# Mitogen-activated Protein Kinase Routes as Targets in the Action of Diaza-anthracene Compounds with a Potent Growth-inhibitory Effect on Cancer Cells[1]

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## Abstract

**1,8-Diaza-anthracene-tetraones are novel intermediates in the synthesis of the antifolate antibiotic diazaquinomycin A that was found before to have potent antitumor activity. Three of them (CV65, CV66, and CV70) were found to inhibit growth of a panel of several human tumor cell lines. The $IC_{50}$s ranged from 0.05 to 1.5 $\mu$M and are comparable with that of doxorubicin. Among the three drugs, CV70 showed the highest cytotoxic activity. The growth-inhibitory action of these compounds was unrelated to the p53 status of the cells. At micromolar concentrations, all three compounds induced apoptosis, CV70 being the most proapoptotic. The incubation of HeLa cells with CV65, CV66, and CV70, at concentrations between 10 and 20 $\mu$M, inhibited the activation of c-Jun $NH_2$-terminal kinase by various stimuli and prevented growth factor-induced extracellular signal-regulated kinase (ERK) 5 activation. At least one drug, CV65, also inhibited p38. This was surprising because proapoptotic antitumor drugs activate stress signaling pathways. Activation of ERK1/2 by growth factors or phorbol esters was unaffected by preincubation of cells with CV compounds. *In vitro*, CV compounds inhibit the enzyme quinone reductase but not c-Jun $NH_2$-terminal kinase or ERK5. Because doxorubicin also inhibits quinone reductase, we conclude that the inhibitory effect of CV compounds on stress signaling kinases is not a direct effect on the kinases and is likely attributable to upstream elements of the activation cascades.**

## Introduction

Diazaquinomycin A is an antifolate antibiotic of unique structure, with cytotoxic activity against mammalian cell lines (1). Synthesis derivatives with activity against tumor cell lines have been obtained (Refs. 2, 3; Fig. 1).

Diazaquinomycin A, as well as its derivatives and synthetic intermediates, are 1,8-diaza-anthracene-2,7,9,10-tetraones. They share the anthracene ring in common with some anthracyclin antitumor agents such as doxorubicin and mitoxantrone, which are widely used in the treatment of cancer (4). Thus, their effect on cancer cells can be considered in relation to that of anthracyclins.

The antiproliferative activity of doxorubicin has been studied extensively. It is known that doxorubicin inhibits DNA topoisomerase II (5–7) and induces apoptosis in sensitive tumor cells. In addition, it can also undergo redox cycling to produce free radicals that cause DNA cleavage and lipid peroxidation (8, 9). Also, doxorubicin induces quinone reductase (10) and activates JNK[3] (11). More recently a doxorubicin-specific NADH-quinone reductase has been purified from plasma membranes (12). Although the effects of doxorubicin at nuclear level can be easily related to its antiproliferative properties, its membrane and cytoplasmic effects are not yet well defined. Studies of gene disruption in mice have suggested a proapoptotic role for JNK (13). Thus, the activation of JNK by doxorubicin can be related to the induction of an apoptotic pathway. It has also been shown that JNK can activate apoptosis by interfering with mitochondria, resulting in the release of cytochrome *c* (14), and it has been proposed that JNK-mediated phosphorylation of Bcl-2 and Bcl-X could inactivate the death-protective function of these proteins (15). On the other hand, the significance of the effect of doxorubicin on quinone reductase has not yet been established, although the role of this enzyme in the protection from oxidative stress is well known (16).

During the study of a group of new diaza-anthracene-tetraone compounds, we found three drugs, *i.e.,* CV65, CV66, and CV70, that exhibit high levels of cytotoxicity against tumor-derived cell lines and inhibited the activation of some MAPK. This prompted us to study the mechanism of cell death induced by these CV compounds and to characterize their effect at the level of MAPKs.

## Materials and Methods

**Drugs.** CV65, CV66, and CV70 are three diaza-anthracene-tetraones, analogues of the antitumor antibiotic diazaquinomycin A (3). A solution (1 mg/ml) in DMSO was prepared from

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[3] The abbreviations used are: JNK, c-Jun $NH_2$-terminal kinase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; ERK, extracellular signal-regulated kinase; PMSF, phenylmethylsulfonyl fluoride; MBP, myelin basic protein; HA, hemagglutinin; EGF, epidermal growth factor; EGFR, EGF receptor; TNF, tumor necrosis factor; NRG, neuroregulin.

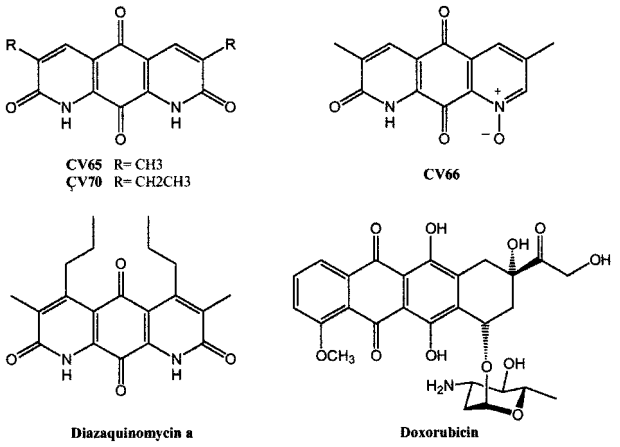


*Fig. 1*. Structure of doxorubicin, diazaquinomycin A, and its derivatives (CVs).

lyophilized samples provided by Instituto Biomar S.A. (León, Spain). Doxorubicin and dicoumarol were purchased from Sigma. Doxorubicin was dissolved in $H_2O$, and dicoumarol was dissolved in 13 mM NaOH.

