis, strongly suggests that interference with MAPK activation is essential in the lethality of this drug combination. MEK1/2 inhibitors attenuated UCN-01-mediated activation of MAPK and promoted mitochondrial damage and cell death. These data are also consistent with studies demonstrating that interdiction of MAPK cascade function potentiates apoptosis in cells exposed to other environmental stresses.¹⁸ Such findings suggest that activation of the MAPK pathway is not essential for cell survival per se, but that it plays a critical role in protecting cells, particularly neoplastic ones, from a variety of noxious stimuli.

Induction of apoptosis, notably by chemotherapeutic drugs, has been linked to mitochondrial damage, including loss of the mitochondrial membrane potential or release of pro-apoptotic proteins from the mitochondria, such as cytochrome c.²⁹ Induction of apoptosis in both the presence or the absence of cytochrome c release has been described and release of cytochrome c is often found to precede loss of the mitochondrial membrane potential, $\Delta\psi_m$.^{30,31} The present findings suggest that cytochrome c release represents one initial event in cells induced to undergo apoptosis when treated with UCN-01 and MEK1/2 inhibitors.

Cytochrome c release can also be triggered by death receptor-related pathways through activation of pro-caspase-8 and cleavage/activation of the pro-apoptotic effector, BID.³² In our studies in malignant hematopoietic cells, we found that the caspase 8 inhibitor IETD was unable to modify apoptosis or cytochrome c release which argued that the extrinsic apoptosis pathway played little role in the death process.²¹ However, in epithelial carcinoma cells, IETD substantially blocked apoptosis induced by the UCN-01 and MEK1/2 inhibitor combination. Thus carcinoma cells appear to require both the intrinsic caspase 9/3 pathway for apoptosis as well as a potential amplification loop involving caspase 8 activation and BID cleavage.

Recent studies have suggested that UCN-01 can promote apoptosis in malignant hematopoietic cells by modulating expression and function of Bcl-2 and related family members.³³ In epithelial carcinoma cells, over-expression of the Bcl-X_L family member has been shown in a variety of cell types, and has been hypothesized to act in a similar manner to Bcl-2 in protecting cells from death.^{34,35,39} Thus it was surprising to discover that over-expression of Bcl-X_L, but not Bcl-2, protected carcinoma cells from combined UCN-01 and MEK1/2 inhibitor-induced apoptosis. Our finding is consistent with previous reports demonstrating that Bcl-2 and Bcl-X_L can exert disparate cytoprotective effects.^{35,39} Over expression of Bcl-X_L abolished the UCN-01 and MEK1/2 inhibitor-induced release of cytochrome c from the mitochondrion, further suggesting that release of cytochrome c into the cytosol is an important pro-apoptotic event. The mechanism(s) by which the UCN-01/MEK1/2 inhibitor drug combination induces cytochrome c release remain to be determined.

UCN-01 was originally developed as a specific inhibitor of "classical" PKC enzymes and several studies have demonstrated that interruption of this pathway by UCN-01 alone may be insufficient to induce apoptosis in tumor cells.¹⁰ There is also evidence that cell cycle perturbations, particularly inappropriate activation of Cdc2 represent a potent inducer of apoptosis.^{25,36} Unexpectedly, we found

that low concentrations of UCN-01, by themselves, very weakly modulated Cdc2 tyrosine 15 phosphorylation or Cdc2 activity in carcinoma cells, and this finding has been observed by other investigators.⁴⁰ Induction of cell death by UCN-01 has been argued to correlate more closely with Cdk activation than with PKC inhibition¹⁰ and the observations that a PKC inhibitor failed to interact with MEK1/2 inhibitors to potentiate cell killing are in agreement with this finding.

In addition to Cdc2, inappropriate activation of G1 and S phase cyclin:Cdk complexes such as cyclin E:Cdk2 and cyclin A:Cdk2 may also be able to play a role in apoptotic processes.^{37,42} MAPK signaling has been implicated in the activation of multiple cyclin:Cdk complexes and in G1/S/G2/M progression.^{38,42,43} We found that only under conditions of the drug combination were significant large activations of either Cdc2 (Fig. 4C) or Cdk2 (Our unpublished results) observed. In addition, it should be noted that the initial cleavage of pro-caspase 9, which occurred at 6-9h after drug treatment, preceded the earliest profound manifestation of Cdc2 dephosphorylation, which occurred at 12-18h. Collectively these findings suggest that Cdc2 and Cdk2 activation, caused by Cdk dephosphorylation, do not represent the primary mechanism by which cells commit to apoptosis. Further studies will be required to determine whether Cdc2 plays any role in the killing process.

It is noteworthy that while both UCN-01 and MEK1/2 inhibitors have been shown to enhance the sensitivity of various cells to radiation,³⁻⁷ addition of radiation exposure to the UCN-01 and MEK1/2 inhibitor combination did not result in a further increase in apoptosis in our cells, except DU145. This finding may be linked to the fact that DU145 cells do not express the pro-apoptotic protein BAX.⁴² UCN-01 as a single agent neither significantly enhanced radiation-induced apoptosis nor further reduced clonogenic survival beyond additivity, except in T47D cells. These findings suggest that the ability of UCN-01 to abrogate the radiation-induced G2/M cell cycle check-point does not significantly reduce survival. Despite being unable to further enhance apoptosis, irradiation of carcinoma cells combined exposure to UCN-01 and MEK1/2 inhibitors resulted in a further loss of clonogenic potential [in agreement with data presented in reference 41]. Together, these findings are consistent with evidence that reproductive cell death may proceed through both apoptotic and non-apoptotic mechanisms, and suggest that the latter mechanism is involved in the loss of clonogenic survival following combined treatment with UCN-01/MEK1/2 inhibitors and radiation.

In summary, the present studies demonstrate that the checkpoint antagonist UCN-01 activates the MAPK pathway in carcinoma cells, and that interference with this process by MEK1/2 inhibitors enhances mitochondrial damage and apoptosis. That non-transformed cells such as human mammary epithelial cells, normal human peripheral blood mononuclear cells, or human CD34⁺ stem cells did not exhibit an enhancement in apoptosis following exposure suggests that this drug combination will not have a large toxicity towards normal tissues in vivo. Further studies will be required to shed light on the mechanisms by which perturbations in signaling and cell cycle regulatory pathways trigger the apoptotic cascade in tumor cells of epithelial origin.

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COMBINED TREATMENT WITH THE CHECKPOINT ABROGATOR UCN-01 AND
MEK1/2 INHIBITORS POTENTLY INDUCES APOPTOSIS IN DRUG-SENSITIVE AND -
RESISTANT MYELOMA CELLS THROUGH AN IL-6-INDEPENDENT MECHANISM

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ABSTRACT

The effects of combined exposure to the checkpoint abrogator UCN-01 and pharmacologic MEK1/2 inhibitors were examined in human multiple myeloma (MM) cell lines. Treatment of RPMI8226, NCI-H929, and U266 MM cells with a minimally toxic concentration of UCN-01 (150 nM) for 24 hr resulted in MAP kinase activation, an effect that was blocked by co-administration of the MEK1/2 inhibitor PD184352. These events were accompanied by enhanced activation of p34^{cdc2} and a marked increase in mitochondrial damage (loss of $\Delta\Psi_m$; cytochrome c and Smac/DIABLO release), PARP cleavage, and apoptosis. PD184352/UCN-01 also dramatically reduced clonogenic survival in each of the MM cell lines. In contrast to As₂O₃, apoptosis induced by PD184352/UCN-01 was not blocked by the free radical scavenger N-acetyl-L-cysteine. Whereas exogenous IL-6 substantially prevented dexamethasone-induced lethality in MM cells, it was unable to protect them from PD184352/UCN-01-induced apoptosis despite enhancing Akt activation. IGF-1 also failed to diminish apoptosis induced by this drug regimen. MM cell lines selected for a high degree of resistance to doxorubicin, melphalan, or dexamethasone, or displaying resistance secondary to fibronectin-mediated adherence, remained fully sensitive to PD184352/UCN-01-induced cell death. Finally, primary CD138⁺ MM cells were also susceptible to UCN-01/MEK inhibitor-mediated apoptosis. Together, these findings suggest that simultaneous disruption of cell cycle and MEK/MAP kinase signaling pathways provides a potent stimulus for mitochondrial damage and apoptosis in MM cells, and also indicate that this strategy bypasses the block to cell death conferred by several other well-described resistance mechanisms.

Introduction

Multiple myeloma, the most common of the plasma cell dyscrasias, is a progressive and generally incurable disorder of mature B-lymphocytes.¹ The mainstay of treatment for myeloma involves chemotherapy, utilizing agents such as steroids (e.g., dexamethasone), alkylating agents (e.g., melphalan), and topoisomerase inhibitors (e.g., doxorubicin).² Although most myeloma patients respond to such approaches, at least initially, the pre-existence or emergence of drug-resistant cells³ represents a formidable barrier to cure.

