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# *N*-Acetylcysteine enhances UV-mediated caspase-3 activation, fragmentation of E2F-4, and apoptosis in human C8161 melanoma: inhibition by ectopic Bcl-2 expression

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**Abstract**

Redox imbalance due to oxidative stress or excessive antioxidant levels can alter apoptotic responses. Recently, antioxidants like *N*-acetylcysteine (NAC) were reported to inhibit $H_2O_2$-mediated necrotic cell death, although they were inactive against apoptosis induced by other agents like etoposide. NAC was also found to kill preferentially tumor cells compared to normal fibroblasts at 20–50 mM, but these concentrations are lethal to normal splenocytes. We now demonstrate that 10 mM NAC, a non-toxic concentration, can enhance the UV radiation-mediated apoptosis of human C8161 melanoma cells. Compared to treatment with UV radiation alone, combination treatment with NAC doubled the ratio of activated caspase-3 to pro-caspase-3 and produced greater fragmentation of the retinoblastoma protein and the E2F-4 transcription factor without affecting the E2F-1 protein. These effects of joint NAC–UV radiation treatment were counteracted by the overexpression of the *bcl-2* gene. To our knowledge, this report is the first to: (i) demonstrate a synergy between DNA-damaging agents, like UV radiation, and antioxidants, like NAC, and (ii) show that a Bcl-2-inhibitable E2F-4 fragmentation occurs concurrently with caspase-3 activation and apoptosis.


*Keywords:* E2F-4 fragmentation; Caspase-3 substrates; E2F-1 induction; Apoptosis

## 1. Introduction

In contrast to necrosis, which causes extensive damage to the plasma membrane [1], apoptosis frequently involves the activation of caspase-3, accompanied by cleavage of substrates like PARP [2], and the cell cycle regulating pRb [3,4]. Antioxidants like NAC, which prevent apoptosis in lymphocytes, neurons, and vascular endothelial cells, were reported previously to induce apoptosis in rat and human smooth muscle cells [5]. Also, a 48-hr treatment with 20 mM NAC was shown to induce p53-mediated apoptosis in the murine papilloma 308 cell line and other transformed mouse fibroblasts [6]. However, the addition of only 5 mM NAC induced $G_1$ cell cycle arrest in the same murine papilloma 308 cell line and in $p21^{WAF1}$-null and *p53*-null cell lines, and shorter treatments with 20 mM NAC caused reversible growth arrest in the murine papilloma 308 cell line [7]. More recently, NAC was found to inhibit $H_2O_2$-mediated necrotic cell death [1,2], although it was inactive against apoptosis induced either by flavonoids like wogonin and fisetin [2] or by VP-16 (etoposide) [1], known to act through pro-oxidant mechanisms towards intracellular thiols [8]. Selective toxicity of NAC at concentrations between 20 and 50 mM against several human tumor cells rather than normal fibroblasts was also reported [9]. However, such concentrations are known to be toxic to normal splenocytes [10]. Many toxic effects of DNA-damaging agents, like ionizing radiation [11] and ultraviolet (UV) radiation [12], are associated with the generation of reactive oxygen species [11,12]. Since NAC counteracts the necrotic effects of pro-oxidants [1,2], we decided to use non-toxic levels of NAC, a scavenger of free radicals [1,2], together with UV radiation, to manipulate the necrotic and apoptotic effects of UV radiation on human C8161 melanoma cells to one that favors apoptosis [13]. Since the apoptotic response to UV radiation specifically involves

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*Abbreviations:* NAC, *N*-acetylcysteine; PARP, poly(ADP ribose) polymerase; pRb, retinoblastoma protein.

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caspase-3 activation and PARP fragmentation [14,15], we used these markers as evidence of the apoptotic response in genetically matched C8161 melanoma cells. These cells were genetically engineered to overexpress the anti-apoptotic *bcl-2* gene [13] and were used in this study to investigate whether changes in PARP fragmentation and caspase-3 activation could be counteracted by the overexpression of this gene [13].

The tumor suppressing pRb is one of the known caspase-3 substrates during apoptosis [3,4]. Since NAC levels capable of causing apoptosis in colorectal carcinoma cells also induce pRb cleavage [16], we investigated whether UV radiation and/or NAC-mediated changes in caspase-3 activation also involved the degradation of E2F-1 and E2F-4; both are known to bind the pRb and to be key regulators of transcription during the cell cycle [17,18]. E2F-4 can switch to an interaction with pRb, when the latter is phosphorylated to decrease its interaction with E2F-1 [18]. Subsequently, the release of E2F-1 results in the formation of an E2F-4/pRb complex, a repressor of E2F-1 mRNA transcription [17,18]. Additionally, binding of E2F-1 and E2F-4 to the pRb protects the latter from degradation [17,18]. E2F-4 and E2F-1 have similar proliferative properties but different apoptotic and oncogenic properties *in vivo* [19,20]. In different cell types exposed to alternative inducers of apoptosis, the level of E2F-1 has been shown to increase with cell death [21,22]. Our results show for the first time that NAC synergizes with UV radiation to induce E2F-4 fragmentation concurrently with caspase-3 activation. These effects are counteracted by the overexpression of the anti-apoptotic *bcl-2* gene [13]. The effect of NAC appeared to be different from that of UV radiation, since the former has shown a distinctive ability to lower the phosphorylation of UV-induced ERK/MAPK and JNK kinases [23], whereas the latter is known to induce apoptosis-associated caspase-3 activation and fragmentation of PARP [14,15].