**Cell Lines and Cell Culture.** K562, Molt4, and P388 cell lines were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS, gentamicin (80 $\mu$g/ml), and 5% $CO_2$ at 37°C. HeLa, A549, 293T, HepG2, and MCF7 cells were grown in DMEM (Life Technologies, Inc.) supplemented with 10% FCS, gentamicin (80 $\mu$g/ml), and 5% $CO_2$ at 37°C.

**Cell Viability Assay.** Cells were seeded at a concentration of 7,500 cells/well for the K562 cell line, 15,000 for Molt4, 10,000 for P388, and 5,000 for the MCF7 and A549 cell lines in 100 $\mu$l of culture medium containing various concentrations of CVs compounds in microtiter plates (tissue culture grade, 96 wells, flat-bottomed). After 48 h of incubation, 10 $\mu$l of WST-1 reagent (Roche) were added per well, and the cells were incubated for 3 h at 37°C. The absorbance was measured at 405 nm in an ELISA reader (EL340 microplate, Biokinetics reader; Bio Tek Instruments).

**Apoptosis Determination.** Binding of annexin V to the cell surface was carried out by flow cytometry with an annexin V-FITC kit following the manufacturer's instructions (PharMingen). The fraction of cells bound to annexin V was measured with a Becton-Dickinson Excalibur cytometer.

**MAPK Assays.** ERK5 activation was analyzed by gel shift of activated ERK5 as described previously (17). In brief, HeLa cells were plated in 100-mm dishes and cultured until 80% confluence. Cells were preincubated with the indicated concentrations of the CV compounds for 60 min before stimulation with EGF (10 nM) or sorbitol (0.4 M) for 15 min. Then monolayers were washed with PBS and lysed in ice-cold lysis buffer [140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% NP40, 20 mM Tris (pH 8.0), 1 mM pepstatin, 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin, 1 mM PMSF, and 1 mM sodium orthovanadate]. After scraping the cells from the dishes, samples were centrifuged at 10,000 $\times$ *g* at 4°C for 10 min, and supernatants were transferred to new tubes with the anti-ERK5 COOH-terminal antibody and protein A-Sepharose. Immunoprecipitations were performed at 4°C for at least 2 h, and the immune complexes recovered by a short centrifugation, followed by three washes with 1 ml of cold lysis buffer. Samples were then boiled in electrophoresis sample buffer and loaded in 6% SDS-PAGE gels. After transfer to polyvinylidene difluoride membranes, filters were blocked for 1 h in TBST and then incubated for 2–16 h with the anti-ERK5 COOH-terminal antibody. After washing with TBST [150 mM NaCl; 0.05% Tween 20; 20 nM Tris-HCl (pH 7.6)], filters were incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min, and bands were visualized by a luminol-based detection system with *p*-iodophenol enhancement. For *in vitro* kinase assays, immunoprecipitates were obtained essentially as before, washed twice with 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 2 mM DTT, and 0.05% Tween 20; and then the precipitates were divided into two parts. The one used for the kinase assay was washed once with a buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 2 mM DTT, and 1 mM PMSF; and then incubated for 30 min at 30°C in 15 $\mu$l of a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 $\mu$M ATP, 10 $\mu$g of MBP, and 2 $\mu$Ci of [$\gamma$-$^{32}$P]ATP. The reactions were quenched by addition of an equal volume of 2$\times$ Laemmli sample buffer and were run in a 10% SDS-PAGE. The gels were then exposed to a Fuji IIIS sensitive screen, and radioactivity in gels was digitally analyzed using a BAS1500 apparatus. Dried gels were also exposed to X-ray films to obtain autoradiographs.

JNK was determined by the immune complex kinase assay as described (18). Cells were grown as before, and after preincubation with CV compounds for different periods of time, the cells were stimulated with 400 J/m$^2$ UV light, 10 $\mu$g/ml anisomycin, 0.6 M sorbitol, 10 ng/ml TNF-$\alpha$, 5% ethanol, 2 mM orthovanadate, 500 nM okadaic acid, or heat shock (42°C for 20 min). Cells were lysed in 20 mM HEPES-NaOH (pH 7.6), 10 mM EGTA, 25 mM $MgCl_2$, 1% NP40, 1 mM DTT, 40 mM $\beta$-glycerophosphate, 100 $\mu$M sodium orthovanadate, 0.5 mM PMSF, 1 $\mu$g/ml of aprotinin, and 1 mg/ml of leupeptin. Lysates were cleared by centrifugation for 10 min at 12,000 rpm at 4°C. Extracts were immunoprecipitated with a rabbit polyclonal anti-JNK 1 antibody from Santa Cruz (sc-474), and immune complexes were recovered with protein G-Sepharose (Amersham-Pharmacia). Beads were sequentially washed three times with 1% NP40 and 2 mM sodium orthovanadate in PBS and twice with kinase buffer. One-fifth of the total immunocomplex was separated for the kinase determinations, and the rest was Western blotted and incubated with the anti-JNK antibody as a loading control. Phosphorylation reactions were performed in a 30-$\mu$l volume containing kinase buffer, 20 $\mu$M ATP, 0.5 $\mu$Ci [$\gamma$-$^{32}$P]ATP, and 1 $\mu$g of glutathione *S*-transferase-c-Jun at 30°C for 30 min, stopped by the addition of 4$\times$ Laemmli sample buffer, and resolved in 10% SDS polyacrylamide gel. The phosphorylated form of JNK was detected with a mouse monoclonal antibody from Promega.