Recently, attempts to understand the pathophysiology of multiple myeloma has focused on apoptosis, a genetically regulated program of cell suicide that is particularly involved in hematopoietic cell homeostasis.⁴ For example, there is accumulating evidence that IL-6, a growth and survival factor for myeloma cells, acts, at least in part, by blocking apoptosis.⁵ While the downstream signaling cascades responsible for the anti-apoptotic actions of IL-6 remain to be fully elucidated, JNK/SAPK-, Stat-, NF κ B-, PI3K/Akt-, Mcl-1-, and Bcl-x_L-related pathways have been implicated.⁵⁻⁹ Recently, attention focused on the p42/44 mitogen-activated protein kinase (MAP kinase) cascade as a key regulator of neoplastic cell survival.¹⁰ MAP kinase represents one of a family of parallel signaling modules, which includes the c-Jun N-terminal kinase (JNK) and the p38 MAP kinase.¹¹ Although exceptions exist, activation of JNK and p38 MAPK is generally associated with pro-apoptotic actions,^{10,12} whereas MAP kinase exerts cytoprotective functions.^{10,13} The observation that in MM cells, IL-6- and vascular endothelial growth factor (VEGF)-related proliferative actions involves MAP kinase activation^{14,15} argues that the latter signaling pathway may play an important role in MM cell survival. The potential clinical implications of such findings are highlighted by the recent development of MEK1/2 inhibitors (e.g., PD184352) that display activity *in vivo*¹⁶, and the introduction of PD184352 into Phase I trials¹⁷.

UCN-01 (7-hydroxystaurosporine) is a staurosporine derivative with *in vivo* activity that was originally developed as a selective protein kinase C (PKC) inhibitor.¹⁸ Subsequently, UCN-01 has been reported to act as an inhibitor of Chk1 and an abrogator of the G₂M checkpoint.¹⁹ In human leukemia cells, UCN-01 interacts synergistically with antimetabolites such as ara-C²⁰ and gemcitabine²¹, and induces apoptosis in human leukemia cells, including those of lymphoid origin.²² Phase I/II trials of UCN-01 are currently underway,²³ and preliminary evidence suggests that it may have activity, particularly when combined with other agents, in B-cell malignancies²⁴. The potential activity of UCN-01 against multiple myeloma cells remains largely unexplored.

In a recent communication, we reported that exposure of multiple human myeloid leukemia cell lines to sub-toxic, pharmacologically achievable UCN-01 concentrations (i.e., ~150 nM) induced activation of MAP kinase, and that interference with the latter process (e.g., by co-administration of MEK1/2 inhibitors) resulted in a dramatic potentiation of mitochondrial damage and apoptosis.²⁵ In view of evidence that MAP kinase activation plays an important role in multiple myeloma cell proliferation/survival,¹⁴ the notion that combined exposure of myeloma cells to UCN-01 and MEK1/2 inhibitors might lead to enhanced apoptosis appeared plausible. To test this possibility, the effects of co-treatment with UCN-01 and MEK1/2 inhibitors on survival have been examined in a variety of multiple myeloma cell lines. Our results indicate that concurrent exposure of myeloma cells to UCN-01 and MEK1/2 inhibitors synergistically induces mitochondrial damage, caspase activation, and apoptosis, and that these lethal effects are undiminished by administration of exogenous IL-6 or IGF-1. Moreover, myeloma cells selected for resistance to dexamethasone, melphalan, or doxorubicin, or displaying cell adherence-related

drug resistance, retain full susceptibility to UCN-01/MEK1/2 inhibitor-induced lethality. Taken together, these findings suggest that combined treatment with a checkpoint abrogator and MEK1/2 inhibitor may warrant further investigation as a therapeutic strategy in multiple myeloma.

Materials and methods

Cells and reagents

Human MM cell lines RPMI8226, NCI-H929, and U266 were purchased from ATCC. The dexamethasone-sensitive (MM.1S) and -resistant (MM.1R) human MM cell lines²⁶ were maintained in RPMI 1640 medium containing 10% FBS, 200 units/ml penicillin, 200 µg/ml streptomycin, minimal essential vitamins, sodium pyruvate, and glutamine. Doxorubicin-(Dox40) and melphalan (LR5)-resistant sublines^{27,28} of 8226 cells were maintained in RPMI 1640 medium as described above containing 400 nM doxorubicin and 5 µM melphalan, respectively.

The selective MEK inhibitor PD184352 was purchased from Upstate Biotechnology (Lake Placid, NY), and PD98059 and UO126 were supplied by Calbiochem (San Diego, CA) as powders. The inhibitors were dissolved in sterile DMSO, and stored frozen under light-protected conditions at -20°C. UCN-01 was kindly provided by Dr. Edward Sausville (Developmental Therapeutics Program/CTEP, NCI), dissolved in DMSO at a stock concentration of 1 mM and stored at -20°C, and subsequently diluted with serum-free RPMI medium prior to use. Arsenic trioxide (As₂O₃) was supplied by Sigma Chemicals (St. Louis, MO), dissolved in 1.65M NaOH at 50mM as a stock solution. N-acetyl-L-cysteine (L-NAC, Calbiochem) was prepared in sterile water immediately before use. Recombinant human IL-6 and IGF-1 were purchased from Sigma and R&D Systems (Minneapolis, MN), rehydrated in PBS and 10 mM acetic acid, respectively, both of which contained 0.1% BSA, aliquoted and stored at -80 °C. The PI3 kinase inhibitor LY294002, dexamethasone, and doxorubicin, were purchased from Sigma, dissolved in DMSO, aliquoted and stored at -20 °C. Melphalan (Sigma) was dissolved in HCl-ethanol (47:1000), aliquoted and stored at -80 °C. In all experiments, the final concentration of DMSO did not exceed 0.1%.

Experimental format

All experiments were performed utilizing logarithmically growing cells (4-6 x 10⁵ cells/ml). Cell suspensions were placed in sterile 25 cm² T-flasks (Corning, Corning, NY), and incubated with PD184352 for 30 min at 37°C. At the end of this period, UCN-01 was added to the suspension, and the flasks placed in 37°C/5% CO₂ incubator various intervals, generally 24 hr. In some studies, IL-6 or IGF-1 were added concurrently with PD184352. After drug treatment, cells were harvested and subjected to further analysis as described below.

Assessment of apoptosis

The extent of apoptosis was evaluated by assessing Wright-Giemsa stained cytospin slides under light microscopy and scoring the number of cells exhibiting classic morphological features of apoptosis. For each condition, 5-10 randomly selected fields per slide were evaluated, encompassing at least 800 cells. To confirm the results of morphologic analysis, in some cases cells were also evaluated by TUNEL staining and Annexin V-FITC Staining.

For TUNEL staining²⁹, cytospin slides were fixed with 4% formaldehyde/PBS for 10min, treated with acetic acid/ethanol (1:2) for 5 min, and incubated with terminal transferase reaction mixture containing 1x terminal transferase reaction buffer, 0.25U/l terminal transferase, 2.5 mM CoCl₂, and 2 pmol fluorescein-12-dUTP (Boehringer Mannheim, Indianapolis, IN) at 37°C for 1 hr. The slides were mounted with Vectashield with propidium iodide (Vector Laboratories, Burlingame, CA) and visualized using fluorescence microscopy.

For Annexin V-FITC staining, 1×10^6 cells were washed twice with cold PBS and then resuspended in 1x binding buffer (10mM Hepes/NaOH, pH7.4, 140mM NaOH, 2.5mM CaCl₂). The cells were incubated with Annexin V-FITC (BD PharMingen, San Diego, CA) and 5 µg/ml propidium iodide (PI), and incubated for 15 min at room temperature in the dark as per the manufacturer's instructions. The samples were analyzed by flow cytometry within 1 hr to determine the percentage of cells displaying Annexin VI staining (early apoptosis) or both Annexin V and PI staining (late apoptosis).

Mitochondrial membrane potential ($\Delta\Psi_m$) assay

After drug treatment, 2×10^5 cells were incubated with 40 nM 3,3-dihexyloxacarbocyanine (DiOC₆, Molecular Probes Inc., Eugene, OR) in PBS at 37°C for 20 min, and then analyzed by flow cytometry.³⁰ The percentage of cells exhibiting low level of DiOC₆ up-take, which reflects loss of mitochondrial membrane potential, was determined using Becton-Dickinson FACScan (Becton-Dickinson, San Jose, CA).

