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Carbazolequinone induction of caspase-dependent cell death in Src-overexpressing cells

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Abstract

We previously reported that RSV-transformed quail neuroretina cells (QNR-ts68) were highly resistant to apoptosis provoked by serum withdrawal, and that this property was due to v-Src kinase activity. The present study investigates the cytotoxic effect and the functional mechanism of carbazolequinone-mediated cell death in this system. QNR-ts68 cells were subjected to carbazolequinone treatment and both growth inhibition and cell death induction were examined using formazan assays. Cell death mechanism (both apoptosis and necrosis) was confirmed through phosphatidyl serine exposure and propidium iodide incorporation. Furthermore, the effect of active carbazolequinone was inhibited by a pan caspase inhibitor. Cytofluorimetric and immunofluorescence data demonstrated the activation of caspase-3 and the involvement of mitochondria. Therefore, this study clearly indicates that carbazolequinones could induce cell death in transformed cells displaying high levels of antiapoptotic tyrosine kinase activity. Further investigations would be necessary to elucidate the mechanisms by which these carbazolequinones act as antitumor agents.

Keywords: Transformation; *v-src*; Carbazolequinones; Cytotoxicity; Necrosis; Apoptosis; Caspase

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Abbreviations: PARP, poly-ADP-ribose-polymerase; FACS, fluorescence activated cell sorter; PBS, phosphate-buffered saline; RSV, rous sarcoma virus; FCS, fetal calf serum; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; XTT, (sodium 3,3′-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}bis(4-methoxy-6-nitro)-benzene sulfonic acid); OD, optical density; FACS, fluorescence activated cell sorting; PI, propidium iodide; $\Delta\psi_m$, mitochondrial transmembrane potential; DMSO, dimethyl sulfoxide; QNR, quail neuroretina cells; ERK, extracellular regulated kinase; MEK, MAPK/ERK kinase; Z.VAD.FMK, Z-Val-Ala-Asp-(Ome)-CH2F; CMXRos, chloromethyl-X-rosamine; TS, thymidylate synthase; DBC, 7*H*-dibenzo[*c*,*g*]carbazole; ROS, reactive oxygen species; B1, 9-ethyl-1,4-dihydrocarbazole-1,4(9*H*)-dione; B2, 9-ethyl-6-hydroxy-1,4-dihydrocarbazole-1,4(9*H*)-dione; B4, 6-ethyl-3-methyl-6*H*-pyrido[3,2-*b*]carbazole-5,11-dione; B5, 10-ethyl-7-hydroxy-3-methyl-10*H*-pyrido-[2,3-*b*]carbazole-5,11-dione; B7, 10-ethyl-3-methoxy-10*H*-pyrido[3,4-*b*]carbazole-5,11-dione; B9, 10-ethyl-2*H*-pyrido[3,4-*b*]carbazole-3,5,11(10*H*)-trione; B10, 6-ethyl-2*H*-pyrido[4,3-*b*]carbazole-3,5,11(6*H*)-trione; B11, 9-methyl-1,4-dihydrocarbazole-1,4(9*H*)-dione; B12, 9-benzyl-1,4-dihydrocarbazole-1,4(9*H*)-dione; B13, 3-ethoxy-10-ethyl-4-methyl-10*H*-pyrido[2,3-*b*]carbazole-5,11-dione; B14, 3-bromo-9-ethyl-1,4-dihydrocarbazole-1,4(9*H*)-dione; B15, 10-ethyl-7-hydroxy-4-methyl-10*H*-pyrido[2,3-*b*]carbazole-5,11-dione; B17, 6-ethyl-2-methyl-2*H*-pyrido[4,3-*b*]carbazole-3,5,11(6*H*)-trione.

1. Introduction

The $p60^{c\text{-}src}$ protein, a member of the non-receptor tyrosine kinase family, plays crucial roles in cell cycle progression and cell differentiation [1,2]. Its activity is tightly regulated and its disregulation could lead to cell transformation. Indeed, a high $p60^{c\text{-}src}$ kinase activity is found in a number of tumor cell lines [3]. Moreover, tumors display either high levels of c-src expression, particularly those of the colon [4] or a mutated version of c-Src being central for tumor progression [5]. Increased c-src mRNA and protein levels tightly correlate with kinase activity [6]. The viral counterpart of the $p60^{c\text{-}src}$ protein, namely $p60^{v\text{-}src}$, is held by the rous sarcoma virus, an avian transforming retrovirus (RSV). In contrast to its cellular homolog, activity of $p60^{v\text{-}src}$ remains constitutively high, irrespective of the cellular context. Thus, infection by RSV leads to rapid v-src-mediated tumor formation *in vivo*. A number of experimental models has been depicted for the study of cell transformation by the v-src oncogene [7,8]. Among them, quail neuroretina cells (QNR) in culture have

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several interesting features. In particular, these cells still exhibit typical features of differentiated nerve cells [9,10]. In addition, while $p60^{v\text{-}src}$ and growth factors act in cooperation to induce cell proliferation [11], v-Src activity on its own is sufficient to promote cell survival independently of serum [12]. Indeed $p60^{v\text{-}src}$ has the ability in QNR cells infected by the NYts68 strain of RSV (QNR-ts68 cells) to prevent cell death upon serum withdrawal, contrary to the products of other oncogenes like, e.g. Myc [13] or E1A [14].

Necrosis and apoptosis are two major mechanisms of cellular death. Necrotic cell death is an unregulated process resulting from severe extracellular damage and is characterized by ATP depletion, cell swelling, lysis and the release of intracellular contents, resulting in tissue inflammation [15–17]. In contrast, apoptosis is a highly regulated, energy-dependent event that leads to the elimination of excess or damaged cells from tissues. There are numerous pathological and physiological stimuli of apoptotic cell death including death factors (e.g. cytokines), reactive oxygen species, and genotoxic agents [18,19]. Apoptosis is a non-random process characterized by cell shrinkage, nuclear condensation, membrane ''flipping,'' internucleosomal DNA cleavage, and cytoplasmic budding [15,20]. Apoptosis and necrosis frequently coexist following insult [21–23]. Apoptosis in tumor cells occurs spontaneously near areas of necrosis, as well as in response to radiation and chemotherapeutic drug exposure, the classic cancer therapy regimen [24]. Recently, several investigations have demonstrated that the induction of apoptosis and necrosis is highly dependent upon the intensity of the initial exposure [21,25].

While the pharmacology of most anticancer agents is well understood, the biochemical mechanisms by which drugs ultimately induce cell death remain poorly characterized. Many anticancer agents have been shown to mediate their cytotoxic effects *via* DNA damage and subsequent induction of apoptosis [26,27]. In addition, anticancer drug resistance cannot be explained entirely by changes upstream of the primary drug–target interaction but may also be mediated *via* alterations in the machinery governing cell death [28,29]. A better understanding of drug-induced cell suicide may permit manipulation of the key components of the apoptotic pathway in order to enhance the therapeutic index of anticancer drugs.