For p38 activity determinations, HeLa cells were transfected with a HA-tagged form of the kinase using Lipofectin (Life Technologies, Inc.) as transfection reagent. Tagged p38 was then immunoprecipitated using an anti-HA 12CA5 monoclonal antibody, and p38 activity was measured as

*Table 1* $IC_{50}$ ($\mu$M) of CV compounds and doxorubicin on different cancer cell lines[a]

| | P388 | K562 | Molt4 | A549 | MCF7 | HeLa |
|---|---|---|---|---|---|---|
| CV65 | 0.50 ± 0.30 | 0.91 ± 0.50 | 0.03 ± 0.03 | 0.40 ± 0.15 | 0.20 ± 0.10 | 1.10 ± 0.4 |
| CV66 | 0.97 ± 0.32 | 0.47 ± 0.25 | 0.03 ± 0.03 | 0.35 ± 0.10 | 0.22 ± 0.05 | 0.65 ± 0.20 |
| CV70 | 0.53 ± 0.05 | 0.13 ± 0.10 | 0.04 ± 0.02 | 0.03 ± 0.01 | 0.05 ± 0.04 | 0.30 ± 0.10 |
| Doxorubicin | | 0.65 ± 0.2 | | | | 0.37 ± 0.2 |

[a] The data are the mean values ± SD from three independent experiments.

described above for JNK, using a glutathione *S*-transferase-ATF2 fusion protein as substrate.

**Quinone Reductase Assay.** HeLa cells were grown until 80% confluence, washed twice in 1 ml of ice-cold PBS, scraped, snap-frozen, and stored at −70°C until use. Upon thawing, the cells were resuspended in 300 $\mu$l of PBS and lysed by freezing and thawing in liquid nitrogen. Then, 0.5 ml of 0.25 M sucrose was added, and the cells were centrifuged at 2,000 × *g* for 5 min. Twenty % of the total volume of 0.1% of $CaCl_2$ was added to the supernatants, and the samples were set on ice for 15 min. The supernatants were then removed for enzyme activity. Quinone reductase activity was measured by a spectrophotometric assay with NADH, menadione, and cytochrome *c* as described (19). The increase in absorbance at 550 nm was recorded continuously, and quinone reductase activity was calculated as nmol/min/$\mu$g of protein in the presence or absence of inhibitor. An extinction coefficient of 18.5 $mM^{-1}cm^{-1}$ was used. Protein was determined by the method of Bradford (20).

## Results

A number of analogues of the antitumor antibiotic diazaquinomycin A, were generated (Ref. 3; Fig. 1). Because diazaquinomycin A and synthetic derivatives were found to have potent antitumor activity,[4] we decided to test the cytotoxic properties of the compounds against a panel of tumor cell lines.

**Proliferation Inhibition by CV Compounds.** These compounds were tested at concentrations ranging from 0.01 to 10 $\mu$M on a panel of six tumor-derived cell lines: human myeloid leukemia K562, human lymphoid leukemia Molt4, human cervix carcinoma HeLa, mouse myeloid leukemia P388, human lung carcinoma A549, and human breast carcinoma MCF7.

The growth rate was determined by the WST-1 reduction assay, and the calculated $IC_{50}$ for the different compounds is indicated in Table 1. Doxorubicin was included in the assay as a reference of anthracyclin drug used in cancer chemotherapy. The results indicate that these compounds had a potent cytotoxic activity on most cell lines, with the $IC_{50}$ ranging from 0.05 $\mu$M (CV70 in MOLT-4) to 1 $\mu$M (CV65 in HeLa). CV65 was also tested on a number of additional cell lines (prostate carcinoma PC3, acute myeloid leukemia HL60 and NB4, Burkitt lymphoma Raji, and chronic myeloid leukemia MEG01), and the $IC_{50}$s were in the range of those reported in Table 1 (not shown). In general, cells derived from

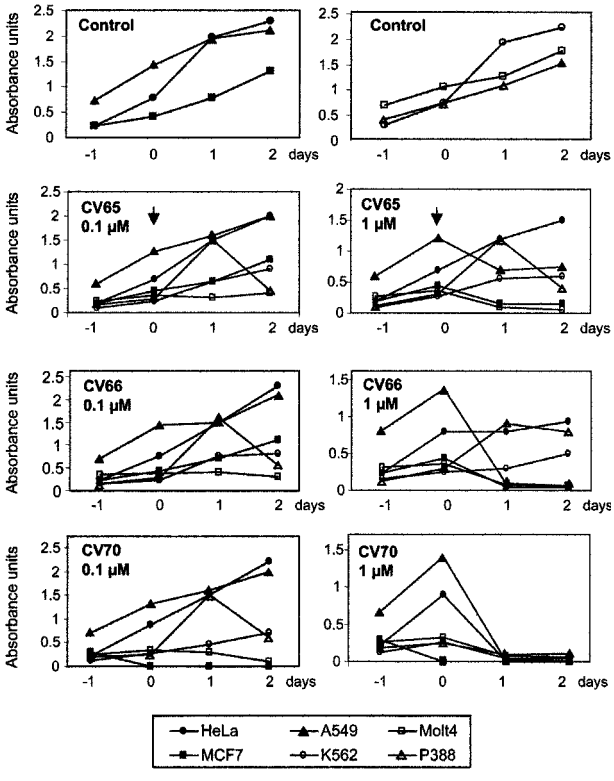


*Fig. 2.* Effects of CV65, CV66, and CV67 on cell growth. Growth of the indicated cell lines was determined by the WST-1 colorimetric assay as described in "Materials and Methods." The drugs were added at the indicated final concentrations at day 0 (*arrow*).

human leukemia were more sensitive than those derived from solid tumors, and CV70 was the most potent of the three compounds. It is noteworthy that the potency of the drugs was not related to the p53 status of the cells because K562, HL 60, NB4, and HeLa lack an active p53 protein.

To further assay the cytotoxic effect of the drugs, we measured growth by cell counting at 24 and 48 h of treatment and different drug concentrations of CV65, CV66, and CV70. The three compounds completely inhibited growth of all cell lines at 5 $\mu$M or higher concentrations. The results with the lower drug concentrations assayed (0.1 and 1 $\mu$M) are shown in Fig. 2 and indicate that: (*a*) there is dose- and time-dependent inhibition of growth for all cell lines; (*b*) CV70 was the most potent inhibitor, because 1 $\mu$M CV70 was the only drug capable of inhibiting growth of the six cell lines; and (*c*) MOLT-4 was the most sensitive cell line.