Western blot assay

After drug treatment, whole-cell pellets were lysed by sonication in 1x sample buffer (62.5 mM Tris base, pH6.8, 2% SDS, 50 mM DTT, 10% glycerol, 0.1% bromophenol blue, and 5 µg/ml each chymostatin, leupeptin, aprotinin, pepstatin, and soybean trypsin inhibitor) and boiled for 5 min. For analysis of protein phosphorylation, 1mM each Na vanadate and Na pyrophosphate was added to 1x sample buffer. Protein samples were harvested as the supernatant following centrifugation of the samples at 12,800g for 5 min, and amount of protein quantified using Coomassie Protein Assay Reagent (Pierce, Rockford, IL). Equal amount of protein (30 µg) were separated by SDS-PAGE and electro-transferred onto nitrocellulose membrane. For blotting phospho-proteins, no SDS was included in the transfer buffer. The blots were blocked with 5% milk in PBS-Tween 20 (0.1%) at room temperature for 1 hr and probed with the appropriate dilution of primary antibody in 5% BSA/PBS-Tween 20 overnight at 4°C. The membranes were washed twice in PBS-Tween 20 for 30 min and then incubated with a 1:2000 dilution of HRP-conjugated secondary antibody (Kirkegaard & Perry, Gaithersburg, MD) in 5% milk / PBS-Tween 20 at room temperature for 1 hr. After washing twice in PBS-Tween 20 for 30 min, the blots were visualized by Western Blot Chemiluminescence Reagent (NEN Life Science Products, Boston, MA). For blots of phospho-proteins, TBS was used instead of PBS throughout. Where indicated, the blots were reprobed with antibodies against β -actin (BD PharMingen) to ensure equal loading and transfer of proteins. The following antibodies were used as primary antibodies: phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (1:1000, rabbit polyclonal, Cell Signaling Technology, Beverly, MA), p44/42 MAP kinase antibody (1:1000, rabbit polyclonal, Cell Signaling Technology), phospho-cdc2 (Tyr15) antibody (1:1000, rabbit polyclonal, Cell Signaling Technology), cdc2 antibody (1:1000, rabbit polyclonal, Cell Signaling Technology), phospho-cdk2 (Thr160) antibody (1:500, rabbit polyclonal, Cell Signaling Technology), cdk2 antibody (1:1000, mouse monoclonal, Transduction Laboratories, Lexington,

KY), PARP antibody (1:2500, mouse monoclonal, Biomol Research Laboratories, Plymouth Meeting, PA), phospho-Akt (Ser473) antibody (1:1000, rabbit polyclonal, Cell Signaling Technology), Akt antibody (1:1000, rabbit polyclonal, Cell Signaling Technology).

Analysis of cytosolic cytochrome c and Smac/DIABLO

After drug treatment, 4×10^6 cells were washed in PBS and lysed by incubating for 30 seconds in lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4 , 1 mM NaH_2PO_4 , 1 mM EDTA, and 350 $\mu\text{g/ml}$ digitonin). The lysates were centrifuged at 12,000g for 1 min, and the supernatant was collected in an equal volume of 2x sample buffer.³¹ The proteins were quantified, separated by 15% SDS-PAGE, and subjected to Western blot as described above. Cytochrome c antibody (1:500, mouse monoclonal, Pharmingen) and Smac/DIABLO antibody (1:500, rabbit polyclonal, Upstate Biotechnology) were used as primary antibody.

CDK kinase assay

Following drug treatment, 1×10^7 cells were disrupted in RIPA buffer by repeated aspiration through a 21 gauge needle. Cell extracts were collected by centrifugation at 10,000g for 10 min and protein levels quantified. For each condition, 200 μg protein was immunoprecipitated with 20 μl of cdc2 p34 (mouse monoclonal, Santa Cruz Biotechnology Inc, Santa Cruz, CA) or cdk2 agarose conjugate (rabbit polyclonal, Santa Cruz Biotechnology Inc.) at 4°C overnight. The agarose was washed 4 times with RIPA buffer and incubated in Assay Dilution Buffer containing 400 $\mu\text{g/ml}$ histone H1 (Upstate Biotechnology), 100 μM ATP, 15 mM MgCl_2 and 2 μCi [γ - ^{32}P]ATP at 30°C for 20 min. An equal volume of 2x sample buffer was added in the reaction mixture and boiled for 3 minutes. [γ - ^{32}P]-histone H1 was separated by 12% SDS-PAGE and visualized by exposure of the dried gels to X-ray film (KODAK) at -80 °C for 1 hr.

Cell survival and clonogenic assays

For cell viability assays, CellTiter 96® AQueous One Solution (Promega, Madison, WI) was used as per the manufacturer's instructions, and the absorbance at 490 nm was recorded using a 96 well plate reader (Molecular Devices, Sunnyvale, CA).

Colony forming ability following drug treatment was evaluated using a soft agar cloning assay as described previously.³⁰ Briefly, cells were washed three times with serum-free RPMI medium. Subsequently, 500 cells/well were mixed with RPMI medium containing 20% FBS and 0.3% agar, and plated on 12 well plates (three wells per condition). The plates were then maintained in a 37°C/5% CO_2 , fully-humidified incubator. After 10 days incubation, colonies consisting of >50 cells were scored using an Olympus Model CK inverted microscope, and colony formation for each condition calculated in relation to values obtained for untreated control cells.

Assessment of effects of fibronectin adherence on drug-induced apoptosis

96-well plates were coated with 50 $\mu\text{g/ml}$ human cellular fibronectin (FN, Sigma) overnight, and the wells were washed twice with serum-free RPMI 1640 media. 4×10^4 cells/well were added to FN-coated plates and incubated at 37°C/5% CO_2 for 1 hr in serum-free media, and nonadherent cells were removed by washing the wells twice with serum-free media as previously described.³² FN-adhered cells were then treated with the drugs for 24 hr. The

percentage of apoptotic cells was determined by assessing Wright-Giemsa stained cytospin slides as described above.

Isolation of CD138⁺ myeloma cells

Bone marrow mononuclear cells were obtained with informed consent from patients with multiple myeloma undergoing routine diagnostic aspirations. Cells were collected in syringes containing preservative-free heparin and diluted 1:4 in RPMI 1640 medium. The mononuclear cell fraction was isolated by centrifugation at 400g for 38 min over Histopaque-1077 (Sigma Diagnostics). The interface layer was extracted with a Pasteur pipette and the cells washed twice in buffer (PBS containing 2mM EDTA and 0.5% BSA). The cells were incubated with MACS CD138 Microbeads (Miltenyi Biotec, Auburn, CA) at 4°C for 15 min. CD138⁺ and CD138⁻ cells were separated using an MS⁺/LS⁺ column and a magnetic separator according to the manufacturer's instructions (Miltenyi Biotec).³³ The purity of isolated CD138⁺ cells was assessed by CD138-PE staining and flow cytometry and was determined to be > 90% positive. Viability of the cells was regularly > 95% by trypan blue exclusion. CD138⁺ cells were cultured in RPMI 1640 medium containing 10% FCS in 96-well plates under the same condition described above. In addition, parallel studies were performed utilizing the CD138⁻ cell population. Following drug treatment, the percentage of apoptotic cells was determined by examining Wright-Giemsa stained cytospin slides under light microscopy.

Statistical analysis

For morphological assessment of apoptotic cells, analysis of $\Delta\Psi_m$, and clonogenic and cell survival assays, experiments were repeated at least three times. Values represent the means $\pm$ SD for at least three separate experiments performed in triplicate. The significance of differences between experimental variables was determined using the student's T test.

Results

UCN-01 and PD184352 interact synergistically to promote mitochondrial damage and apoptosis in MM cells

To assess the effects of combined exposure to UCN-01 and MEK inhibitors on MM cell survival, three MM cell lines (8226, H929, and U266) were exposed to 150 nM UCN-01 $\pm$ 10 μ M PD184352 for 24 hr, after which apoptosis was evaluated by morphologic criteria and loss of $\Delta\Psi_m$ determined by monitoring DiOC₆ uptake (Table 1).

Table 1. Effects of Combined Exposure to UCN-01 and PD184352 on Apoptosis and Loss of $\Delta\Psi_m$ in MM Cells

Apoptotic cells (%)	"Low" $\Delta\Psi_m$ (% cells)
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8226		
Control	5.9 ± 2.7	2.8 ± 1.0
PD184352	12.7 ± 2.6	12.6 ± 1.6
UCN-01	9.1 ± 5.1	8.4 ± 1.3
PD+UCN	61.9 ± 5.9	39.6 ± 2.7
H929		
Control	3.6 ± 2.6	3.5 ± 1.4
PD194352	10.0 ± 4.5	8.7 ± 0.1
UCN-01	5.9 ± 1.9	6.8 ± 0.2
PD+UCN	73.8 ± 2.9	44.8 ± 0.9
U266		
Control	3.5 ± 0.8	3.2 ± 0.4
PD194352	10.5 ± 1.7	8.1 ± 0.3
UCN-01	7.5 ± 2.2	8.6 ± 0.1
PD+UCN	56.5 ± 11.6	37.8 ± 8.0

8226, H929, and U266 MM cells were exposed for 24 hr to 10 μ M PD184352 + 150 nM UCN-01 after which the percentage of morphologically apoptotic cells was determined by evaluating Wright Giemsa-stained cytospin preparations as described in Methods (left column). Alternatively, cells were treated as above, after which loss of mitochondrial membrane potential ($\Delta\Psi_m$) was monitored by analyzing DiOC₆-treated cells by flow cytometry as described in Methods (right column). Values correspond to the percentage of cells displaying "low" DiOC₆ uptake. In each case, values represent the means $\pm$ S.D. for three separate experiments performed in triplicate.