Following the reports on the synthesis and antitumor activity of ellipticine, numerous studies focused on pyridocarbazole and ellipticine derivatives [30,31], including structure–activity relationships, mechanisms of action, and the design and synthesis of new analogues. In contrast, the cytotoxic activities of carbazole derivatives have been only evaluated in a few tumor cell lines [32]. To our knowledge, no data is currently available about the pattern of death following carbazolequinone exposure of transformed cells displaying high levels of tyrosine kinase activity. The objectives of this study were to (1) determine whether carbazolequinones were directly cytotoxic to $p60^{v\text{-}src}$-transformed cells, (2) analyze the mechanism of cell death involved in carbazolequinones cytotoxicity. The understanding of carbazolequinones biological activity may contribute to their clinical application in cancer treatment.

2. Materials and methods

2.1. Cells and viruses

Neuroretina cells (QNR) were prepared from 7-day-old quail embryos (*Coturnix coturnix japonica*) and infected with the RSV strain NYts68 (QNR-ts68). Cells were grown and passaged in Eagle's basal medium (EBME, Biomedia) supplemented with 10% heat inactivated fetal calf serum glutamine, pyruvate and penicillin–streptomycin as previously described [11].

2.2. Chemical preparation

Carbazolequinones were obtained from H. Fillion, Laboratoire de Chimie Organique, Lyon, France: B15, B2, B12, B5 [33], B7, B9, B10, B17 [34], B1, B11, B4, B14, B13 [35]; 100 mM stock solutions were prepared in DMSO and diluted to various concentrations with serum-free culture medium.

2.3. Proliferation and cytotoxicity assays

Cell density and cytotoxicity were measured, respectively, using the XTT assay (cell proliferation kit II, XTT, Roche) and the LDH cytotoxicity detection kit (Takara Biochemicals) following manufacturers instructions.

2.4. Immunodetection of activated caspase-3 and nuclear staining

Rabbit polyclonal anticaspase-3 antibody CM1 was a kind gift of Srinavasan from Idun Pharmaceuticals. Activated form of caspase-3 was detected as previously described [12]. The Hoechst staining procedure that gives a clear representation of nuclear morphology was detailed previously [12]. Apoptotic cells were identified as those showing condensed chromatin or apparently fragmented nuclei. Normal cells showed an even distribution of the staining throughout the nucleus with flocculated chromatin.

2.5. Analysis of mitochondrial transmembrane potential ($\Delta\psi_m$)

To measure $\Delta\psi_m$, cells were loaded with Mitotracker Red CMXRos (Molecular Probes) as previously described

[12] and subsequently analyzed for fluorochrome incorporation in a FACS Calibur™ cytofluorometer. Mitotracker Red CMXRos is a vital dye that is preferentially taken up by the mitochondria, as a function of mitochondrial transmembrane potential $\Delta\psi_m$.

2.6. Quantification of apoptosis

Propidium iodide incorporation (necrosis) and phosphatidyl serine exposure (apoptosis) were measured essentially as previously described [12,36]. Briefly, cells were seeded at the density of 5×10^5 cells per 60 mm diameter cell culture dish in EBME containing 10% FCS. After a 24 hr culture at 36.5°, cells were incubated with DMSO (MOCK) or the specified chemical for 18 hr in EBME without serum. Floating and adherent cells were collected and placed in cold PBS for immediate analysis of propidium iodide incorporation and Annexin-V-FITC labeling (according to the manufacturer's instructions, Annexin-V-Fluos staining kit, Roche) using, respectively, FL2 and FL1 channels of the FACS Calibur™ cytofluorometer. In all cell death assays, there were no significant differences in the number of apoptotic cells between untreated and solvent-treated (DMSO) control cultures (data not shown).

3. Results

We and others previously showed that cells displaying high levels of v-Src kinase activity are highly resistant to apoptosis following serum withdrawal [12,37]. In particular, serum-starved QNR-ts68 cells were able to survive for at least 3 days and to proliferate for at least 24 hr when cultured at 36.5°. We made use of this system to test the death-inducing and antiproliferative properties of carbazolequinones on apoptosis-resistant cancer cells.

3.1. Effects of carbazolequinones on LDH release from QNR-ts68 cells

LDH measurement was used to assess the cytotoxicity of many chemicals *in vitro* [21,38]. The release of LDH is a more sensitive marker for cytotoxicity compared to the trypan blue exclusion method and has been used as an indicator of necrosis [21,39]. This non-morphological cell toxicity assay is based on the principle that dead cells (necrotic, late apoptotic) lose the ability to maintain their plasma membrane integrity, permitting intracellular constituents, such as LDH, to leak out of the cell into culture media. Data obtained from this assay were used to determine for each compound the range of concentration that was overtly cytotoxic. In a first set of experiments, carbazolequinones were applied for different durations and an optimal period of exposure was determined (data not shown). At 18 hr, a concentration-dependent increase in formazan production, indicating decreased viability of QNR-ts68 cells was observed (Fig. 1A). Cytotoxicity reached 50% for approximately 50 μM of B1, B2, B12 and B14, and 75 μM of B11 (see Fig. 2 for the nomenclature of the compounds used in this study).

3.2. Antiproliferative effect of carbazolequinones on QNR-ts68 cells

The effect of carbazolequinones on cell proliferation was evaluated by the XTT assay. This test is based on the principle that living (metabolically active) cells reduce tetrazolium salts. This semi-automated method has been chosen for its rapidity and good reproductibility. In this test, the decrease in LDH content of the cell population after drug exposure reflects cytotoxicity. When carbazolequinones were applied for 18 hr, inhibition of proliferation reached 50% for approximately 12.5 μM of B14, 20 μM of B2, 50 μM of B12, 60 μM of B1, and 70 μM of B11, other compounds exhibited poor activity if any (Fig. 1B). There was no significant effect on cell proliferation and viability for concentrations below 10 μM. For concentrations above 100–200 μM, depending on the compound, cells could not be cultured more than 3–6 hr without detachment from the substratum. Taken together, these data show that most carbazolequinones inhibit cell growth at concentrations which do not seem to affect cell metabolism and viability.