The potent antiproliferative activity of CVs on K562 was most interesting, because these cells have been shown to be

[4] Patent application EPO695752, 1966.

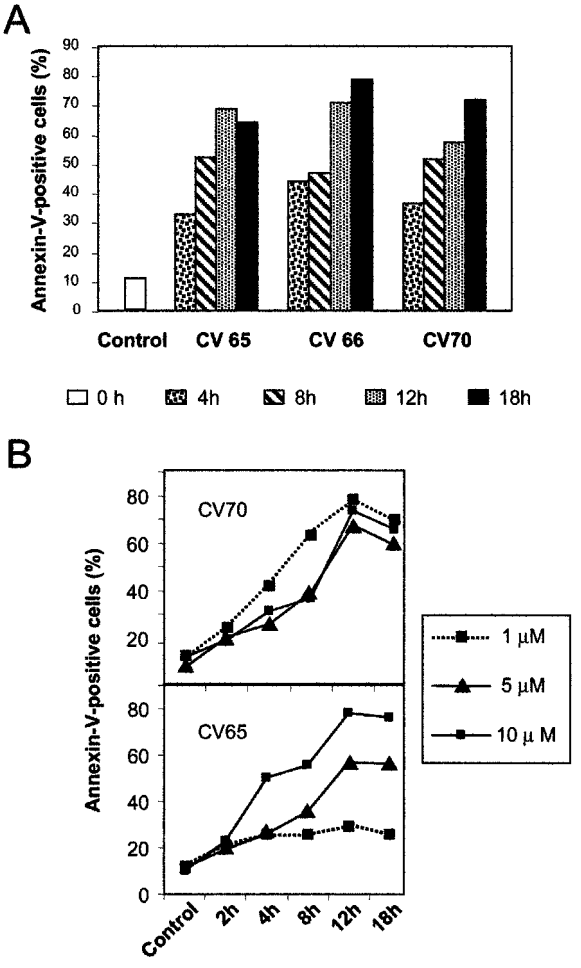


*Fig. 3*. Apoptosis induced by CV65, CV66, and CV70 in K562 cells. *A,* apoptosis assayed by annexin V binding after 4, 8, 12, and 18 h of treatment with 5 μM CV65, CV66, and CV70. *B,* apoptosis after treatment for 2–18 h with the indicated concentrations of CV65 and CV70.

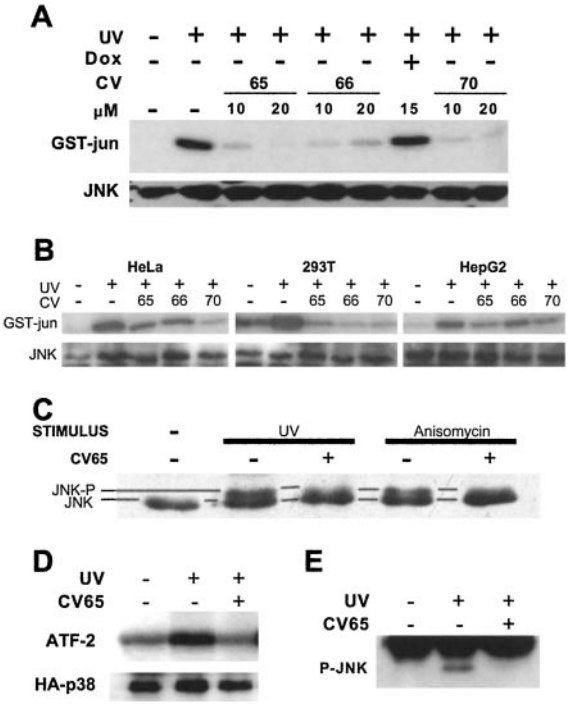


*Fig. 4*. CV compounds inhibit the activation of JNK and p38. *A,* CV65, CV66, and CV70 were added at the indicated concentrations in DMSO to HeLa cells growing at 80% confluence and incubated for 3 h before treating the cells with 400 mJ/m$^2$ UV light. After an additional 30-min incubation, cells were lysed, and JNK activity was determined. *B,* effect of three CV compounds on the activation of JNK in different cell lines. Cells were preincubated with CV compounds at the 15 μM concentration for 2 h before UV stimulation and treated as in *A*. *C,* CV65 prevents the appearance of slower migrating phosphorylated forms of JNK. JNK was immunoprecipitated from HeLa cells stimulated with either UV light or anisomycin in the presence or absence of CV65. JNK was then identified with an anti-JNK antibody. *D,* CV65 blocks JNK phosphorylation induced by UV light. An anti-phospho-JNK antibody was used to detect the phosphorylated kinase in JNK immunocomplexes from HeLa extracts. *E,* UV activation of p38 is also blocked by CV65. A plasmid coding for a HA-tagged form of p38 was transfected into HeLa cells and then treated with UV and/or CV65 as stated. p38 activity was determined in anti-HA immunocomplexes as indicated in "Materials and Methods."

resistant to apoptosis by many drugs (21, 22), a feature that has been correlated to the absence of p53 and the expression of the Bcr-Abl tyrosine kinase (23–25). Therefore, we chose K562 to further test the effects of CVs on cell growth and apoptosis. The inhibition of K562 cell proliferation provoked by CVs, as determined by cell counting, was similar to that of doxorubicin and consistent with the data obtained by WST-1 reduction (not shown). At concentrations of 5 μM and higher, K562 and other cells treated with CVs for 24 h or longer showed a morphology consistent with apoptosis (not shown). To quantify the apoptosis induced by the compounds, we performed annexin V binding assays in K562 and HeLa cells. This assay detects the translocation of phosphatidyl serine to the outer layer of cell membrane, a marker of apoptosis. The results demonstrate that, in both cell lines, the three CVs induced apoptosis at 5 μM in a time-dependent manner (Fig. 3*A*). However, the determination of apoptosis at different concentrations of CV65 or CV70 showed that CV70 was the most cytotoxic agent, inducing maximal apoptosis with the lowest concentration assayed (1 μM), whereas this concentration of CV65 did not induce apoptosis (Fig. 3*B*). This result agrees with the highest growth inhibitory activity displayed by CV70 in all cell lines assayed.