In each cell line, drugs administered individually were minimally toxic, whereas combined treatment resulted in a substantial increase in cell death and mitochondrial damage. Concordant results were obtained when apoptosis was monitored by Annexin V/PI staining (data not shown) or the TUNEL assay (Figure 1). In separate studies, sequential exposure to these agents also resulted in an increase in cell death, but simultaneous exposure yielded optimal lethality (data not shown). Similarly, when MM.1S myeloma cells were exposed to other MEK1/2 inhibitors (e.g., 20 μ M U0126 or 50 μ M PD98059) or to 150 nM UCN-01 individually for 24 hr, the percentage of annexin V/PI+ cells was < 10%. However, combined treatment of cells with UCN-01 + PD98059 or U0126 resulted in an increase in the percentage of Annexin V-stained cells to 62 and 77% respectively (data not shown). Consistent with these findings, combined (but not individual) treatment of MM cells with PD184352 and UCN-01 resulted in PARP degradation into an 85 kDa cleavage fragment and release of the pro-apoptotic proteins cytochrome c and Smac/DIABLO into the cytosolic S-100 fraction (Figure 2A). Together, these findings indicate combined treatment with UCN-01 and MEK1/2 inhibitors represents a potent stimulus for mitochondrial damage and apoptosis in MM cells.

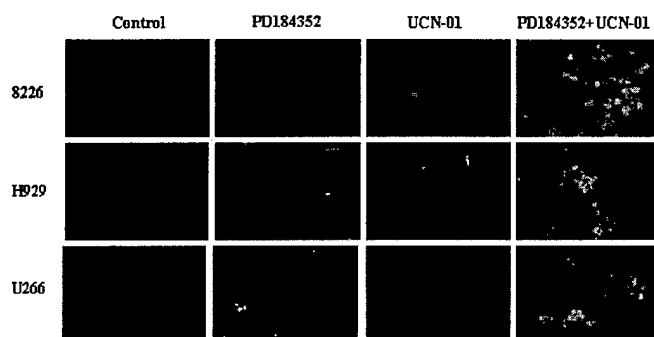


Figure 1. UCN-01 and PD184352 interact synergistically to induce apoptosis in MM cells. Logarithmically growing 8226, H929, and U266 MM cells were exposed to 10 μ M PD184352 $\pm$ 150 nM UCN-01 for 24 hr, after which cytospin preparations were obtained and apoptosis assessed by TUNEL assay as described in Methods. Slides were viewed under fluorescence microscopy under 60x magnification. An additional experiment yielded equivalent results.

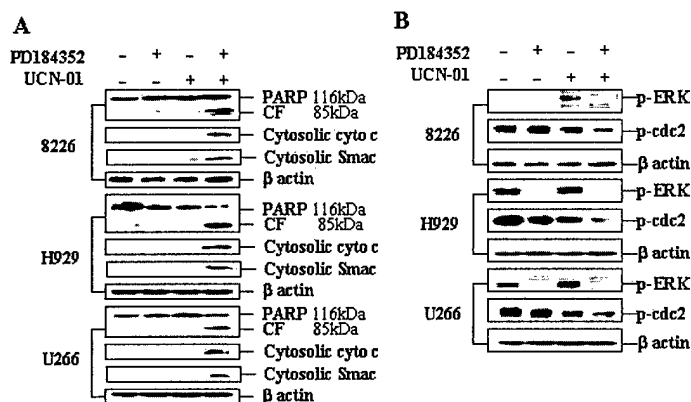


Figure 2. Co-administration of PD184352 and UCN-01 in MM cells results in enhanced cytochrome c and Smac/DIABLO release, PARP degradation, and p34^{cdc2} dephosphorylation, but diminished ERK activation. Logarithmically growing 8226, H929, and U266 MM cells were exposed to 10 μ M PD184352 $\pm$ 150 nM UCN-01 for 24 hr, after which cells were lysed, the proteins separated by SDS-PAGE, and Western blot analysis performed to monitor expression of PARP (A), phospho-ERK (B), or phospho-p34^{cdc2} (B) as described in Methods. Alternatively, S-100 cytosolic fractions were obtained as described in Methods, and expression of cytochrome c and Smac/DIABLO assessed by Western blot analysis (A). For each condition, lanes were loaded with 30 μ g of protein; blots were subsequently stripped and reprobed for expression of β actin to ensure equivalent loading and transfer of protein. The results of a representative experiment are shown; an additional study yielded equivalent results. CF = PARP cleavage fragment.

MEK1/2 inhibition blocks UCN-01-mediated ERK phosphorylation while promoting p34^{cdc2} activation in MM cells

Exposure of 8226 cells to 150 nM UCN-01 for 24 hr resulted in a striking increase in ERK activation, whereas in H929 and U266 cells, which displayed constitutive ERK phosphorylation, the increase was more modest (Figure 2B). However, co-exposure to PD184352 abrogated ERK activation in each cell line, comparable to results initially observed in human myeloid leukemia cells²⁵. UCN-01 also induced dephosphorylation of p34^{cdc2}, consistent with its role as an inhibitor of Chk1¹⁹. Moreover, in each MM cell line, co-administration of PD184352 resulted in a pronounced increase in p34^{cdc2} dephosphorylation. Similar findings were obtained when other MEK1/2 inhibitors (e.g., U0126 and PD98059) were employed (data not shown). These findings indicate that MEK1/2 inhibition blocks UCN-01-mediated ERK activation and markedly potentiates the capacity of UCN-01 to induce dephosphorylation of p34^{cdc2} in MM cells.

To confirm that the diminished phosphorylation of p34^{cdc2} was specific for this CDK and corresponded to increased activity, p34^{cdc2} and CDK2 activities were assessed in drug-treated U266 and MM.1S cells (Figure 3). Consistent with the previous results in which PD184352 was employed, co-administration of U0126 and UCN-01 resulted in a marked decrease in p34^{cdc2} phosphorylation, but no change in total p34^{cdc2} expression. Moreover, immune kinase assays demonstrated a clear increase in p34^{cdc2} activity in cells exposed to both drugs. In contrast, combined exposure to U0126 and UCN-01 did not modify CDK2 phosphorylation status or activity.

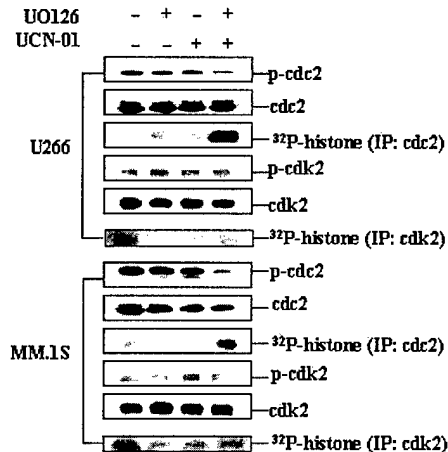


Figure 3. Co-administration of U0126 and UCN-01 in MM cells results in dephosphorylation and increased activation of p34^{cdc2}, but not CDK2. U266 and MM.1S cells were exposed to 20 μ M U0126 $\pm$ 150 nM UCN-01 for 24 hr, after which cells were lysed, the proteins separated by SDS-PAGE, and total/phosphorylated p34^{cdc2} and CDK2 were monitored by Western blot analysis. For each condition, lanes were loaded with 30 μ g of protein. Alternatively, kinase assays were performed after immunoprecipitation with p34^{cdc2}- and CDK2-specific antibodies as described in Methods. The activity of p34^{cdc2} and CDK2 was determined

by monitoring incorporation of γ - ^{32}P into histone H1. The results of a representative experiment are shown; an additional study yielded equivalent results.

Combined exposure to UCN-01/PD184352 potently inhibits the self-renewal capacity of MM cells

To gain insights into the combined effects of UCN-01 and MEK1/2 inhibitors on the survival and self-renewal capacity of MM cells, parallel studies were performed utilizing a pharmacologically relevant concentration of As_2O_3 (i.e., 1 μM), an agent that has shown promising activity against MM cells³⁴ (Figure 4). For each of the endpoints examined (i.e., induction of apoptosis, MTS dye reduction, loss of clonogenicity), the MM cell lines displayed a differential sensitivity to a 48-hr exposure to As_2O_3 , with H929 cells the most sensitive, 8226 cells the least sensitive, and U266 cells displaying an intermediate susceptibility (Figure 4). In particular, 8226 cells were quite resistant to As_2O_3 , in that apoptosis was induced in only ~20% of cells after 48 hr exposure, and clonogenicity was reduced by only ~40%. This general sensitivity pattern was also observed in cells exposed to UCN-01/PD184352. However, in each of the lines, the UCN-01/PD184352 combination was highly toxic, inducing apoptosis in the large majority of cells, and essentially abrogating clonogenic survival.