3.3. Structure–activity relationships

Carbazolequinone B2, which is similar to B1, except in an hydroxyl group present on the first aromatic cycle is more efficient in inducing cell death, suggesting that this OH group may be important for enhancing the activity of carbazolequinones. The OH group may allow the establishment of critical hydrogen bonds or favor nucleophilic attacks. A *Me* group instead of a bulkier *Et* or *Bn* groups on the second cycle renders the molecule less toxic (compare B11 with B1 or B12). Addition of a *Br* group on the third aromatic cycle has poor effect on the activity of the drug (compare B14 and B1). Carbazolequinones sharing four cycles (B4, B5, B7, B9, B10, B13, B15 and B17) with a fused pyridine nucleus on the quinone ring, when soluble in DMSO (B5 and B15), have no cytotoxic effect on QNR-ts68 cells. This additional ring may prevent the 1,4-addition of nucleophilic cell residues to the quinone. An alternative explanation would be that addition of such a group modifies the oxidant capability of the quinones.

3.4. Suppression of carbazolequinones effects by caspase inhibitor Z.VAD.FMK

The MTT and XTT assays are convenient screening assays for the measurement of cell death and proliferation

but not discriminate between necrosis and apoptosis. Activation of the caspase family of proteases is a central biochemical event occurring in apoptotic cells. To investigate whether inhibition of caspase activation prevents QNR-ts68 cells from carbazolequinone-induced cell death, we treated QNR-ts68 cells with carbazolequinones in the presence or absence of the pan caspase inhibitor Z.VAD.FMK for 18 hr. Carbazolequinones were used at concentrations (25 μM) determined from pilot experiments (not shown). Fig. 3A shows QNR-ts68 cultured in presence

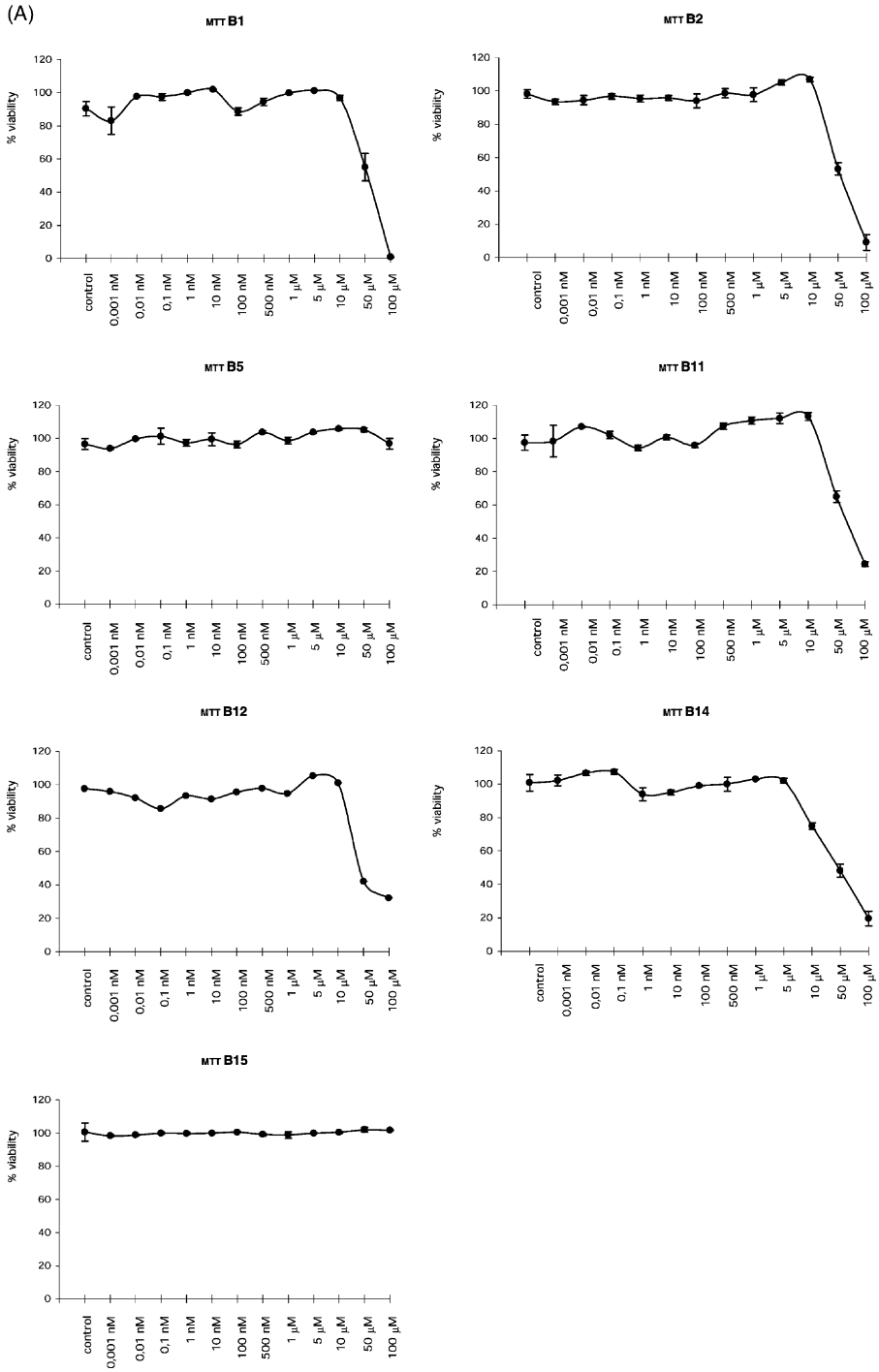


Fig. 1. (A) Carbazolequinone induces QNR-ts68 cell death. Cultures were treated for 18 hr with various concentration of chemical before being assayed for LDH release using the viability MTT test. Each bar represents the mean value for eight measurements; each value was compared to control without chemical. Control experiments were performed by exposing cells to the same concentrations of DMSO. DMSO alone has no cytotoxic effect. (B) Antiproliferative effect of carbazolequinones on QNR-ts68 cells. Cultures were treated for 18 hr with various concentrations of chemical before being assayed for LDH activity using the cell growth XTT test. Each bar represents average value for eight measurements; each value was compared to control without chemical. Control experiments were performed by exposing cells to the same concentrations of DMSO solvent. DMSO alone has no growth inhibitory effect.