Because many anticancer drugs, in particular anthracyclines, activate stress-signaling cascades and this effect has been related to apoptosis, we studied the effect of CV compounds on the JNK/p38 signaling cascades. The drugs were incubated with HeLa cells for 3 h at two different concentrations (10 and 20 μM) in media with serum. After UV light stimulation, cells were lysed, and JNK activity was determined in immunoprecipitates as described in "Materials and Methods." All three CV compounds were found to inhibit JNK at both concentrations (Fig. 4*A*). Inhibition was already high at the 10 μM concentration and increased (in CV65) or was maintained (in CV66 and CV70) at 20 μM. In contrast, doxorubicin did not change or increased the level of activation of JNK. To find out whether the effect observed in HeLa cells could be extended to other cell lines, a similar experiment was carried out using cell lines 293T and HepG2. In all cell

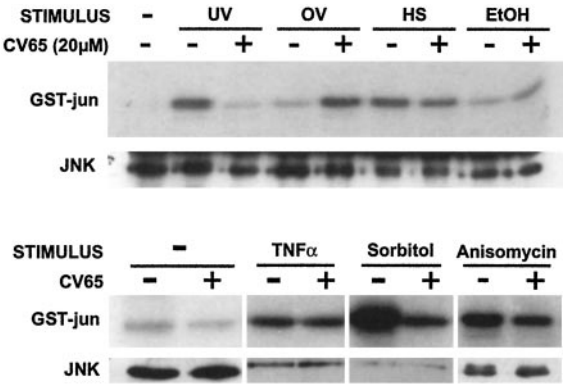


*Fig. 5*. Effect of CV65 on the activation of JNK by various stimuli. HeLa cells were incubated for 10 min in the presence of CV65 or vehicle as indicated and then stimulated with UV light (*UV*), orthovanadate (*OV*), heat shock (*HS*), 5% ethanol (*EtOH*), TNF-$\alpha$ (*TNF*$\alpha$), sorbitol, or anisomycin as described. JNK was immunoprecipitated from the cell extracts, and JNK activity was determined. Four-fifths of each determination's immunocomplex was used for the loading control, shown below each figure.

lines tested, CV compounds were found to inhibit JNK activation. The degree of inhibition in 293T was higher than in HeLa cells (Fig. 4*B*). Taking CV65 as a prototype, the inhibitory effect of CV65 could also be detected in Western blotting by the disappearance of a double band that was detected in SDS-PAGE after stimulation. The double band is likely attributable to the slightly different migration of the unphosphorylated (*lower band*) and phosphorylated form of JNK (*upper band*) that disappears after incubation with CV65 (Fig. 4*C*). This was confirmed, using an anti-phospho-JNK antibody, by the disappearance of the band corresponding to the phosphorylated form of JNK after preincubation with CV65 (Fig. 4*D*).

In HeLa cells transfected with the HA-*p38* gene, p38 kinase activity was determined in immunoprecipitates using ATF-2 as substrate. The activation of p38 by UV light was also inhibited by preincubation with CV65 at the 20 $\mu$M concentration (Fig. 4*E*).

It is known that both JNK and p38 can be activated by a number of different stimuli. To study the effect of CV65 on JNK after activation with different agents, HeLa cells were stimulated with UV, orthovanadate, heat shock, ethanol, TNF-$\alpha$, sorbitol, or anisomycin. As shown in Fig. 5, JNK activation by orthovanadate, heat shock, ethanol, or TNF-$\alpha$ was not inhibited by preincubation with CV65 at the 20 $\mu$M concentration. However, a significant inhibition was observed when we stimulated HeLa cells with UV, sorbitol, or anisomycin

The former results suggested that CV65 might not directly inhibit JNK. To test this, CV65 (5–20 $\mu$M) was added to JNK immunoprecipitates of HeLa cell extracts, and JNK activity was determined. *In vitro*, CV65 failed to inhibit JNK at the concentrations that were found to inhibit JNK *in vivo* (not shown). This suggested that an upstream component of the cascade rather than JNK could be the target of the inhibitory action of this compound.

**CV Compounds Inhibit ERK5 but not ERK 1/2.** We also investigated whether CV compounds could affect ERK5, a MAPK that is activated by growth factors and participates in proliferation in response to several growth factors. Analysis of ERK5 activation was performed by Western blotting, with antibodies that recognize the COOH terminus of ERK5. As reported previously, addition of EGF to HeLa cells caused a retardation in the mobility of ERK5 as a result of the dual phosphorylation of ERK5 at its TEY microdomain (Fig. 6*A, top panel*). In parallel, Western blotting of lysates of these cells using the anti-p-ERK1/2 antibody indicated that EGF also efficiently activated ERK1/2 in these cells (Fig. 6*A, bottom panel*). As shown in Fig. 6, *B–D,* preincubation of HeLa cells with the CV compounds caused a dose-dependent inhibition of EGF-induced ERK5 activation. In contrast, activation of ERK1/2 induced by EGF was largely unaffected by preincubation with these compounds. The lack of an inhibitory effect of CV65 on ERK1/2 activity stimulated by EGF or phorbol esters was confirmed in a functional assay (Fig. 6*E*). The action of CV70 on ERK5 was also evaluated in MCF7 cells treated with the EGF-like ligand NRG. Addition of NRG resulted in activation of ERK5, and this effect was prevented by preincubation with CV70. In addition, CV70 was also able to decrease the resting elevated level of ERK5 activity in BT474 cells and prevented further activation triggered by NRG (Fig. 7*A*). The inhibitory effect of CV70 on ERK5 activation was compared with the effect of preincubating HeLa cells with doxorubicin. Although CV70 readily prevented EGF-induced ERK5 activation, doxorubicin at equimolar concentration failed to prevent the action of EGF in these cells (Fig. 7*B*). The inhibitory action of CV70 was also evident when HeLa cells were treated with either optimal or suboptimal concentrations of EGF (Fig. 8*A*).