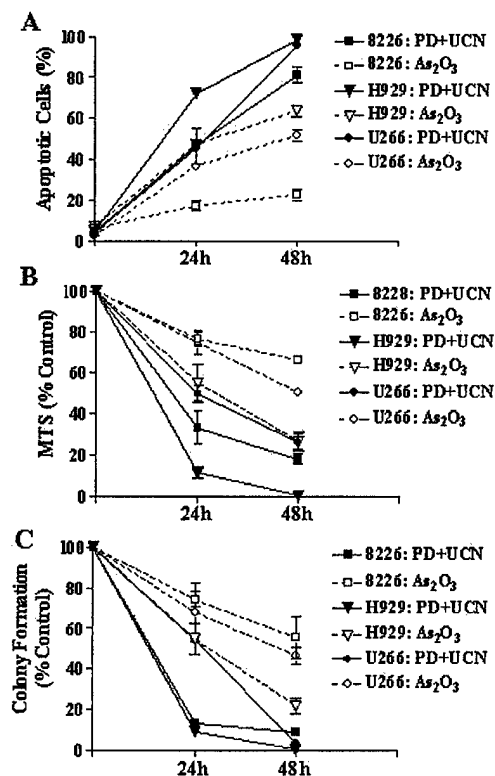


Figure 4. Co-administration of PD184352 and UCN-01 potently induces loss of viability and clonogenic survival in MM cells. 8226, H929, and U266 MM cells were exposed to either 1 μ M As_2O_3 or 10 μ M PD184352 + 150 nM UCN-01 for 24 or 48 hr, after which the extent of morphological apoptosis (A) or loss of viability, reflected by MTS dye reduction (B), was determined as described in Methods. Alternatively, cells were washed free of drug and clonogenic assays performed as described in Methods (C). Values represent the means $\pm$ S.D. for three separate experiments performed in triplicate.

UCN-01/MEK1/2 inhibitor-mediated apoptosis in MM cells, in contrast to that induced by As_2O_3 , is not antagonized by a free radical scavenger

The results of previous studies suggested that As_2O_3 induced apoptosis in MM cells through generation of reactive oxygen species (ROS).³⁵ To determine whether a similar mechanism might be responsible for UCN-01/MEK1/2 inhibitor-induced apoptosis, MM cells were exposed to these agents for 48 hr in the presence or absence of the free radical scavenger L-NAC³⁶, after which apoptosis was assessed (Figure 5). Co-administration of L-NAC essentially abrogated the lethal effects of As_2O_3 in each of the MM cell lines, consistent with the results of earlier studies³⁵. In marked contrast, L-NAC exerted no effect on UCN-01/MEK1/2 inhibitor-mediated lethality. These findings suggest that the lethal actions of UCN-01/MEK1/2 inhibitors in MM cells involve factors other than or in addition to generation of reactive oxygen species.

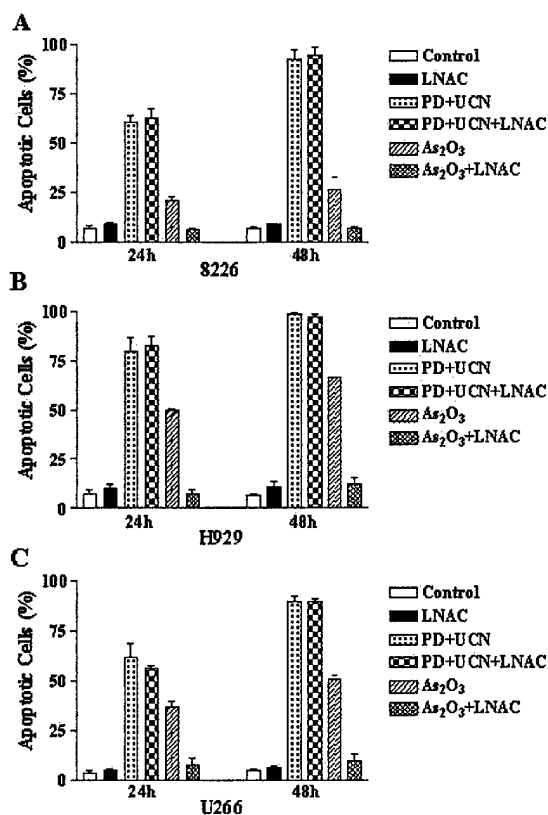


Figure 5. The free radical scavenger L-NAC blocks As_2O_3 - but not PD184352/UCN-01-induced apoptosis in MM cells. 8226 (A), H929 (B), and U266(C) MM cells were exposed to either 1 μM As_2O_3 or 10 μM PD184352 + 150 nM UCN-01 for 24 or 48 hr after 2 hr pretreatment of 10 mM L-NAC, after which the percentage of apoptotic cells was determined by evaluating Wright-Giemsa-stained cytopsin preparations as described in Methods. Values represent the means $\pm$ S.D. for three separate experiments performed in triplicate.

UCN-01/MEK1/2 inhibitor-induced apoptosis proceeds through IL-6- and IGF-1-independent pathways

In addition to its role in promoting MM cell survival, IL-6 has also been shown to protect MM cells from the lethal actions of cytotoxic agents, including dexamethasone.³⁷ To determine whether and to what extent IL-6 might exert a similar function in cells exposed to UCN-01/MEK1/2 inhibitors, MM cells were exposed to these agents for 72 hr in the presence or absence of 100 ng/ml IL-6 (Figure 6). Consistent with earlier results³⁷, exogenous IL-6 essentially abrogated the lethal effects of 10 μM dexamethasone in each of the MM cell lines, reflected by loss of viability as determined by the MTS assay. Similar results were obtained when apoptosis was monitored (data not shown). In marked contrast, IL-6 failed to attenuate UCN-01/PD184352-mediated lethality in any of the MM cell lines. Similarly, IGF-1 (400 ng/ml), which as also been shown to act as survival factor in MM³⁷, was unable to block apoptosis in UCN-01/MEK1/2 inhibitor-treated cells (Figure 6D). These findings indicate that the lethal actions of the UCN-01/MEK1/2 inhibitor combination in MM cells operate downstream of IL-6 and IGF-1 cytoprotective pathways.

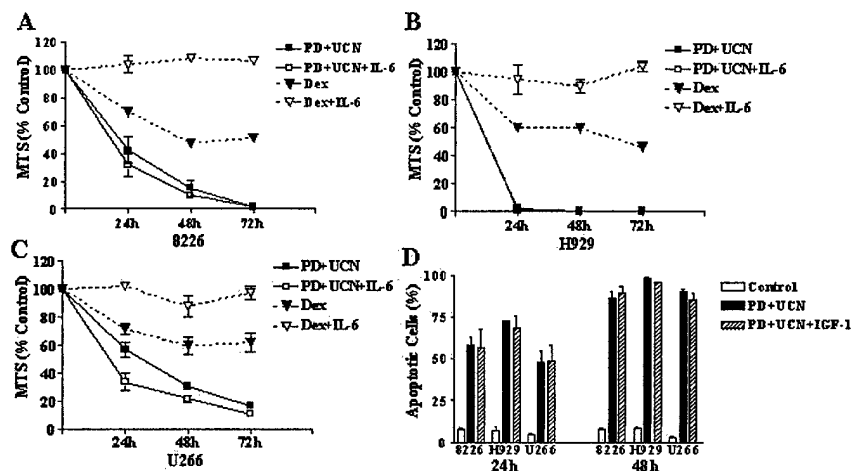


Figure 6. PD184352/UCN-01-induced apoptosis proceeds via IL-6- and IGF-1-independent pathways in MM cells. 8226 (A), H929 (B), and U266 (C) MM cells were exposed to 10 μM PD184352 + 150 nM UCN-01 or 10 μM dexamethasone for 24 to 72 hr in the presence or

absence of 100 ng/ml IL-6, after which the loss of viability, reflected by MTS dye reduction, was determined as described in Methods. Alternatively, cells were exposed to PD184352 + UCN-01 for 24 or 48 hr in the presence or absence of 400 ng/ml IGF-1, after which the percentage of apoptotic cells was determined by examining Wright-Giemsa-stained specimens as described in Methods (D). In all cases, values represent the means $\pm$ S.D. for three separate experiments performed in triplicate.

IL-6 fails to block UCN-01/MEK1/2 inhibitor-induced mitochondrial damage in MM cells

To determine whether the failure of IL-6 to attenuate UCN-01/PD184352-induced apoptosis in MM cells originated at the mitochondrial level, cytochrome c and Smac/DIABLO release were monitored in cells exposed to these agents (Figure 7A). In each of the MM cell lines, co-administration of IL-6 with UCN-01/PD failed to attenuate cytochrome c and Smac/DIABLO release, or PARP degradation. These findings suggest that UCN-01/MEK1/2 inhibitor-induced mitochondrial damage occurs independently of IL-6-related cytoprotective signaling pathways.