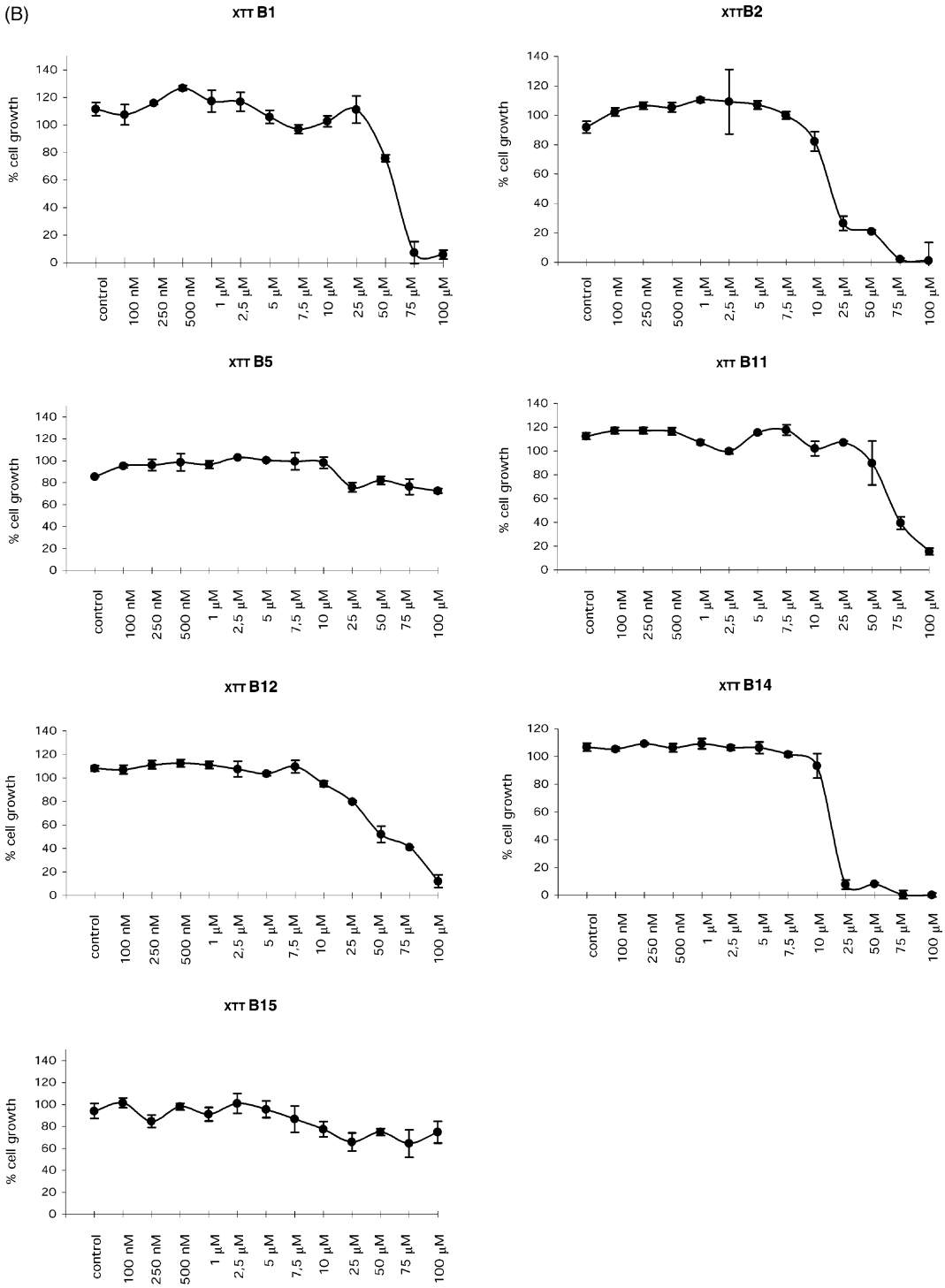


Fig. 1. (*Continued*).

or absence of carbazolequinones by phase contrast microscopy. Morphologically, QNR-ts68 cells rounded up, detached and floated 12–14 hr after carbazolequinone addition, indicating cell death (Fig. 3B). The inhibitory activity of the wide range inhibitor Z.VAD.FMK on cell death of QNR-ts68 could be clearly observed, indicating that caspase activation is necessary for carbazolequinone-induced cell death in QNR-ts68 cells.

3.5. Detection of activated caspase-3 in carbazolequinone-treated QNR-ts68 cells

To further confirm that the cells were dying *via* a caspase-dependent cell death, we looked if caspase-3 was activated in QNR-ts68 cells, using the CM1 antibody raised against the active form of caspase-3 [40]. Fig. 4 shows that cells treated with carbazolequinones displaying a pycnotic