The above data raised two important questions, *i.e.,* whether CV compounds unspecifically affected kinases upstream of ERK5 and whether the inhibitory action of the CV compounds extended to other stimuli that activate ERK5 through stress routes that are independent of growth factor receptor activation. To answer the first question, we investigated the effect of CV70 on the tyrosine phosphorylation of the EGFR in response to EGF concentrations that ranged from suboptimal (0.1 nM) to maximal (>5 nM) concentrations. As shown in Fig. 8*A,* EGF induced ERK5 activation in a dose-dependent fashion, and CV70 efficiently inhibited EGF-induced ERK5 activation over that range of concentrations tested. In contrast, CV70 failed to inhibit EGFR tyrosine phosphorylation in response to EGF, even at suboptimal concentrations of the growth factor. Furthermore, CV70 increased EGF-induced tyrosine phosphorylation of the EGFR. This effect of CV70 was more evident at suboptimal EGF concentrations (Fig. 8*B*).

To investigate whether CV compounds may also affect activation of ERK5 triggered by other stimuli, we used sorbitol, which causes osmotic stress and that has been reported to activate ERK5 in HeLa cells. As shown in Fig. 8, sorbitol efficiently triggered ERK5 activation, and its effect was largely prevented by preincubation with CV70. Parallel analysis of EGFR tyrosine phosphorylation indicated that sorbitol only slightly increased EGFR tyrosine phosphorylation. The degree of activation of the EGFR by sorbitol was equivalent to that obtained by suboptimal (1–2.5 nM) con-

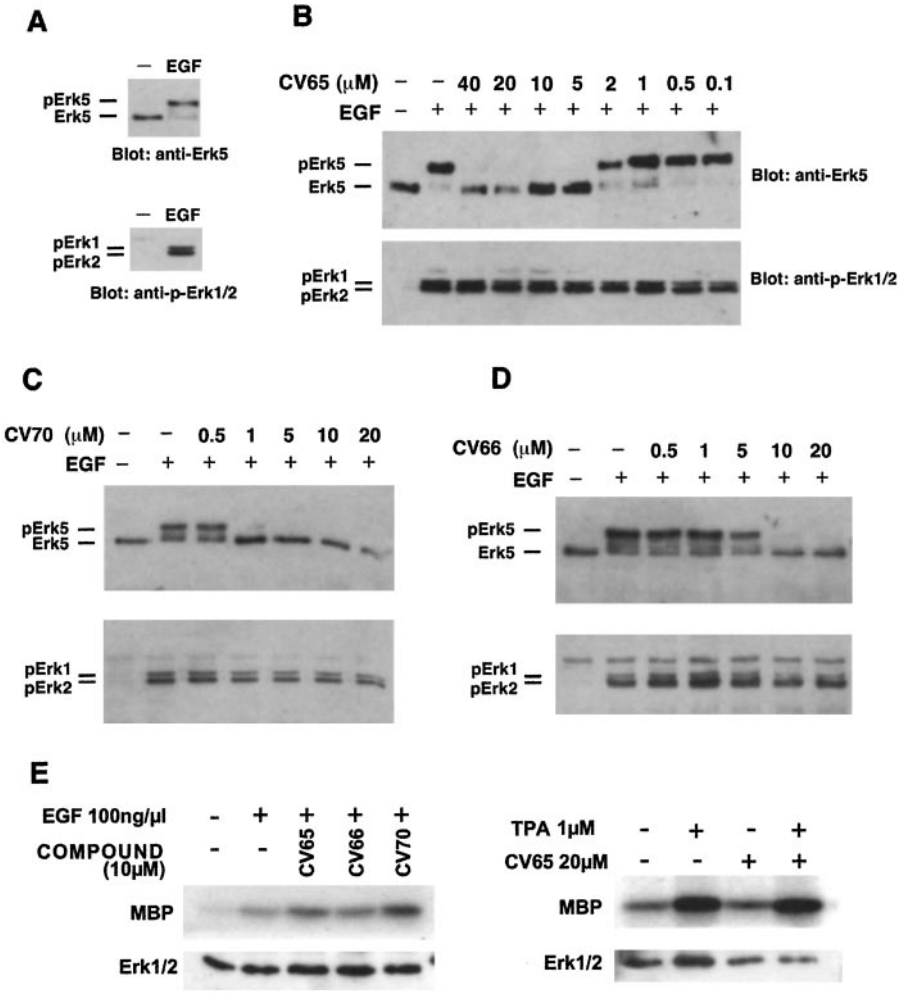


*Fig. 6*. Effect of CV compounds on the activation of ERK5 and ERK 1/2. *A,* HeLa cells were treated with EGF (10 nM) for 15 min, and then cells extracts were prepared for immunoprecipitation with the anti-ERK5 antibody, followed by Western blotting with the same antibody (*top panel*). An aliquot of the cell extracts was run on 12% SDS-PAGE gels, and the blot was probed with the anti-ERK1/2 antibody. *B,* dose-response effect of CV65 on EGF-induced ERK5 and ERK 1/2 activation. HeLa cells were preincubated for 60 min with the indicated concentrations of CV65, and then lysates were subjected to immunoprecipitation and Western blotting with the anti-ERK5 antibody as above. In parallel, pERK1/2 was determined in cell lysates as described above. Dose-response of the effect of CV70 (*C*) and CV66 (*D*) on EGF induced ERK5 and ERK 1/2 activation in HeLa cells. Treatments and Western blots were performed as above. *E, left panels, in vitro* kinase assay of the action of CV compounds on EGF-induced ERK 1/2 activation. Cells were preincubated with the inhibitors for 60 min before treatment with EGF. Then lysates were immunoprecipitated with anti-ERK2 antibodies, and kinase assays were performed using MBP as a substrate. ERK2 loading is shown at the bottom. *Right panels,* effect of CV65 on TPA-induced ERK1/2 activation. HeLa cells were treated with PMA for 15 min, and then lysates were prepared for immunoprecipitation with anti-ERK2 antibodies. *In vitro* kinase activity of ERK2 was probed using MBP as the substrate, and the amount of ERK2 immunoprecipitated was probed with anti-ERK2.