UCN-01/MEK1/2 inhibitor-induced apoptosis occurs in IL-6-treated MM cells despite Akt activation

Recent studies suggest that in MM cells, IL-6-related cytoprotective effects involve activation of the PI3K/Akt cascade.⁹ Consistent with these results, exposure of each of the MM lines to IL-6 resulted in Akt phosphorylation (Figure 7B). However, despite the failure of IL-6 to attenuate UCN-01/MEK1/2 inhibitor-induced apoptosis in MM cells (Figure 5), IL-6 administration continued to increase Akt phosphorylation over basal levels in UCN-01/PD184352-treated cells. IL-6 also increased ERK activation in each MM cell line, but this effect was abrogated in cells exposed to UCN-01/PD184352 (Figure 7B). Lastly, co-administration of the PI3K inhibitor LY294002 (10 μ M) failed to modify UCN-01/PD184352-mediated lethality in any of the MM cell lines, although slight increases in UCN-01-induced apoptosis were noted (data not shown). Together, these findings argue that factors other than or in addition to perturbations in the PI3K/Akt pathway are responsible for MM cell death following exposure to UCN-01/MEK1/2 inhibitors.

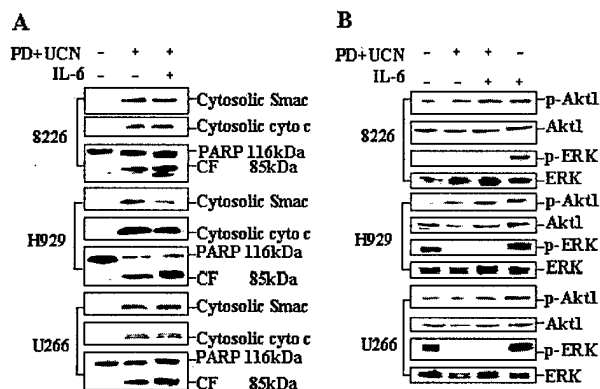


Figure 7. IL-6 fails to block PD184352/UCN-01-mediated mitochondrial damage despite enhancing Akt phosphorylation. (A) 8226, H929, and U266 MM cells were exposed to 10 μ M PD184352 + 150 nM UCN-01 in the presence or absence of 100ng/ml IL-6 for 24 hr after which cells were lysed and Western blot analysis performed to monitor PARP cleavage (whole cell lysates) or cytochrome c and Smac/DIABLO release (S-100 fractions) as described in Methods. (B) Alternatively, Western blot analysis was performed to assess the effects of these agents on phosphorylation of Akt and ERK as well as total ERK expression. In each case, lanes were loaded with 30 μ g of protein. The results of a representative experiment are shown; a second study yielded equivalent results.

UCN-01/PD184352 effectively induces apoptosis in MM cells resistant to doxorubicin or melphalan

To determine whether MM cells resistance to standard chemotherapeutic drugs would extend to the UCN-01/MEK1/2 inhibitor combination, doxorubicin-resistant (8226/Dox40)²⁷ and melphalan resistant (8226/LR5)²⁸ cells were treated with 10 μ M PD184352 ∇ 150 nM UCN-01 for 24-72 hr, after which the extent of apoptosis was assessed (Figure 8). Whereas sensitive cells (8226/S) were highly susceptible to 400 nM doxorubicin and 5 μ M melphalan (Figure 8A), the corresponding resistant lines were essentially immune to these agents (Figures 8B and 8C). Equivalent results were obtained when loss of $\Delta\Psi_m$ was monitored (data not shown). In marked contrast, both 8226/Dox40 and 8226/LR5 cells were as sensitive to the PD184352/UCN-01 combination as parental cells, with approximately 100% of cells undergoing apoptosis after 72 hr. Western analysis confirmed that induction of cytochrome c/Smac/DIABLO release and PARP degradation by the combination of UCN-01 and PD184352 in 8226/Dox40 and 8226/LR5 cells (Figure 8D) was equivalent to effects observed in parental cells (Figure 3A). These findings indicate that MM cells highly resistant to doxorubicin or melphalan remain fully sensitive to mitochondrial injury and apoptosis induced by UCN-01/PD184352.

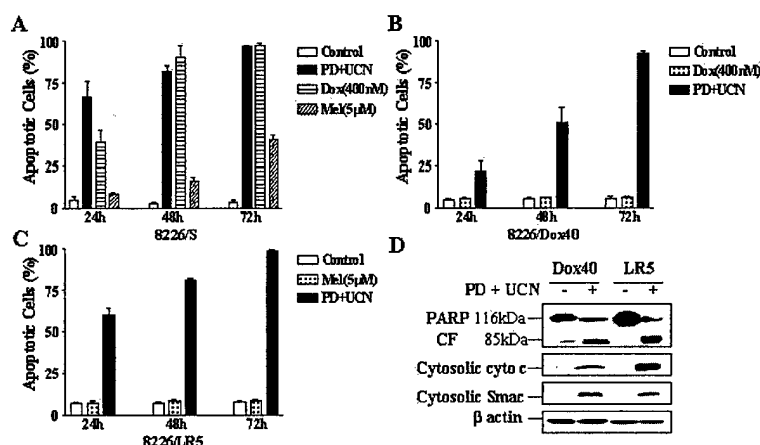


Figure 8. MM cells resistant to doxorubicin or melphalan retain full sensitivity to UCN-01/PD184352. Logarithmically growing parental 8226 cells (8226/S; A), a doxorubicin-resistant

cell line (8226/Dox40, B), and a melphalan resistant line (8226/LR5, C) were exposed to 10 μ M PD184352 + 150 nM UCN-01, 400 nM doxorubicin (Dox), or 5 μ M melphalan (Mel) for 24-72 hr, after which the percentage of apoptotic cells was determined by examining Wright-Giemsa-stained cytopsin preparations as described in Methods. values represent the means $\pm$ S.D. for three separate experiments performed in triplicate. (D) 8226/Dox40 and 8226/LR5 cells were exposed to PD184352 + UCN-01 as above for 48 hr and 24 hr, respectively, after which Western blot analysis was employed to monitor degradation of PARP into an 85 kDa fragment (CF) or release of cytochrome c and Smac/DIABLO into the cytosolic S-100 fraction as described above. Lanes were loaded with 30 μ g of protein; blots were subsequently stripped and reprobbed for expression of β actin to ensure equivalent loading and transfer of protein. The results of a representative experiment are shown; an additional study yielded equivalent results.

MM cells highly resistant to dexamethasone retain their sensitivity to combined treatment with UCN-01 and PD184352

Using a previously described dexamethasone-resistant myeloma cell line (MM.1R)²⁶, attempts were made to determine whether cross-resistance to the lethal effects of UCN-01/PD184352 would occur. As shown in Figures 9A and 9B, MM.1R cells were highly resistant to dexamethasone-induced apoptosis, but retained full sensitivity to apoptosis induced by 150 nM UCN-01 + 10 μ M PD184352. Furthermore, while IL-6 significantly attenuated dexamethasone-induced apoptosis, it had no effect on PD184352/UCN-01-induced cell death in either cell line. Similar results were obtained when U0126 was employed instead of PD184352 (data not shown). Concordant results were also obtained when the reduction in $\Delta\Psi_m$ was monitored (Figure 9C).

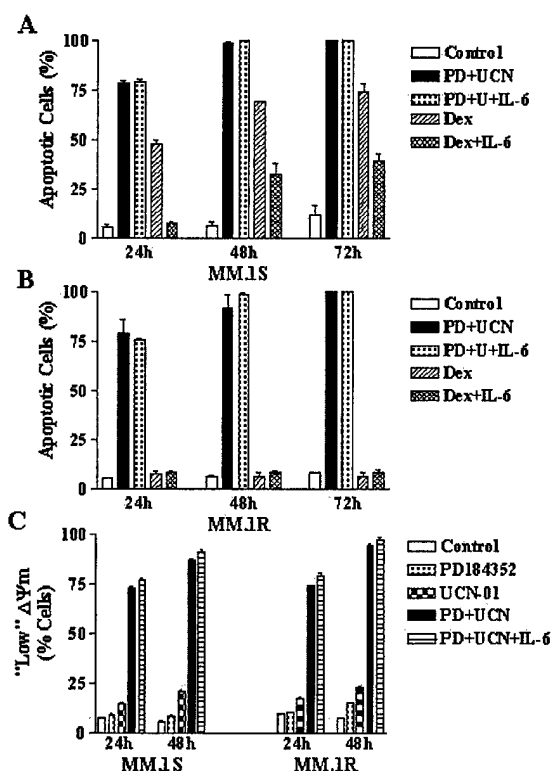


Figure 9. IL-6 fails to protect dexamethasone-sensitive and -resistant MM cells from the lethal actions of UCN-01/PD184352. Logarithmically growing dexamethasone-sensitive (MM.1S; Figure 9A) and -resistant MM cells (MM.1R; Figure 9B) were exposed to 10 μ M PD184352 + 150 nM UCN-01 or 10 μ M dexamethasone in the presence or absence of 100 ng/ml IL-6 for 24-72 hr, after which the percentage of apoptotic cells was determined by examining Wright-Giemsa-stained cytopsin preparations. Alternatively, MM.1S and MM.1R cells were treated as above for 24 or 48 hr, after which the percentage of cells displaying a reduction in $\Delta\Psi_m$ was determined as described in Methods (Figure 9C). Values represent the means $\pm$ S.D. for three separate experiments performed in triplicate.