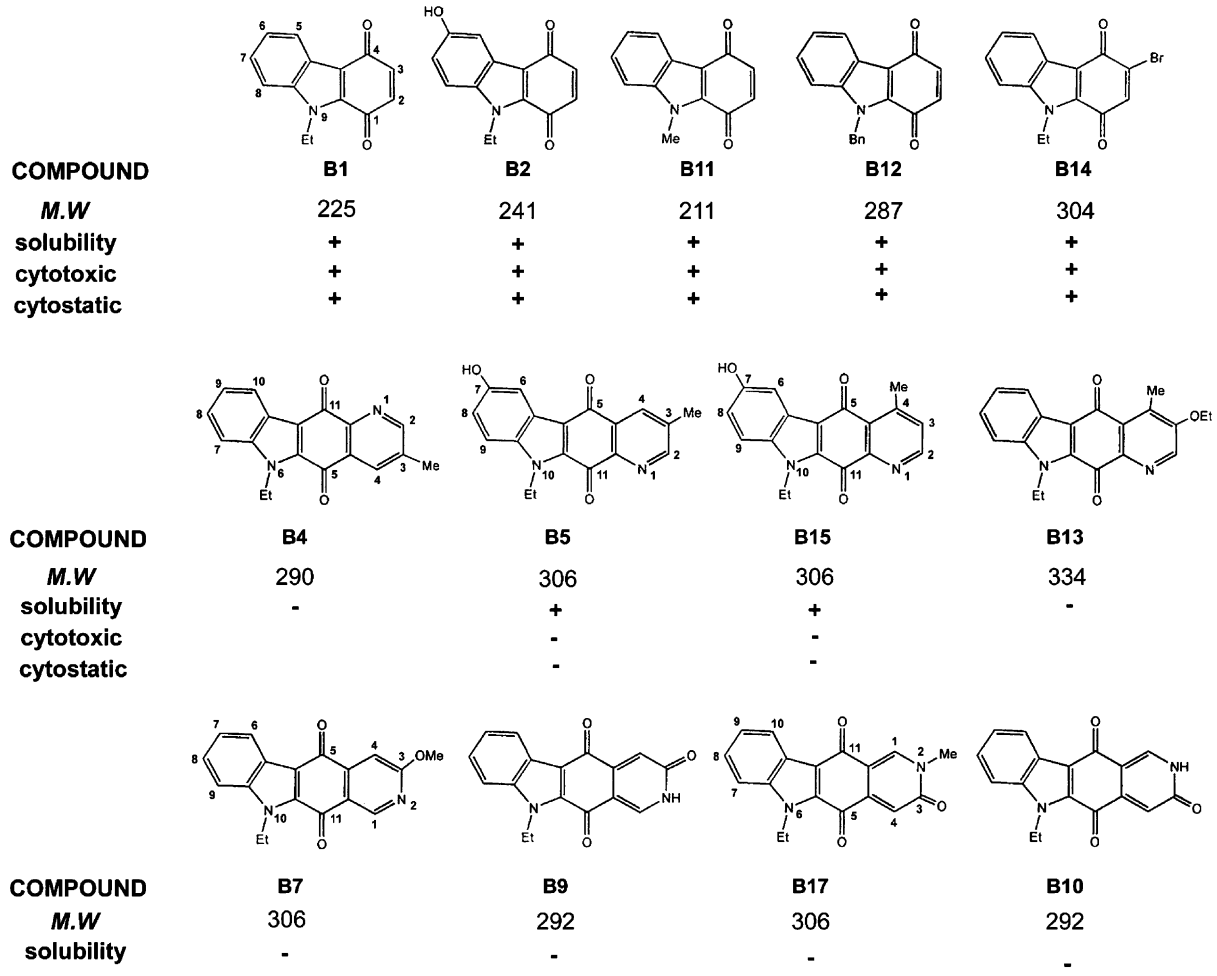


Fig. 2. Semi-developed formula of the carbazolequinones used in the study. Molecular weight (M.W.), solubility in DMSO and biological effects (cytotoxicity and growth inhibitory activity) are indicated for each compound.

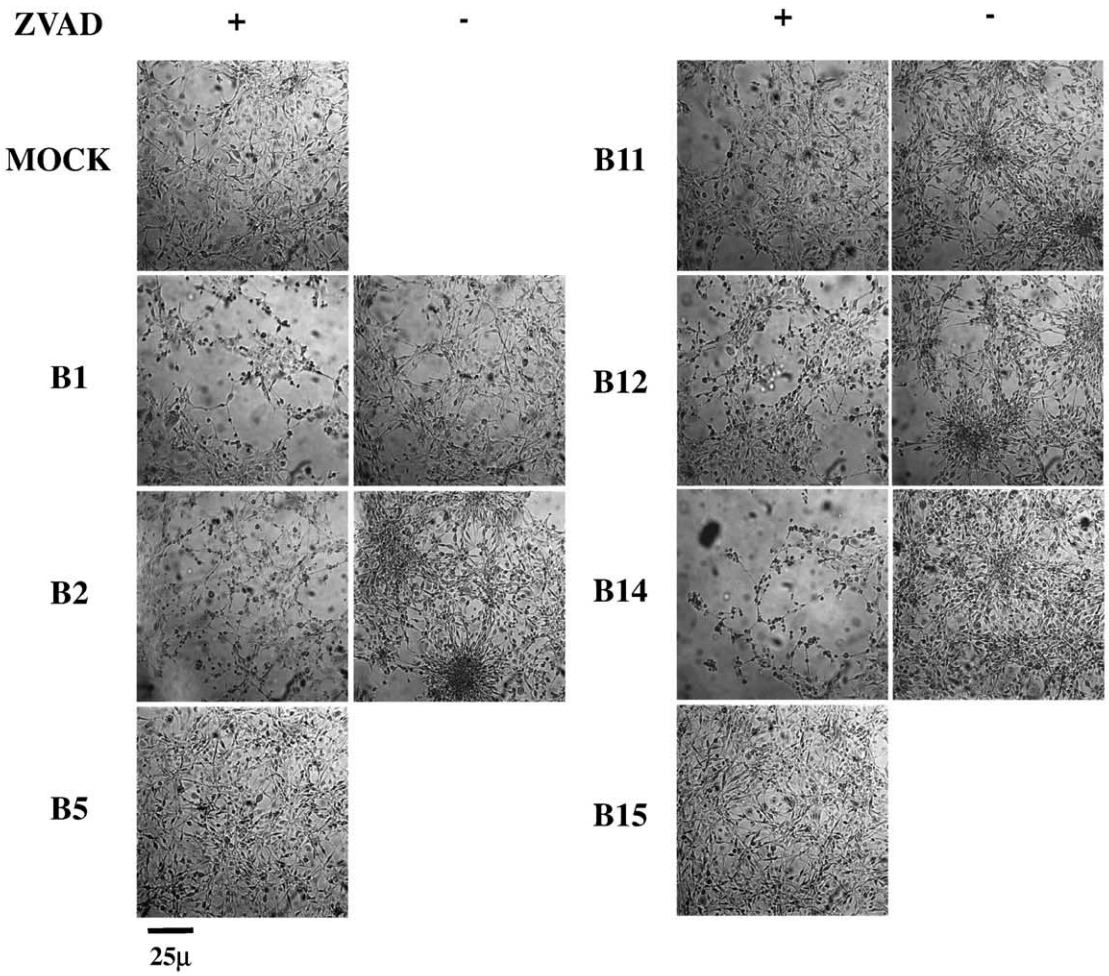
nucleus were labeled with CM1. These results, together with the data obtained with the broad-spectrum caspase inhibitor Z.VAD.FMK, clearly demonstrated that caspase-3 activation is a requisite for cell death in this system.

3.6. Carbazolequinones induce both apoptosis and necrosis in QNR-ts68 cells

To characterize further cell death occurring after carbazolequinones exposure, we looked at typical events of apoptosis and necrosis: presence of phosphatidylserine at the outer face of the plasma membrane and propidium iodide incorporation, respectively. As shown in Fig. 5, loss of cell viability in most treated cells was due to both necrosis and apoptosis. However, although the relative contribution of apoptosis and necrosis to cell death depended on the chemical. B2 and B14 induced preferentially necrosis while B1 and B11 induced both apoptosis and necrosis. Interestingly, incubation with Z.VAD.FMK completely abrogated cell death, indicating that cells incorporating propidium iodide were actually late apoptotic or secondary necrotic in most cases.