centrations of EGF that slightly activated ERK5. Therefore, it is quite likely that most of the action of sorbitol on ERK5 activation occurs by an EGF-independent route.

The prevention of ERK5 activation by CV70 indicated that this compound inhibited kinases upstream of ERK5 rather than directly inhibiting ERK5. To further substantiate this point, we performed *in vitro* kinase assays of ERK5 in the presence of CV70. Addition of EGF resulted in a substantial increase in ERK5 autophosphorylation. As expected, CV70 at a 10 μM concentration readily prevented ERK5 activation *in vivo* and failed to affect the kinase activity of ERK5 *in vitro* (Fig. 8*C*). A similar result were obtained when the related anthracycline doxorubicin was used instead of CV70.

**Quinone Reductase Inhibition of CV Compounds.** The activity of stress signaling kinases JNK and p38 is commonly regulated by the actual redox environment of the cell. Quinone reductase inhibitors such as dicoumarol are known to inhibit JNK stimulated by UV light or anisomycin. Because the chemical structure of CV compounds suggested similarity with some quinone reductase inhibitors, CV compounds were tested as potential quinone reductase inhibitors in cell-free extracts of HeLa cells using menadione as a H acceptor. Table 2 shows that at the concentration of 15 μM, both CV65 and CV66 inhibit quinone reductase significantly (46 and 59%, respectively), whereas CV70 inhibits only 34% at the concentration of 40 μM. Doxorubicin also inhibits quinone

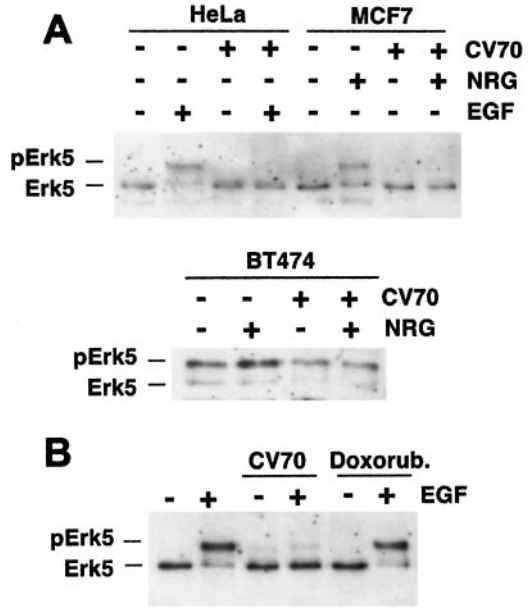


*Fig. 7.* *A,* effect of CV70 on the activation of ERK5 in different cell lines. Cell lines HeLa, MCF7, and BT474 were preincubated with CV 70 (10 $\mu$M) for 30 min and then incubated with EGF (10 nM) or NRG (10 nM) for an additional period of 5 min. ERK kinase was then immunoprecipitated and detected by Western blotting as indicated. *B,* as in *A,* except that HeLa cells were preincubated either with CV70 (10 $\mu$M) or doxorubicin (*Doxorub.,* 10 $\mu$M).

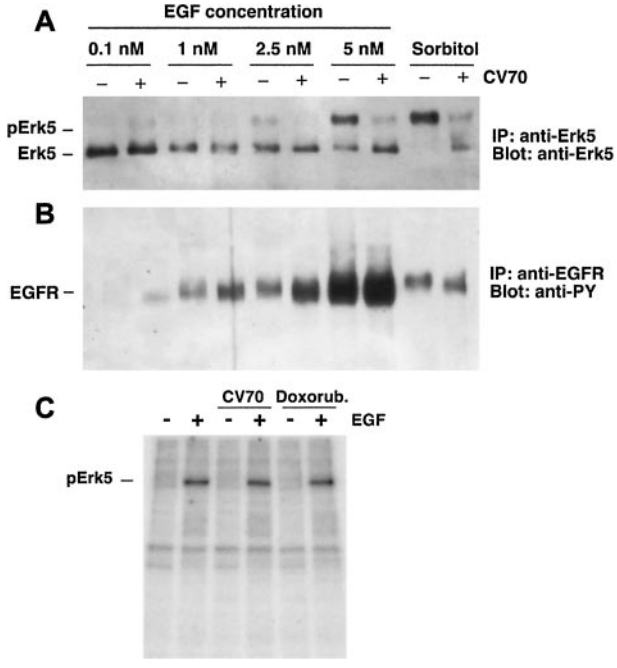


*Fig. 8.* Effect of CV70 on the activation of ERK5 by EGF and sorbitol. *A,* HeLa cells were preincubated with CV70 (10 $\mu$M) for 60 min before treatment with EGF (indicated concentrations) for 15 min. Cell lysates were prepared, and ERK5 activation was identified by Western blotting with the anti-ERK5 COOH-terminal antibody. *IP,* immunoprecipitation. *B,* the supernatants from the anti-ERK5 immunoprecipitation (*IP*) were reprecipitated with an anti-EGFR antibody, and tyrosine phosphorylated EGFR was detected with anti-pTyr antibodies. *C,* HeLa cells were treated with EGF (10 nM) for 15 min, and ERK5 kinase was immunoprecipitated. The immunoprecipitates were subjected to *in vitro* kinase assay to which CV70 (10 $\mu$M) or doxorubicin (*Doxorub.,* 10 $\mu$M) were added, where indicated. Reactions were stopped by the addition of sample buffer, and 10% SDS-PAGE gels were run. Dried gels were exposed to autoradiography film.

reductase by 24% at the latter concentration. Thus, although quinone reductase could be a common target in the action of CV compounds and doxorubicin, it is not likely that this effect could account for the differences in MAPK inhibition.