Finally, consistent with results obtained in other MM cell lines (Figure 2B), both MM.1S and MM.1R cells displayed activation of MAP kinase following UCN-01 exposure, an effect that was abrogated by PD184352. Moreover, the combination of UCN-01 and PD184352, but not drugs administered alone, induced an equivalent increase in cytosolic release of cytochrome c and Smac/DIABLO as well as PARP degradation in dexamethasone-sensitive and resistant MM cells (data not shown). These results indicate that a high level of resistance to dexamethasone provides MM cells essentially no protection from the mitochondrial injury and lethality of the UCN-01/MEK1/2 inhibitor combination.

The UCN-01/PD18432 regimen is active against MM cells exhibiting cell adherence-associated drug resistance

Based upon recent evidence that induction of MM cell adherence (e.g., by fibronectin) confers resistance to multiple classes of cytotoxic agents³², attempts were made to determine whether such treatment would protect MM cells from exposure to UCN-01/PD184352. As shown in Figure 10, treatment of MM.1S and MM.1R with fibronectin significantly reduced the toxicity of both doxorubicin and melphalan, consistent with results previously reported by Damiano et al., in 8226 MM cells³². However, in marked contrast to these findings, fibronectin-adhered cells remained fully sensitive to PD184352/UCN-01-mediated apoptosis. These observations suggest that the lethal effects of checkpoint abrogation/MEK1/2 inhibition, unlike conventional cytotoxic agents, are minimally attenuated by cytoprotective signaling events associated with the adherent phenotype.

Co-administration of UCN-01 with a MEK1/2 inhibitor displays selective lethality toward primary bone marrow CD138⁺ MM cells

To determine whether the UCN-01/MEK inhibitor strategy would be lethal toward primary MM specimens, CD138⁺ MM cells were isolated from the bone marrow of MM patients as previously described³³. Samples #1 and #2 were obtained from patients who had progressed following therapy, whereas sample #3 was obtained from a newly-diagnosed patient. The cells were then exposed to U0126 $\pm$ UCN-01 for 24 hr, after which the extent of apoptosis was determined by morphological analysis of Wright Giemsa-stained cytopsin preparations. As shown in Figure 11A-C, co-administration of U0126 + UCN-01 resulted in a clear increase in apoptosis in each of the three primary specimens evaluated. Interestingly, U0126 + UCN-01 exerted relatively minimal toxicity toward the CD138⁻ cell population, raising the possibility that

primary MM cells may exhibit selective sensitivity to this regimen. Representative photomicrographs of pre- and post-treatment MM specimens (obtained from patient #2; Figure 11D) illustrate the striking increase in MM cell death following *ex vivo* UCN-01/U0126 exposure.

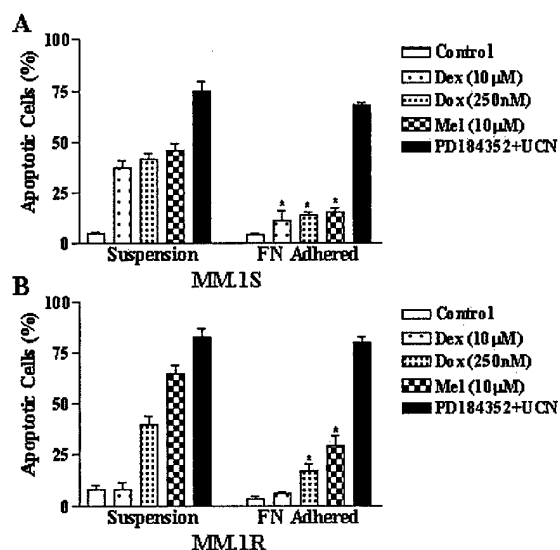


Figure 10. Fibronectin-adhered MM cells remain sensitive to UCN-01/PD184352-induced apoptosis. MM.1S and MM.1R cells were seeded into 96 well plates coated with fibronectin, and cells remaining in suspension removed as described in Methods. Adherent cells and cells in suspension were separately exposed to 250 nM doxorubicin, 10 μM melphalan, 10 μM dexamethasone, or 10 μM PD184352 + 150 nM UCN-01 for 24 hr, after which the extent of apoptosis was determined by evaluating Wright Giemsa-stained cytospin preparations as described previously. Values represent the means $\pm$ S.D. for three separate experiments performed in triplicate. * = significantly lower than values for cells in suspension; $P < 0.01$.

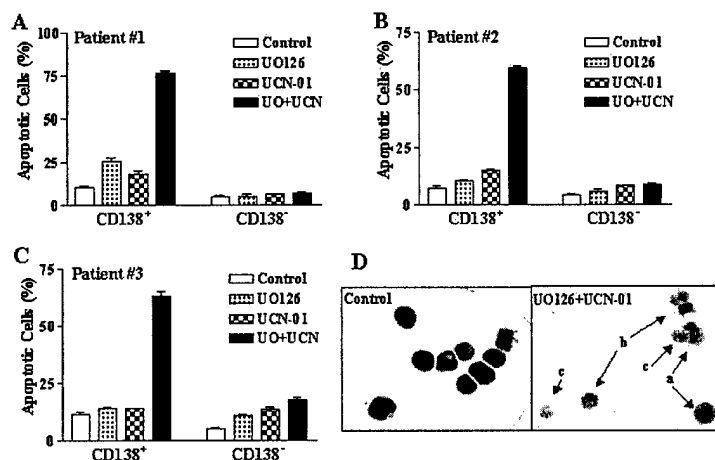


Figure 11. Primary CD138⁺ MM cells display selective sensitivity to UCN-01/UO126-induced apoptosis. CD138⁺ and CD138⁻ cells were isolated from the bone marrow of MM patients as described in Methods. The cells were exposed individually or in combination to drugs as follows, UO126 (A: 25 μ M; B and C: 20 μ M); UCN-01 (A: 150 nM; B: 250 nM; C: 100 nM). After 24 hr treatment, the extent of apoptosis was determined by evaluating Wright Giemsa-stained cytospin preparations as described previously. Values represent the means $\pm$ S.D. for more than 20 randomly selected fields encompassing more than 300 cells. Representative microphotographs of cells obtained from patient #2 are shown in D, a: early apoptotic cells; b: late apoptotic cells; c: post-apoptotic (ghost) cells.

Discussion

The present results indicate that combined exposure to a clinically relevant concentration of UCN-01 in conjunction with pharmacologic MEK1/2 inhibitors potently induces programmed cell death in MM cells, including those selected for resistance to a variety of agents useful in the treatment of this disease, including melphalan, doxorubicin, and dexamethasone. Despite recent advances in our understanding of the molecular pathogenesis of MM, survival of patients with this disorder has not changed appreciably over the last several decades.^{1,2} Consequently, attention has begun to focus on the development of novel agents that target specific MM survival pathways. These have included thalidomide, an inhibitor of the VEGF signaling cascade³⁸, proteasome inhibitors such as PS341, which interfere with NF κ B cytoprotective functions³⁹, and As₂O₃, which promotes the formation of lethal free radical species in MM cells³⁵. However, the concept of combining novel signal transduction and cell cycle inhibitors in MM or other hematologic malignancies remains relatively unexplored. The data presented here indicate that combined treatment with UCN-01, a checkpoint abrogator in clinical evaluation²³ and pharmacologic MEK1/2 inhibitors, including PD184352, which is now in Phase I trials¹⁷, represents an extremely potent stimulus for mitochondrial injury and apoptosis in MM cells. Taken in conjunction with observations involving myeloid leukemia cells²⁵, these findings raise the possibility that malignant hematopoietic cells may be particularly susceptible to a strategy in which cell cycle regulatory and cytoprotective signaling pathways are simultaneously interrupted.

While the mechanism(s) by which MEK1/2 inhibition potentiates UCN-01-induced apoptosis in MM cells remains to be defined, it is tempting to speculate that inappropriate activation of p34^{cdc2} may be involved. For example, consistent with its role as an inhibitor of Chk1¹⁹, UCN-01 induced dephosphorylation (activation) of p34^{cdc2} in each of the MM cell lines (Figure 2). Moreover, this event was enhanced by co-administration of PD184352. As unscheduled activation of p34^{cdc2} in hematopoietic cells represents a potent stimulus for apoptosis⁴⁰, the cytoprotective actions of MAP kinase activation may serve to limit this process. Such a concept could help to explain the observed potentiation of p34^{cdc2} dephosphorylation and activation in UCN-01/PD184352-treated MM cells. In this context, a requirement for MAP kinase activation in G₂M progression, an event that is regularly accompanied by p34^{cdc2} activation⁴¹, would be fully consistent with this model.