3.7. Early mitochondrial permeability transition in QNR-ts68 cells treated with carbazolequinones

To further confirm that carbazolequinones induce apoptosis before necrosis, we made use of the observations obtained with the MTT assay. Indeed, the MTT assay is a colorimetric assay for the non-radioactive quantification of cell viability and is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. The formation of formazan is thought to take place *via* intact mitochondria [41]. A decrease in the number of living cells results in a decrease in the total metabolic activity in the sample. Our MTT assays demonstrated that the reduction of metabolic activity was significant, suggesting an inhibition of mitochondrial respiratory functions by carbazolequinones in this cell system. Mitochondria is a central organelle in the time course of apoptosis. A drop in the mitochondrial potential ($\Delta\psi_m$) is a hallmark of apoptosis after treatment by a number of apoptosis inducers [42]. These observations prompted us to investigate mitochondria metabolism in carbazolequinone-treated QNR-ts68 cells. To determine



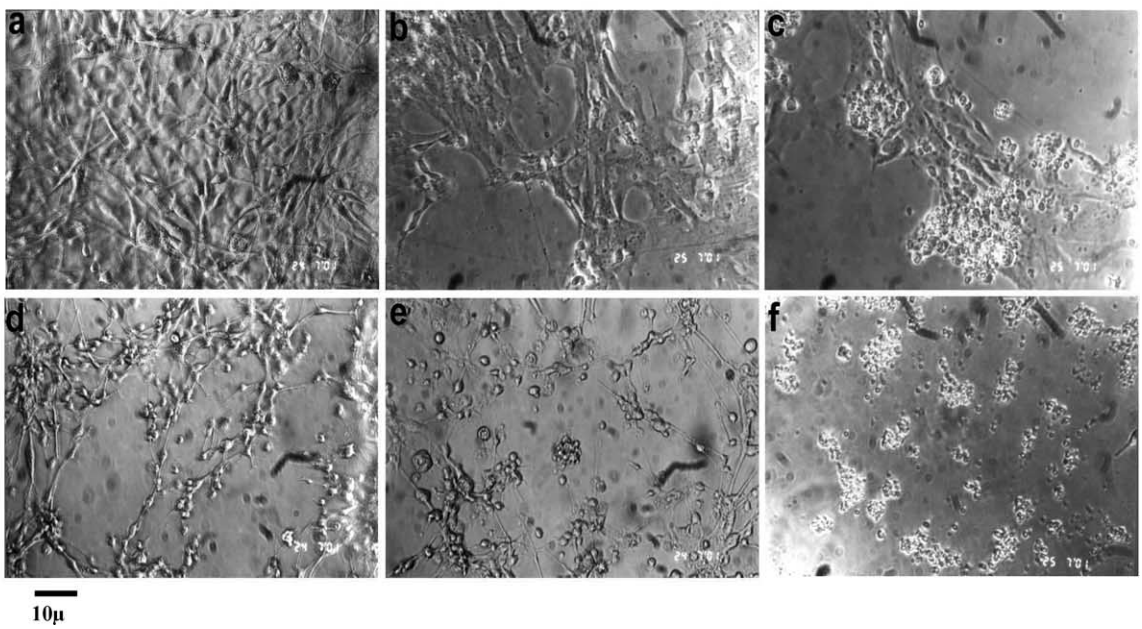


Fig. 3. Carbazolequinone treatment leads to morphological changes typical of cell death that are inhibited by the wide range caspase inhibitor Z.VAD.FMK. The name of each compound is indicated on the left of each panel. (A) Phase contrast microphotographs showing representative fields of QNR-ts68 cells cultured for 18 hr in serum-free medium containing either 25 μM carbazolequinone with (+) or without (−) Z.VAD.FMK or with solvent alone (MOCK). (B) Morphological alterations of QNR-ts68 cells following exposure to carbazolequinones (high magnification). Note the extensive cell shrinkage, cytoplasmic blebbing and other alterations typical of cell death when cells are cultured in the presence of increasing concentrations: 10 μM (d), 50 μM (e–b), 100 μM (f–c) of carbazolequinone B2. Partial recovery could be achieved by incubating cells with 200 μM Z.VAD.FMK (b–c).

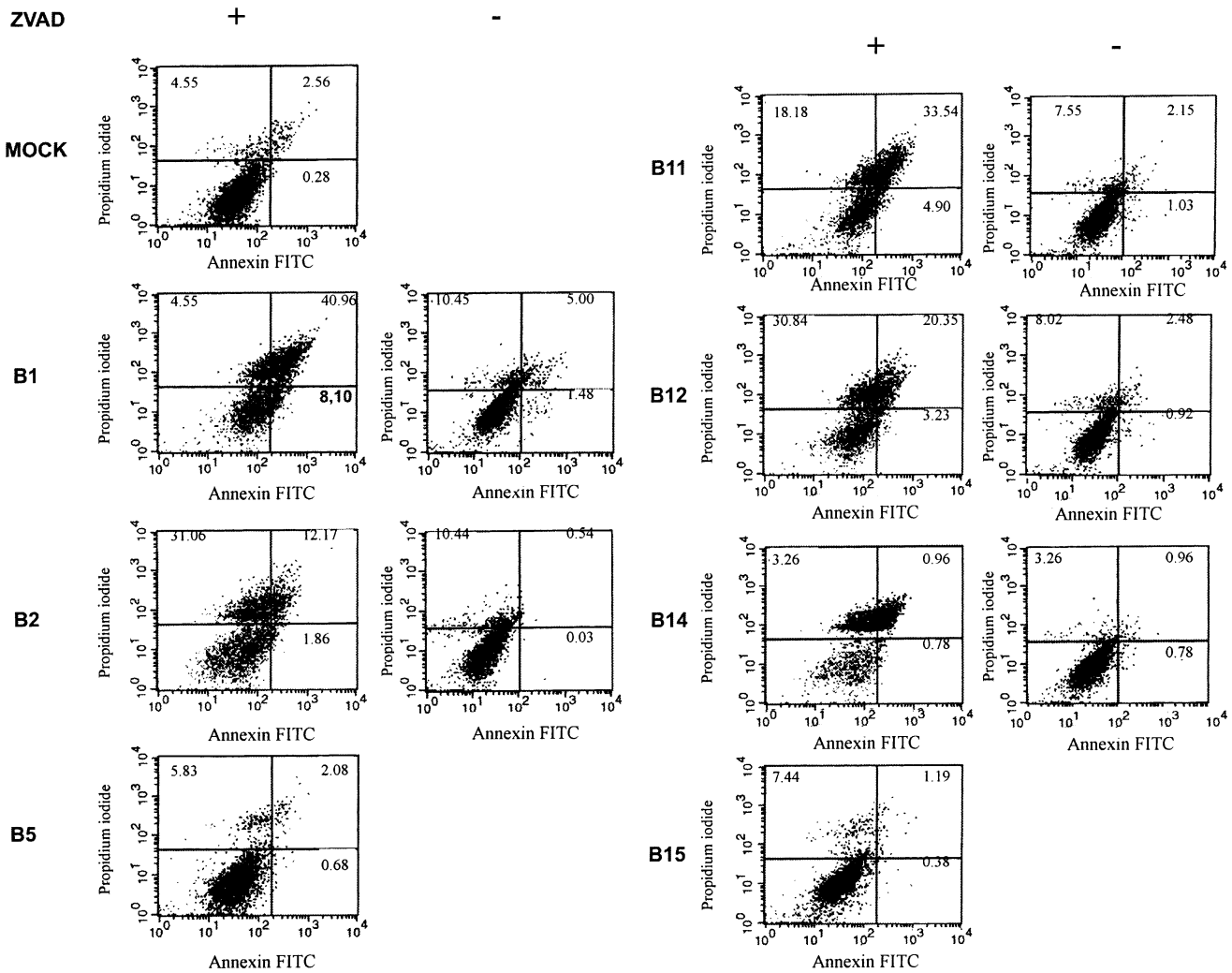


Fig. 4. Carbazolequinones treatment of QNR-ts68 cells induces both apoptotic and necrotic cell death. Apoptosis was detected using Annexin-V labeling test monitoring phosphatidyl serine exposure. Necrosis was quantified by measuring propidium iodide incorporation. QNR-ts68 cells were cultured for 18 hr in serum-free medium containing either 25 μM carbazolequinone with (+) or without (−) Z.VAD.FMK or with solvent alone (MOCK).