## 2. Materials and methods

### 2.1. Cells and treatments

These studies were carried out with human C8161 melanoma cells [13] that were transduced with either the control, puromycin-resistant, LPC retroviral plasmid, or LPC harbouring the *bcl-2* gene (provided by Dr. Scott Lowe of Cold Spring Harbor Laboratory) [24]. These retroviral plasmids were propagated by transfection into Phoenix retroviral-packaging cells (provided by Dr. Gary P. Nolan, Department of Molecular Pharmacology, Stanford University) using the $CaCl_2$ method [24]. The retroviral particles, i.e. the empty LPC vector and LPC containing the *bcl-2* gene, were used to infect the parental C8161 melanoma cells. Following infection, the cells harbouring either of theretroviruses were selected by using 2 μg/mL of puromycin [24].

Subconfluent LPC-*bcl-2*- and LPC vector-transduced melanoma cells were exposed 1 day after seeding for 2 or 24 hr to 10 mM NAC and/or UV-C irradiation (10 J/m$^2$) for comparison with control cultures. UV irradiation was carried out in PBS in a UV crosslinker apparatus (model RPN 2500/2501, Amersham Life Science) that incorporates a sensor to monitor UV energy output at 254 nm. The setting for our experiments was adjusted to provide an output of 10 J/m$^2$.

### 2.2. Assessment of growth arrest and apoptosis

Growth arrest/cytotoxicity was estimated in 96-well tissue culture microtiter plates initially seeded with $7.5 \times 10^3$ cells in 1:1 DME/F12 medium supplemented with 10% fetal bovine serum (Gibco-BRL-Life Technologies). NAC was added to the cultures at concentrations ranging from 2.5 to 20 mM, 4 hr before UV irradiation (10 J/m$^2$). Subsequently, the cells were incubated for an additional 30 hr at 37° before the addition of one-fifth volume of Alamar blue, which was left on the cells for 4 hr. The fluorometric estimation of growth arrest/cytotoxicity was done using a Fluoroskan Accent fluorimeter (Labsystems) set at an excitation wavelength of 530 nm and an emission wavelength of 590 nm [25].

Relative DNA fragmentation [26] was determined using subconfluent cultures in Lab-Tek chamber slides and exposing them to 10 mM NAC and/or UV radiation (10 J/m$^2$). After treatment, the cells were fixed in 4% *p*-formaldehyde in PBS for 20 min. Subsequently, relative nuclear DNA fragmentation was assessed by terminal transferase labeling of free 3′-ends in fragmented DNA with the TUNEL FragEL Kit, as indicated by the manufacturer (Amersham-Calbiochem). The percentage of positive cells was estimated in three different experiments, counting an average of 500 cells per condition tested.

### 2.3. Western blot analysis

Cells were harvested in PBS containing protease and phosphatase inhibitors [13] using a rubber policeman. Extracts were prepared in cell lysis buffer (50 mM Tris–HCl, pH 8, 120 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 5 mM EDTA, 10 μg/mL each of leupeptin, soybean trypsin inhibitor, and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.4% Nonidet P-40). Seventy-five micrograms of protein was loaded into each well of a 12% SDS–polyacrylamide gel and electrophoretically separated. The gels were bi-directionally blotted onto replica nitrocellulose membranes as described previously [13]. After protein transfer, the membranes were blocked with TBS (Tris-buffered saline, pH 7.5) containing 0.1% Tween-20 and 5% nonfat skim milk. Antibodies against caspase-3 and its activated form (SC-7148), pRb (SC-102), E2F-1 (SC-251), E2F-4 (SC-1082), PARP (SC-7150), Bcl-2 (SC-492), p53 (SC-126; DO-1), and phosphorylated JNK

(SC-6254) were all purchased from Santa Cruz Biotechnology. Antibody 9106 to phosphorylated ERK was obtained from Cell Signalling, and antibody 06-984 to MnSOD was obtained from Upstate Biotechnology. Whenever necessary, blot pairs were erased by heating (70°; 30 min) with 62 mM Tris–HCl, 2% SDS, 100 mM β-mercaptoethanol. The stripped blots then were washed extensively with 0.1% Tween-20 in TBS, blocked with skim milk, and exposed to a different set of primary antibodies. In each case, detection was accomplished by reaction with protein A-peroxidase and Super Signal chemiluminescence (Pierce).

### 2.4. Quantitation of western blots

Densitometry was carried out using a Gel Pro Analyzer (version 3.0, Media Cybernetics) with a Fluor-S-Imager and Quantity One image acquisition software (Bio-Rad) as previously indicated [13].