Although activation of the MAP kinase pathway is generally known to exert anti-apoptotic actions¹⁰, in MM cells it appears to play a specific role in IL-6-related cytoprotection. Moreover, the ability of UCN-01/MEK1/2 inhibitors to induce MM cell death in an IL-6-independent manner is entirely compatible with this notion. For example, the finding that the MEK1/2 inhibitor PD98059 prevents IL-6 from protecting OPM-6 MM cells from dexamethasone-mediated cell death³⁷ implicates the MAP kinase pathway in IL-6 survival functions. It also suggests that activation of the latter pathway represents a downstream consequence of IL-6 actions. However, while administration of IL-6 resulted in MAP kinase activation in each of the MM cell lines examined here (Figure 7), this effect was abrogated in UCN-01/PD184352-treated cells. Thus, administration of MEK1/2 inhibitors blocked MAP kinase activation by exogenous IL-6, UCN-01, as well as the combination of these agents. It should be noted that recently histone deacetylase inhibitors (HDIs) have been shown to trigger apoptosis in MM cells in an IL-6-independent manner⁴². The relationship between this phenomenon and effects on MAP kinase activation remains to be defined. Finally, the cytoprotective effects of IL-6 and IGF-1 in MM cells have recently been related to the PI3K/Akt pathway⁹. The ability of UCN-01/PD184352 to trigger MM cell apoptosis in the presence of IL-6 and despite Akt activation suggests that these agents act independently of, or at a point downstream of Akt actions.

The inability of the free radical scavenger L-NAC to block UCN-01/PD184352-induced apoptosis in MM cells argues that this process proceeds through an ROS-independent mechanism. Although ROS generation has been implicated in the induction of apoptosis by various cytotoxic agents⁴³, in some cases it may represent a secondary event stemming from mitochondrial injury and disruption of the mitochondrial respiratory chain⁴⁴. It is noteworthy that As₂O₃, which also potently induces apoptosis in MM cells, including those resistant to conventional cytotoxic agents, appears to trigger cell death through a ROS-dependent process³⁵. The finding that UCN-01/PD184352 also effectively induced cell death in drug-refractory MM cell lines indicates circumvention of conventional drug resistance in MM cells can involve apoptotic pathways operating independently of ROS.

Previous studies have demonstrated that in MM cells, cell death induced by different stimuli can elicit distinct forms of mitochondrial injury. For example, in MM.1S cells, ionizing radiation primarily induced cytochrome c release, whereas dexamethasone triggered release of Smac/DIABLO⁴⁵. In contrast, UCN-01/PD184 induced, although to varying extents, cytosolic distribution of both cytochrome c and Smac/DIABLO in each of the MM cell lines. The relative contributions of these proteins to UCN-01/PD184352-induced lethality is unclear. In this context, recent studies in prostate cancer cells suggest that Smac/DIABLO release following PI3K/Akt inhibitor LY294002 exposure is necessary for cytochrome c-mediated activation of the apoptotic cascade⁴⁶. Whether similar events occur in MM cells remains to be determined.

Resistance of 8226/Dox40 cells to doxorubicin has been attributed to increased expression of P-glycoprotein (Pgp), leading to reduced intracellular drug accumulation²⁷. Although enhanced expression of Pgp has been shown to reduce, albeit weakly, uptake of small molecule inhibitors such as flavopiridol⁴⁷, it has been found to be somewhat more effective in diminishing the toxicity of UCN-01 in MCF-7 breast cancer cells⁴⁸. However, the finding that Dox40 MM cells were fully sensitive to UCN-01/PD184352 toxicity argues that Pgp-related mechanisms do not play a major role in determining the response of MM cells to either of these agents. Resistance of MM.1R cells to dexamethasone has been attributed to a mutation in the steroid receptor²⁶. In view of the presumed distal site of action for the UCN-01/PD184352

combination (i.e., downstream of IL-6/MEK/MAP kinase signaling), it seems likely that this regimen simply bypasses the block to steroid action. The basis for alkylating agent resistance in MM or other neoplastic cells may be multifactorial, including diminished drug uptake, decreased DNA binding, increased GSH levels, or enhanced repair mechanisms, among others⁴⁹. The unimpaired ability of UCN-01/PD184352 to induce apoptosis in 8226/LR5 cells suggests that none of these mechanisms is involved in conferring resistance to this drug combination. Finally, the phenomenon of cell adhesion-mediated drug resistance (CAM-DR) has recently been described in MM cells, suggesting that integrins can raise the threshold for drug-induced apoptosis³². Although the mechanism underlying this phenomenon remains to be elucidated, the present findings indicate that the lethal effects of the UCN-01/PD184352 combination operate independently of adhesion-related survival pathways.

In summary, the present findings indicate that combined treatment with the checkpoint abrogator UCN-01 and the MEK1/2 inhibitor PD184352, both of which are currently in clinical trials, represents a highly potent trigger for mitochondrial damage and apoptosis in MM cells. These events are associated with inhibition of UCN-01-mediated activation of MAP kinase, and enhanced activation of p34^{cdc2}. They also indicate that UCN-01/PD184352-mediated cell death proceeds in an IL-6-independent manner, and in contrast to As₂O₃, is not inhibitable by free radical scavengers. In addition, this drug combination effectively induces apoptosis in MM cells that are resistant to three cytotoxic agents widely used in the MM treatment i.e., dexamethasone, melphalan, and doxorubicin, as well as in cells displaying adhesion-related drug resistance. Finally, this strategy is active against at least some primary MM cell samples *ex vivo*. A theoretical model that might provide a framework for understanding the present findings is illustrated in Figure 12. UCN-01, by inhibiting Chk1, spares cdc25C from proteasomal degradation, thereby activating p34^{cdc2}, which is known to be a potent apoptotic stimulus⁴⁰. The compensatory activation of MEK1/2 and ERK1/2, which lie downstream of IL-6, IGF-1, and integrin-stimulated PKC/Ras/Raf-related pathways, may permit cells to survive the inappropriate activation of p34^{cdc2} in UCN-01-treated cells. Conversely, abrogation of the compensatory ERK1/2 response might render cells particularly sensitive to UCN-01-induced mitochondrial injury and apoptosis. Such a model could also account for the failure of effectors such as IL-6, IGF-1, and integrins, which signal upstream of the Ras/Raf/MEK/MAP kinase cascade, to circumvent UCN-01/MEK1/2 inhibitor-associated lethality. However, the possibility that MEK inhibitors may induce perturbations in NFκB, which has been implicated in MM cell survival decision³⁹, cannot be excluded, nor can a direct effect of MEK inhibitors on cdc25C activity be ruled out. In any case, the present findings suggest that MM cells may be highly sensitive to a therapeutic strategy in which cell cycle regulatory and survival signaling pathways are simultaneously disrupted. However, the issue of therapeutic selectivity will clearly play an important role in determining the ultimate utility of this approach. In this regard, we have recently observed that UCN-01/MEK1/2 inhibitor regimens exhibit relatively modest toxicity toward normal human peripheral blood mononuclear cells, bone marrow CD34⁺ cells, primary rodent hepatocytes, and in this study, CD138⁺ bone marrow cells.⁵⁰ In view of these considerations, efforts to develop this strategy in MM and possibly in other B-cell malignancies appear warranted.

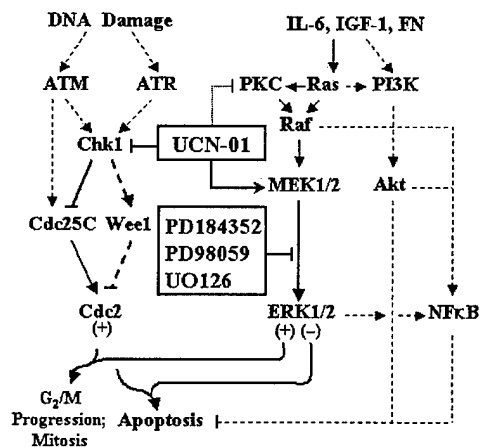


Figure 12. Model of UCN-01 and MEK1/2 inhibitor interactions in MM cells. The DNA damage response genes ATM and ATR activate Chk1, which phosphorylates the Cdc25C phosphatase, leading to its proteasomal degradation. Inhibition of Chk1 phosphorylation results in activation of p34^{cdc2}, which, if unscheduled, leads to apoptosis. UCN-01, by inhibiting Chk1 phosphorylation, spares Cdc25C, which in turn promotes activation (dephosphorylation) of p34^{cdc2}. The putative pro-apoptotic actions of activated p34^{cdc2} may be opposed by a compensatory activation of the cytoprotective Raf/MEK/MAP kinase cascade, which is also stimulated by several MM survival factors, including IL-6, IGF-1, and integrins. Blocking the MEK/MAP kinase cascade (e.g., by pharmacologic MEK1/2 inhibitors) downstream of IL-6-, IGF-1-, and integrin-related actions (e.g., fibronectin, FN) may thus render MM cells particularly vulnerable to the lethal actions of UCN-01. The contribution of the NFκB axis, which is linked to both ERK1/2 and PI3K/Akt (dashed lines), to these events remains to be fully elucidated. Finally, the possibility that MEK1/2 inhibitors act directly on cdc2 regulatory molecules (i.e., cdc25C or Wee1) cannot be excluded.

Acknowledgements

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