whether carbazolequinones trigger $\Delta\psi_m$ drop, we examined the $\Delta\psi_m$-dependent uptake of the fluorochrome Mitotracker Red CMXRos after treatment of QNR-ts68 cells with the lead compounds previously characterized in the course of this work. As shown in Fig. 6, QNR-ts68 cells treated with 25 μM of each carbazolequinone exhibit a depressed $\Delta\psi_m$ compared to control cells, this effect being strictly correlated with the respective ability of the specified carbazolequinones to induce cell death. Together, these results clearly establish that the $\Delta\psi_m$ drop is an early event during carbazolequinone-induced cell death in QNR-ts68 cells.

4. Discussion

Few reports are currently available concerning the cytotoxic effects of carbazolequinone on transformed cells. Synthetic 2-methyl- or 3-methyl-carbazolequinone derivatives with various substituents in the A-ring were evaluated by Itoigawa *et al.* [43] on several established cell lines, 3-methyl-carbazolequinone demonstrating significant cytotoxicity. 7*H*-Dibenzo[*c*,*g*]carbazole (DBC) produced DNA damage in HepG2 cells as measured by the formation of DNA strand breaks [44]. The cytotoxicity of DBC *in vitro* has been studied previously [45,46]. We report here that carbazolequinones induce cell death in a time and dose-dependent manner in avian nerve cells transformed by the RSV. These cells are rendered highly resistant to apoptosis by the $p60^{v\text{-}src}$ tyrosine kinase activity [12]. We undertook this investigation to determine the mechanisms of carbazolequinones-induced cytotoxicity in this cellular model. Our results indicate that carbazolequinones could induce some of the morphological and biochemical changes associated with apoptosis including cell shrinkage, DNA fragmentation, caspase activation and $\Delta\psi_m$ drop.

While rapidly phagocytosed *in vivo*, apoptotic cells are not eliminated *in vitro*, and eventually undergo secondary necrosis, leading to the development of a 'leaky' membrane over time in culture [21,47]. This was noted for most

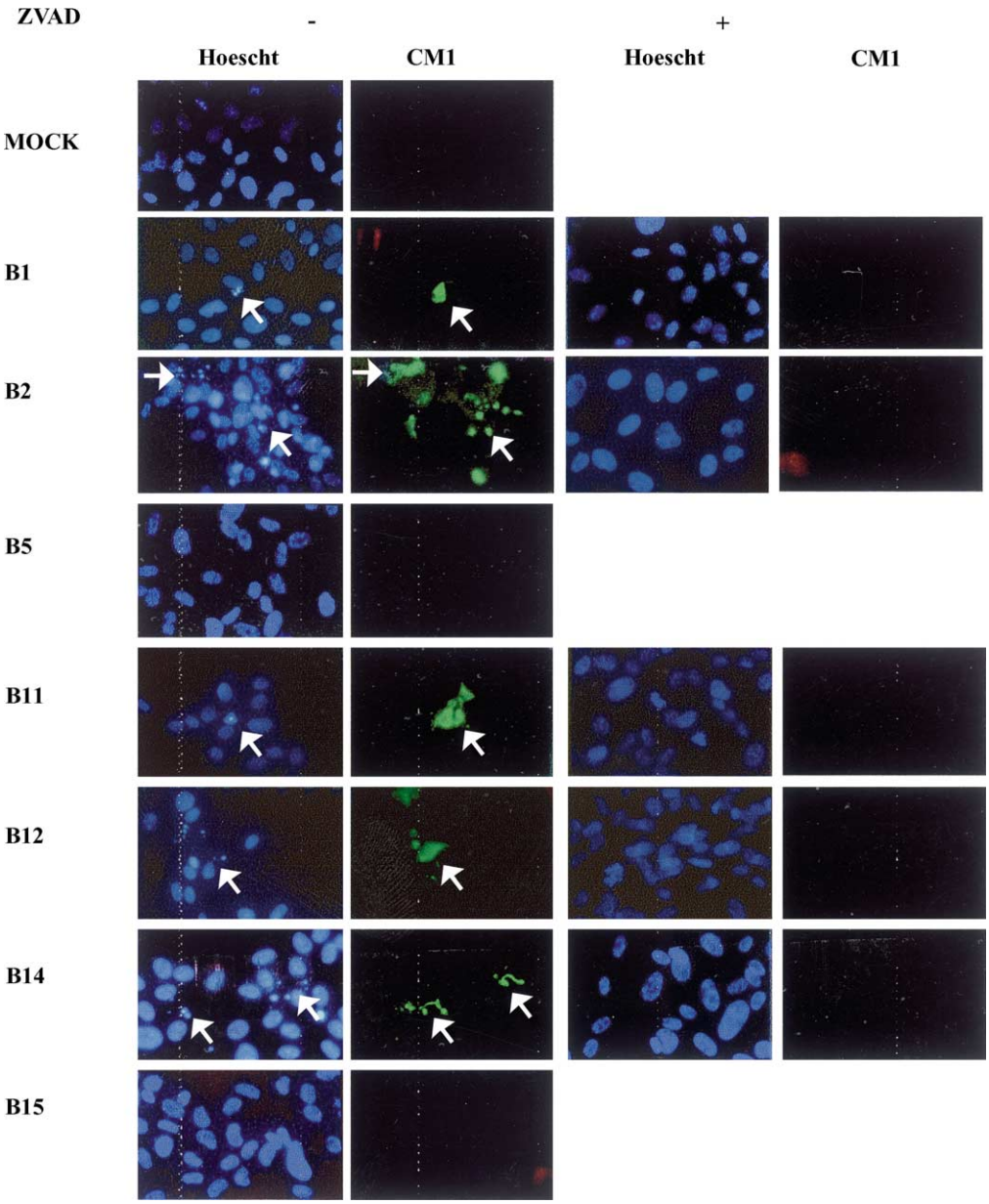


Fig. 5. Carbazolequinones induce activation of caspase-3 in QNR-ts68 cells. QNR-ts68 cells were cultured for 18 hr in serum-free medium containing either 25 μM carbazolequinone with (+) or without (−) Z.VAD.FMK or with solvent alone (MOCK). Cells were fixed for immunofluorescence and labeled using the CM1 antibody, which specifically recognizes the activated form of caspase-3 (CM1 panels) and were counter-stained with Hoechst 33258 to detect nuclei (Hoechst panels). Cells positively labeled with CM1 exhibit pycnotic nuclei (see arrows). Activation of caspase-3 is prevented by Z.VAD.FMK (right panels).