## 3. Results

### 3.1. Non-toxic concentrations of NAC synergize with UV radiation to increase PARP fragmentation and apoptosis

NAC at 20–50 mM was reported recently to preferentially induce p53-mediated apoptosis in transformed cells rather than in normal fibroblasts [9]. Since these concentrations were toxic for normal splenocytes [10], we set out to first determine a non-toxic concentration for human C8161 melanoma cells. After a 30-hr treatment with 2.5–20 mM NAC, cell viability decreased significantly only with 20 mM NAC, as determined by Alamar blue fluorescence (Fig. 1, upper panel). This decrease in cell viability, interpreted as toxicity, correlated with an increase in apoptosis-associated PARP fragmentation [14,15] (Fig. 1, middle panel). We therefore used 10 mM NAC, a non-toxic concentration, to determine whether exposure to NAC synergized the effect of UV radiation (10 J/m$^2$) on C8161 cells. These studies showed that PARP fragmentation was negligible in control cells as well as in those exposed to 10 mM NAC. In contrast, UV radiation induced the partial fragmentation of PARP, while the combined NAC/UV treatment caused complete PARP cleavage (Fig. 1, lower panel).

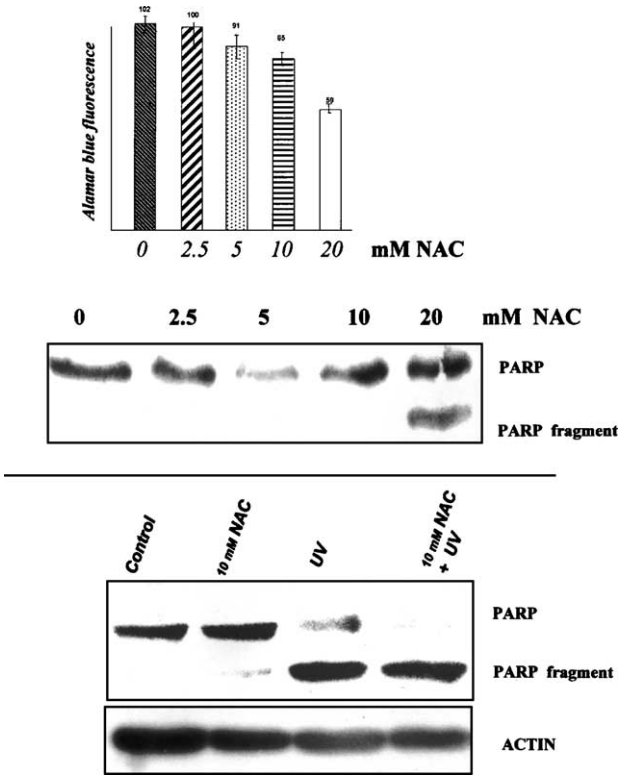


Fig. 1. Concentration-dependent effect of NAC on cell viability and on UV-mediated PARP cleavage. *Upper panel*: Alamar blue fluorescence in 96-well microtiter tissue culture plates [11] monitored in a microplate reader in triplicates, 30 hr after exposing parental C8161 melanoma cells [10] to 2.5–20 mM NAC. *Middle panel*: Proteins extracted from cells treated as indicated above were subjected to SDS–PAGE and transferred to nitrocellulose membranes; PARP cleavage was detected immunologically. *Lower panel*: Two hours after exposure of parental C8161 cells to 10 mM NAC or the vehicle control, parental C8161 cells were either UV- (10 J/m$^2$) or mock-irradiated and cultured for an additional 24 hr. Cells were harvested for SDS–PAGE and western blot analysis using anti-PARP and anti-actin antibodies. Actin levels were used as loading controls. Note the enhancement of UV-mediated PARP cleavage by 10 mM NAC. This experiment was repeated three times with comparable results.

### 3.2. Caspase-3 activation induced by UV radiation is enhanced by NAC and decreased by Bcl-2 in human C8161 melanoma cells

UV radiation-induced apoptotic signalling is known to cause caspase-3 activation, which leads to PARP fragmentation [14,15]. This apoptotic pathway was investigated in control LPC vector-transformed C8161 melanoma cells and their *bcl-2*-transduced counterparts, which overexpress the anti-apoptotic *bcl-2* gene. Western blot analysis with the SC-7148 antibody, which reacts with intact caspase-3 as well as its p20 and p11 activated forms, was used to determine the ratio of activated caspase-3 to that of pro-caspase-3. When the relative band densities were digitized and quantified, it became apparent that a low or negligible amount of caspase-3 activation was induced by NAC, compared with the UV-irradiated LPC vector-transformed cells (Fig. 2). In spite of the very low caspase-3 activation by NAC, the latter clearly synergized with UV irradiation to increase caspase-3 activation by 2-fold. These effects were evident only in LPC vector-transduced melanoma cells as they were clearly diminished in their Bcl-2 over-expressing counterparts (Fig. 2, middle two panels). The lower blot, from the same experiment, shows that there are similar MnSOD levels in LPC vector- and LPC/*bcl-2*-transduced cells that were treated with both NAC and UV radiation, although caspase-3 activation was clearly lower in the latter cells (Fig. 2, lower panel).

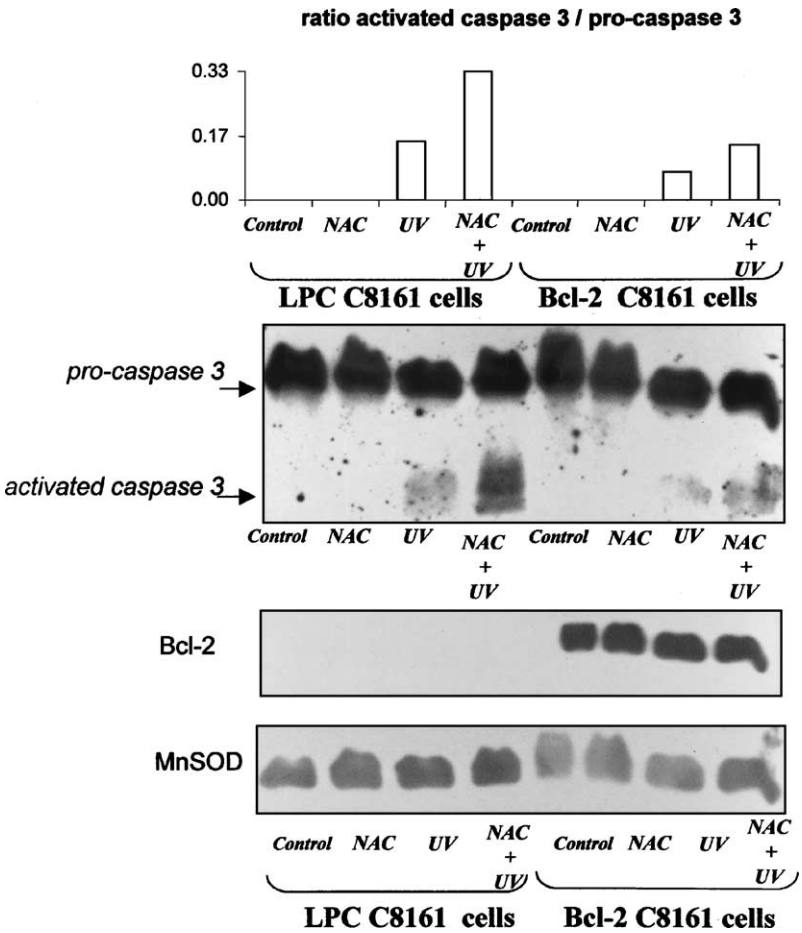


Fig. 2. Effect of NAC and Bcl-2 expression on UV-mediated caspase-3 activation. *Upper panel*: LPC vector-transduced control C8161 melanoma cells and their *bcl-2* transduced counterparts were exposed for 2 hr to 10 mM NAC or the vehicle control. The cells then were either UV- (10 J/m$^2$) or mock-irradiated and cultured for an additional 24 hr before lysis. Aliquots of the lysates were analyzed by SDS–PAGE and were transferred bi-directionally to replicate nitrocellulose membranes. Activated caspase-3 was detected immunologically on the membranes. *Middle panel*: Confirmation of Bcl-2 overexpression in cells with attenuated caspase-3 activation. *Lower panel*: Shows that MnSOD levels in LPC vector-transduced cells and their bcl-2-tranduced counterparts are similar after treatment with the NAC/UV combination, although caspase-3 activation is much lower in Bcl-2 overexpressing cells, following this treatment. These results are representative of three experiments.

### *3.3. Non-toxic concentrations of NAC neither induce nor synergize with UV radiation to increase p53*

Since 10 mM NAC increased caspase-3 activation induced by UV radiation, we investigated whether this NAC concentration also increased p53 or enhanced the UV-mediated induction of p53 in LPC vector-transduced control and Bcl-2 overexpressing C8161 melanoma cells. Immunoblotting showed no significant change in p53 expression in control cells or those treated with NAC, whereas the UV-mediated induction of p53 was increased significantly, as expected from cells with wild-type p53 [13]. However, this UV-mediated induction of p53 was not modified by 10 mM NAC, although levels of p53 appeared higher in Bcl-2 overexpressing cells exposed to UV radiation compared to those of similarly treated LPC-C8161 cells (Fig. 3). This suggests that the enhancing effects of a non-toxic concentration of NAC on UV-irradiated C8161 melanoma cells are independent of p53 induction and may be augmented by the overexpression of Bcl-2.

### *3.4. Decreased caspase-3 activation correlates with attenuation of DNA damage in Bcl-2 overexpressing C8161 melanoma cells*

To examine whether the synergistic effect of NAC on UV irradiation was regulated by Bcl-2 and involved DNA damage in C8161 melanoma cells, we used the TUNEL assay to measure free 3′-OH ends in fragmented DNA [26] (Table 1). Genetically matched control LPC vector-transduced C8161 cells and their Bcl-2 overexpressing counterparts were treated for 30 hr with either 10 mM NAC, UV radiation, or a combination of NAC and UV radiation. The TUNEL assay revealed that there was a slight increase in DNA damage in cells exposed to NAC and that this increase was not counteracted by the overexpression of Bcl-2. However, Bcl-2 overexpression did lower the percentage of TUNEL-positive cells evident after treatment with either UV radiation, or combination treatment with NAC and UV radiation, a treatment that produced the highest number of LPC vector-transduced apoptotic cells (Table 1).