carbazolequinones at concentrations between 10 and 100 μM where a trend of increased LDH release was observed. In this concentration range, carbazolequinones induced the greatest degree of apoptosis and the data reflected apoptotic cells that have detached from the dish and lost their membrane integrity. Concentrations above 100–200 μM, concentrations of carbazolequinones resulted in direct induction of necrosis, suggesting that the type of cell death is dependent upon drug concentration. Other compounds have also been shown to display apoptotic and necrotic concentrations [21,44]. Indeed, a number of studies suggested different patterns of cell death by sulfur mustard with respect to the dose and time of exposure. At low concentrations apoptotic death prevailed, in contrast rapid necrotic death was observed at high concentrations [48]. Besides, taxol was demonstrated to produce a biphasic viability curve *in vitro* [25]. Low concentrations of taxol led to mitotic block and apoptosis, while high concentrations inhibited S-phase entry and produced necrosis. These data provide evidence that the mechanism of action of a compound can be heavily dependent on the concentration used. Furthermore, Martin *et al.* [49] suggested that the biochemical cascade of chemotherapy-induced apoptosis is dependent on the severity of the initial insult: modest damage induces arrest while massive damage induces apoptotic cell death. Such a

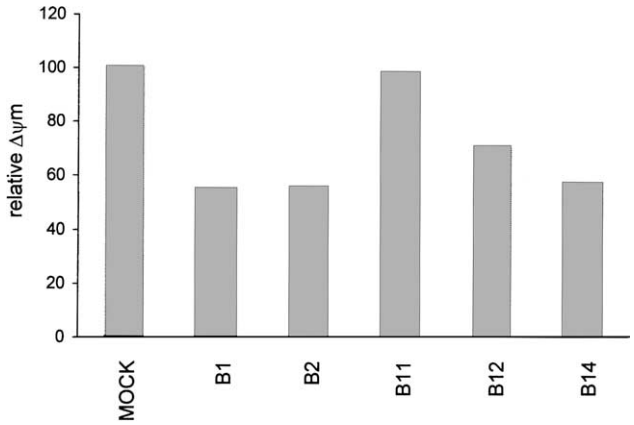


Fig. 6. Loss of mitochondrial $\Delta\psi_m$ in carbazolequinone-treated QNR-ts68 cells. Cells were cultured for 12 hr in presence or absence (MOCK) of carbazolequinone, subsequently loaded for 15 min with fluorescent Mitotracker Red CMXRos and fluorescence intensity was recorded using FACS. Data are representative of three independent experiments.

phenomenon has also been observed following exposure of leukemia cells to Ara-C and anthracyclines [27] or TS inhibitors [50]. Finally, impairment of cell cycle by toxic chemicals usually leads to growth retardation, cytotoxicity and apoptosis [51]. Likewise, our results indicated that increasing concentration of carbazolequinones eventually lead to a shift from apoptosis to necrosis. Moreover, several active carbazolequinones had a cell cycle blocking effect on the quail QM7 cell line (our unpublished results).

Mitochondria contain proteins that are involved in the apoptotic cascade, e.g. cytochrome *c* [52]. The use of cell-free systems demonstrated that the release of these products in the cytoplasm is rate-limiting for the activation of caspases and endonucleases. Functional studies indicated that drug-enforced opening or closing of the mitochondrial megachannel (the permeability transition pore) can induce or prevent apoptosis [18]. Our results demonstrate that a $\Delta\psi_m$ drop occurs in QNR-ts68 cells as early as 12 hr after treatment with active carbazolequinones. This suggests that changes in the mitochondrial permeability is a key event during carbazolequinone-induced apoptosis. However, blocking of caspase activation with Z.VAD.FMK is sufficient to inhibit effective cell death in QNR-ts68 cells, suggesting that caspase-3 activation is downstream of the $\Delta\psi_m$ drop. Thereafter, caspase-3 could rapidly induce the cleavage of PARP, which in turn leads to degradation of DNA into nucleosomal fragments. This time course of events is supported by the observation that pycnotic nuclei and activation of caspase-3 are observed as simultaneous events in carbazolequinone-treated QNR-ts68 cells.

Further experimentation will be necessary to elucidate the precise mechanism of cytotoxicity of carbazolequinones. Recent studies have demonstrated that induction of apoptosis by cytotoxic drugs in primary human leukemic blast specimens correlated closely with loss of $\Delta\psi_m$ [53]. Mitochondria are known for being the major source for endogenous cellular ROS production [54]. Mitochondria have been shown to participate in the process of chemical-induced cell injury that leads to cellular dysfunctions. In addition to ATP synthesis, mitochondria are crucial for the modulation of cell redox status, osmotic regulation, pH control and calcium homeostasis [16,17,55–58]. However, mitochondria are proned to the attack by oxidants, electrophiles and lipophilic cations [59]. Oxidative stress and depletion of mitochondrial glutathion by toxic chemicals may impair the mitochondrial functions and bioenergetics [50,60], leading to dysregulation of cell cycle control, apoptosis and necrosis [61,62]. It is noteworthy that apoptosis is the mechanism of cell death for a diverse group of neuronal lesions and that oxidative stress was shown to play a role in this apoptotic process [63,64]. Oxidative stress is believed to be an important mediator of neuronal cell death and has been postulated to contribute to the pathogenesis of retina degeneration [41]. Because QNR cells exhibit typical features of differentiated nerve cells [9,10], it would be of interest to test whether oxidative stress is involved in the carbazolequinone-induced cell death in these particular cells.

The use of carbazolequinones as antitumor agents is still limited. However, we show here that these compounds can destroy transformed cells displaying high $p60^{v\text{-}src}$ kinase activity which are particularly resistant to apoptosis. Thus, carbazolequinones could be used to kill tumor cells displaying high endogenous $p60^{c\text{-}src}$ tyrosine kinase activity. Together, our results encourage further study on the use of carbazolequinone derivatives to prevent the recurrence of tumors displaying high tyrosine kinase activity.

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