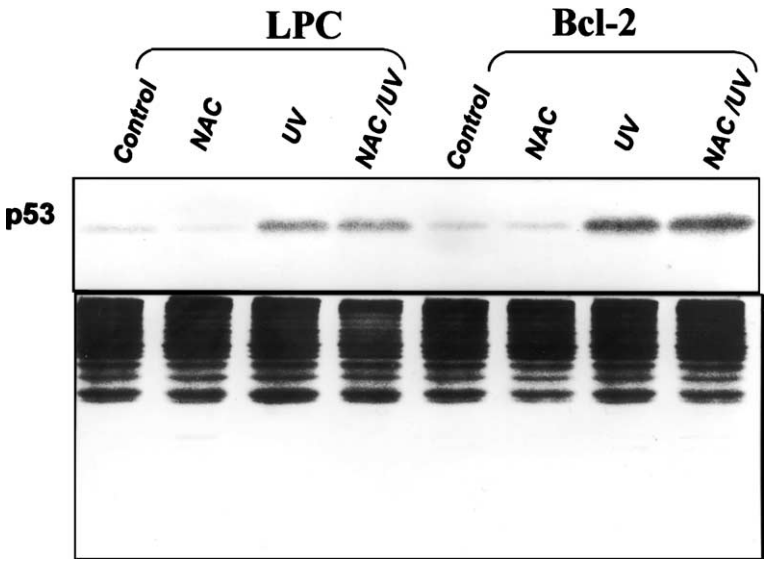


Fig. 3. Effect of NAC on basal and UV radiation-induced p53 levels. LPC vector-transduced control C8161 melanoma cells and their *bcl-2* transduced counterparts were exposed for 2 hr to 10 mM NAC or the vehicle. The cells were then either UV- (10 J/m$^2$) or mock-irradiated and cultured for an additional 24 hr before lysis. Aliquots of the lysates were analyzed by SDS–PAGE and transferred to nitrocellulose membranes for the immune detection of p53 using monoclonal antibody DO-1. Note that the level of p53 did not increase in UV-irradiated C8161 melanoma cells in the presence of a non-toxic concentration of NAC. This experiment was repeated four times with comparable results.

### 3.5. Bcl-2 attenuates pRb fragmentation induced by UV radiation and NAC

During cellular apoptosis the pRb is proteolyzed near its carboxyl terminus by a caspase-3-like protease generating protein fragments of 68 and 48 kDa [3,4]. We investigated whether this cleavage of the pRb occurred also in our C8161 cells following treatment with either the NAC/UV

Table 1
Effect of UV radiation and *N*-acetyl cysteine on nuclear DNA fragmentation

| Cells and treatment | % TUNEL-positive cells |
|---|---|
| Control LPC-C8161 melanoma cells | |
| None | 4.7 ± 1.6 |
| NAC (10 mM) | 14.6 ± 2.9* |
| UV (10 J/m$^2$) | 27.9 ± 2.7* |
| NAC (10 mM) + UV (10 J/m$^2$) | 54.6 ± 4.1* |
| Bcl-2 overexpressing C8161 melanoma cells | |
| None | 3.9 ± 1.4 |
| NAC (10 mM) | 17.6 ± 2.2* |
| UV (10 J/m$^2$) | 22.3 ± 2.1* |
| NAC (10 mM) + UV (10 J/m$^2$) | 35.6 ± 3.8* |

Subconfluent cultures in Lab-Tek chamber slides were exposed to NAC and/or UV, wherever indicated. After the indicated treatments, cells were fixed in 4% *p*-formaldehyde in PBS for 20 min, treated with proteinase K for 15 min, and endogenous peroxidase activity was inactivated by a 10-min exposure to 2% hydrogen peroxide. Subsequently, relative nuclear DNA fragmentation was assessed by terminal transferase labeling of free 3′-OH ends in fragmented DNA with the TUNEL FragEL Kit, as indicated by the manufacturer (Amersham-Calbiochem). The percentage of positive cells was estimated in three different experiments by counting an average of 500 cells per experimental condition. The values shown are means ± SD N = 3.

* $P < 0.05$, vs. "none", by Student's *t*-test.

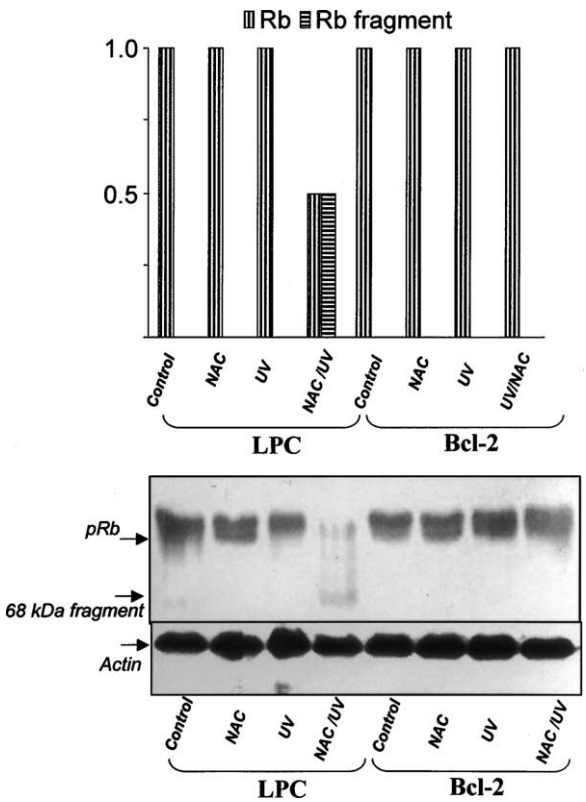


Fig. 4. Effect of UV and NAC treatment and Bcl-2 expression on pRb fragmentation. LPC vector-transduced control C8161 melanoma cells and their *bcl-2*-transduced counterparts were exposed for 2 hr to 10 mM NAC or the vehicle control. The cells were then either UV- (10 J/m$^2$) or mock-irradiated and cultured for an additional 24 hr before lysis. Aliquots of the lysates were analyzed by SDS–PAGE and transferred to nitrocellulose membranes for the immune detection of the pRb and actin. Actin levels were used as loading controls. Note that pRb cleavage occurred only in LPC vector-transduced cells exposed to both NAC and UV radiation. This result was reproducible in three different experiments.

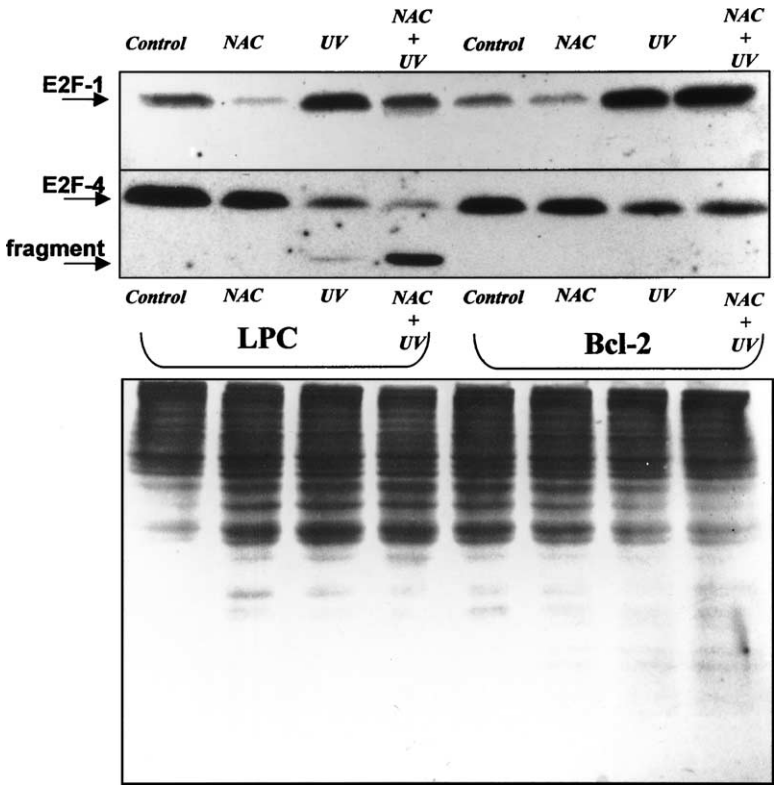


Fig. 5. Different effect of NAC and UV radiation on the expression of E2F-1 and E2F-4. LPC vector control and *bcl-2* transduced C8161 melanoma cells were exposed for 2 hr to 10 mM NAC or the vehicle control. The cells were either UV- (10 J/m$^2$) or mock-irradiated and cultured for an additional 24 hr before lysis. The protein components of the lysates were separated by SDS–PAGE and were bi-directionally transferred to replicate nitrocellulose membranes for the immunological detection of E2F-1 (upper panel) and E2F-4 (middle panel). The loading control consists of a Coomassie blue-stained duplicate gel (lower panel). Note the decline of E2F-1 with NAC treatment and the reciprocal UV-mediated cleavage of E2F-4, enhanced by co-treatment with NAC in the LPC vector-transduced cells. No comparable E2F-4 cleavage was evident in similarly treated Bcl-2 overexpressing cells, which instead showed E2F-1 induction, whenever treated with UV radiation. This experiment was repeated three times with comparable results.

combination or each agent individually. In agreement with the results in Fig. 2 showing maximal caspase-3 activation after the combination treatment, SDS–PAGE and western blot analysis revealed that the NAC/UV combination is required to produce the 68 kDa pRb fragment along with a corresponding decline in the intact pRb. This effect was antagonized in cells overexpressing Bcl-2 (Fig. 4).

### *3.6. Bcl-2-regulated E2F-4 fragmentation correlates with caspase-3 activation*

During cell proliferation, members of the retinoblastoma gene family like pRb can interact with members of the family of E2F transcription factors [18,20] not only to regulate their function in cell proliferation and apoptosis but also to protect them from degradation [21]. Since the data in Fig. 4 showed that the pRb was degraded in LPC vector-transduced C8161 melanoma cells exposed to the NAC/UV combination, we investigated whether loss of the pRb correlated with changes in the expression of either E2F-1 or E2F-4. Previously, increased expression of the transcription factor E2F1 was reported to occur during other forms of apoptosis in various cell types [21,22]. In agreement with such results, we also observed induction of E2F-1 in all UV-irradiated cells regardless of their Bcl-2 status, although the UV inducibility of E2F-1 was attenuated, in part, by NAC in LPC vector-transduced cells (Fig. 5, upper panel). A replica of the same bi-directional blot was used to investigate the relationship between E2F-1 and E2F-4 using the same extracts showing pRb fragmentation in Fig. 4. Expression of E2F-4, known to be constitutive through the cell cycle and expressed in quiescent cells [19], was not augmented by UV radiation like it was for E2F-1. Instead, we detected partial fragmentation of E2F-4 by UV radiation, which was augmented further by the NAC/UV combination in LPC vector-transduced cells (Fig. 5, four left lanes). This E2F-4 cleavage was suppressed by Bcl-2 overexpression (Fig. 5, four right lanes), correlating well with conditions increasing caspase-3 activation in the LPC vector-transduced C8161 melanoma cells and its inhibition by Bcl-2 overexpression (Fig. 2, right side of upper panel).

### *3.7. NAC decreases UV-mediated phosphorylation of ERK and JNK*

Inhibition of the ERK/MAP kinase phosphorylation signalling pathway has been reported to induce apoptosis

in human tumors [23,27]. Also, UV-induced c-jun-N-terminal kinase (JNK) phosphorylation was potently suppressed by Trolox, a water-soluble vitamin E analog antioxidant in primary keratinocytes [28]. Hence, we explored whether early changes in the phosphorylation of ERK [23] or JNK [28] were involved in the effects of NAC and UV radiation on C8161 melanoma cells. This was investigated with control cells and those exposed to 10 mM NAC that were either mock- or UV-irradiated and then analyzed 2 hr later. Analysis of control LPC vector-transduced C8161 cells revealed that JNK was highly phosphorylated, that their exposure to UV radiation augmented JNK phosphorylation (Fig. 6, upper panel, lanes 3 and 7), and that co-treatment with NAC abrogated this increase, irrespective of the Bcl-2 status (Fig. 6, upper panel, lanes 3, 4 and 7, 8). In contrast to the lack of effect of NAC on JNK phosphorylation (Fig. 6, upper panel, lanes 2 and 6 vs. lanes 1 and 5, respectively), NAC treatment decreased ERK phosphorylation, particularly in the LPC vector-transduced cells (Fig. 6, middle panel, lanes 2 and 6 vs. lanes 1 and 5, respectively). Moreover, a comparison of the phosphorylation status of ERK in UV-irradiated LPC vector-transduced cells and their Bcl-2 overexpressing counterparts showed a decline in the phosphorylation state of ERK in the former but an increase in ERK phosphorylation in the latter (Fig. 6, middle panels, lanes 3 and 7 vs. lanes 1 and 5, respectively). Although both of these UV-mediated changes in the phosphorylation state of ERK were counteracted, in part, by co-treatment with NAC, ERK activation remained higher in the Bcl-2 overexpressing cells than in the LPC vector-transduced cells (Fig. 6, middle panels, lanes 4 and 8). All of the densitometric data in Fig. 6 was corrected for relative protein load (lower panel).

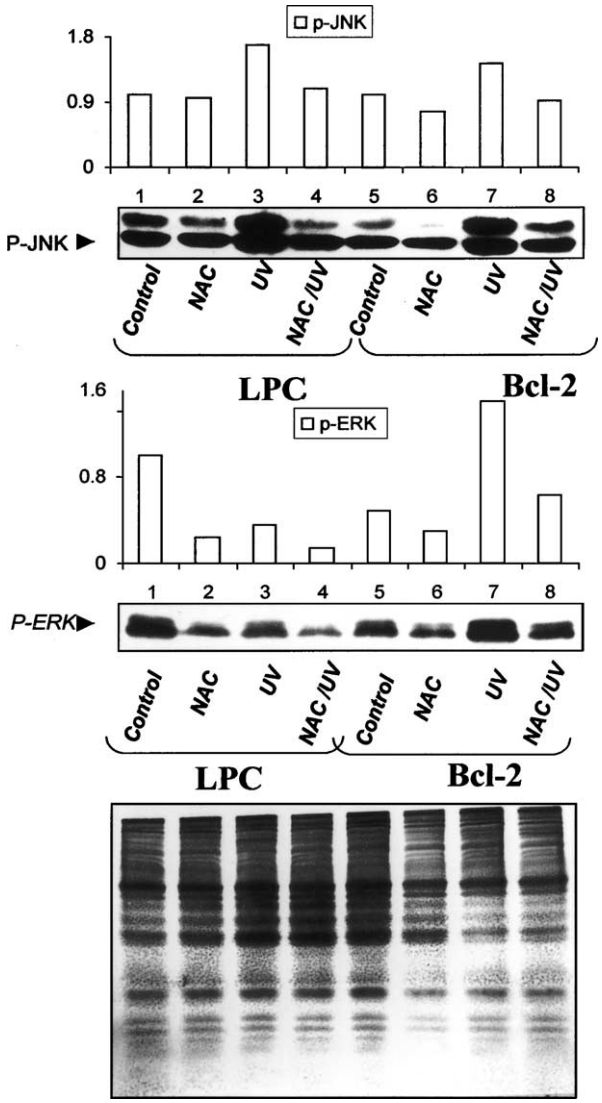


Fig. 6. Unequal effects of NAC and UV radiation on the phosphorylation of ERK and JNK. LPC vector control and *bcl-2*-transduced C8161 melanoma cells were exposed for 2 hr to 10 mM NAC or the vehicle control. The cells then were either UV- (10 J/m$^2$) or mock-irradiated and cultured only for an additional 2 hr before lysis. Aliquots of the lysates were analyzed by SDS–PAGE and bi-directionally transferred to replicate nitrocellulose membranes for the immunological detection of phosphorylated JNK (P-JNK) (upper panel) and phosphorylated ERK (P-ERK) (middle panel). The loading control consists of a duplicate Coomassie blue-stained gel (lower panel). Note that NAC decreased the phosphorylation of ERK in mock-irradiated LPC vector-transduced cells to a greater extent than their *bcl-2*-transfected counterparts. Additionally, NAC attenuated the UV radiation-mediated increase in ERK phosphorylation to a greater degree in the vector-transduced cells than in the *bcl-2* tranfectants. Also note that NAC decreased the phosphorylation of JNK only in the Bcl-2 overexpressing cells. These results were reproducible in three different experiments.

## 4. Discussion

ERK/MAPK survival signalling pathways have been implicated in regulating survival and cell death responses of tumor cells to chemotherapeutic drugs. Moreover, MAPK modulators may have potential as chemotherapeutic drugs themselves or as chemosensitizing agents [27]. The ability of MAPK/ERK kinase (MEK) inhibitors to block survival signalling in specific contexts and promote drug cytotoxicity represents an example of potentially interesting anticancer agents [23,27]. This study demonstrates a novel function for subtoxic concentrations of NAC, as a molecule with the ability to inhibit ERK/MAP kinase phosphorylation. It was reported previously that 20 mM NAC counteracted ERK/MAPK activation by $H_2O_2$, but no study was undertaken to investigate the effect of NAC on ERK/MAP kinase phosphorylation in cells not exposed to $H_2O_2$ [29]. We show that in LPC vector-transduced C8161 melanoma cells, UV radiation decreased viability (Table 1) and decreased ERK/MAP kinase phosphorylation (Fig. 6) and that these effects were enhanced by the addition of 10 mM NAC. In contrast, Bcl-2 overexpressing C8161 melanoma cells, which are less affected by the NAC/UV combination treatment than are the vector-transduced cells, showed a less pronounced decrease in ERK/MAP kinase phosphorylation when exposed to such treatments. This correlates well with the decreased apoptosis observed in these cells.

Another novel result found in these studies was that a non-toxic concentration of NAC was able to enhance the effect of a genotoxic agent like UV radiation on caspase-3 activation and apoptosis. Our results suggest that NAC is partly acting like a non-toxic MEK inhibitor, although it seems less effective in Bcl-2 overexpressing cells [30]. In the absence of Bcl-2 overexpression, this NAC stimulation of UV-mediated C8161 melanoma cell apoptosis led to interference with the key cell cycle-controlling pRb-E2F pathway [17–21]. This interference included disruption of pRb-E2F regulatory interactions because of increased pRb degradation and greater E2F-4 cleavage with no comparable effect on E2F-1. Cleavage of E2F-1-regulating proteins like pRb and Mdm-2 together with activation of E2F-1 is known to occur during CD95-induced death of thymocytes [31]. However, this is the first report indicating that: (i) in response to UV radiation E2F-4 is partially degraded and caspase-3 is activated, and (ii) NAC significantly increases E2F-4 fragmentation, caspase-3 activation, and pRb cleavage induced by UV radiation. These NAC-enhanced UV effects were decreased by Bcl-2 overexpression in C8161 melanoma cells. Bcl-2 has also been shown to inhibit CD95-mediated apoptosis by decreasing mitochondrial destabilization, and the resulting p20/p12 caspase-3 is not active and does not undergo normal autocatalytic processing to form p17/p12 caspase-3 [30]. In summary, we show for the first time that antioxidants like NAC can synergize with known caspase-3 activators, like genotoxic UV radiation [14,15], to enhance caspase-3 processing, cleavage of the E2F-4 transcription factor, and apoptosis in human C8161 melanoma cells. We postulate that the lack of pRb [3,4] and E2F-4 function and the inappropriate activation of E2F-1 [21,22,31] are factors in this apoptotic mechanism. Our data showing enhanced pRb degradation in LPC vector-transduced C8161 melanoma cells in response to combination treatment with NAC and UV radiation are compatible with a report showing pRb cleavage in colorectal cancer cells in which NAC mediates apoptosis [16]. This report showing a role for subtoxic NAC as a novel co-activator of caspases, when used in conjunction with DNA-damaging agents like radiation [11,12], make it an attractive molecule to study, in conjunction with alternative anticancer therapies.

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