## Discussion

The cytotoxicity of the new diaza-anthracene-tetraone compounds was tested in a number of human tumor cell lines in relation to doxorubicin. CV compounds showed antiproliferative activity on all tumor cell lines tested, derived from different solid tumors, leukemia, and lymphoma. The cytotoxicity of CV compounds is of the same order as that of doxorubicin showing an $IC_{50}$ of 0.05–0.5 $\mu$M for the most potent drug (CV70). p53 confers sensitivity to anticancer drugs (26), and mutated or absent p53 confers resistance against chemotherapeutic agents to tumors (27). However, the cytotoxic potency of CVs is not related to the p53 status of the tested cell lines, which is a desirable feature for an antitumoral drug. The three CV compounds readily induced apoptosis on several cell lines and particularly on K562. These cells are known to be particularly resistant to apoptosis induced by many drugs. Altogether these results indicate that CVs are good candidates for further development as anticancer drugs.

Because MAPKs are important signaling cascades in the control of cell proliferation and apoptosis, we studied the involvement of these pathways in the process of apoptosis triggered by CV compounds. It is clear from *in vivo* data with several cells types that, unlike other proapoptotic anticancer drugs, including doxorubicin, CV compounds inhibit rather than activate the stress signaling cascades that involve JNK, ERK5, and p38. Independence of the activation of the stress signaling cascades and apoptosis triggered by doxorubicin has been reported (28). Also, Potapova *et al.* (29) have reported that inhibiting the expression of JNK2 by antisense oligonucleotides induces apoptosis in tumor cells. However, most reports indicate that induction of apoptosis is accompanied by activation of stress signaling kinases and in particular by activation of JNK (30). Thus, although the structure and some cell effects of CV compounds show similarities with those of anthracyclines, there is a clear difference between both groups of compounds at the level of activation of stress signaling kinases. The significance of this difference is not apparent, at least in the long term, because both CV compounds and doxorubicin show a similar growth-inhibitory effect against malignant cell lines and CV compounds similar to doxorubicin also induce apoptosis. It is possible, that the significance of the difference in their action on MAPK has to be analyzed at the short term and at the level of individual target molecules. In this respect, we consider, in the first place, DNA topoisomerase II as the best known target of anthracyclines. We approached this point by studying the inhibition of Taq polymerase by CV compounds. It is

*Table 2* Inhibition of quinone reductase by CV compounds and doxorubicin

Quinone reductase was determined in 1 ml of assay mixture containing 50 mM Tris ClH, 0.08% Triton X-100, 0.5 mM NaOH, 10 μM menadione, and 77 μM cytochrome *c*. The reaction was initiated by addition of 10 μg of protein from HeLa extracts. Inhibitors were added from a concentrated solution in 50% DMSO. The data are presented as mean ± SD of three different experiments.

| Inhibitor | Concentration (μM) | Activity (nmol/min/μg protein) | Inhibition (%) |
|---|---|---|---|
| None | | 254 ± 13 | 0 |
| Dicoumarol | 15 | 44.5 ± 3 | 82.5 |
| CV 65 | 5 | 176 ± 10 | 30.7 |
| | 15 | 137 ± 9 | 46 |
| | 40 | 94.5 ± 4 | 62 |
| CV66 | 5 | 147 ± 12 | 42 |
| | 15 | 104 ± 8 | 59 |
| | 40 | 94.5 ± 5 | 62 |
| CV70 | 5 | 243 ± 12 | 4.4 |
| | 15 | 223 ± 13 | 12 |
| | 40 | 167 ± 7 | 34.3 |
| Doxorubicin | 5 | 278 ± 11 | 0 |
| | 15 | 192.8 ± 9 | 24 |
| | 40 | 195.7 ± 13 | 24 |

believed that inhibition of DNA topoisomerase II by anthracyclines is attributable to interference in the binding of the enzyme to DNA caused by the drugs. Likewise, Taq polymerase (and other DNA binding enzymes), which uses DNA as a template and therefore binds to it, could be inhibited by anthracyclines. In this line, Perry *et al.* (31) have reported that doxorubicin at 10 μM totally inhibits Taq polymerase. Using the same approach, we tested CV compounds as Taq polymerase inhibitors. It was found that CV65 and CV70 inhibit Taq polymerase at the concentration of 100 μM, but none of them were able to inhibit significantly at 10 μM (Fig. 9). This indicates that CV compounds may have some DNA binding activity and may share targets in common with classical anthracyclines at the DNA level. This is not unexpected because of the structural analogies between both types of molecules. Another target of doxorubicin is probably some cellular form of quinone reductase and possibly a plasma membrane enzyme (12). Cross *et al.* (32) had reported that quinone reductase inhibitors inhibit the activation of JNK and produce an increase in apoptosis. More recently, it has been found that the quinone reductase inhibitor dicoumarol suppresses the transient activation of JNK mediated by insulin-like growth factor I receptor as well as protection of apoptosis (33). We show in Table 2 that CV compounds (and to a lower level, doxorubicin) inhibit a quinone reductase present in HeLa cell extracts. Quinone reductase is involved in the regulation of intracellular ROS levels, and these have been shown to mediate activation of MAPK. However, although both CV compounds and doxorubicin inhibit quinone reductase, the small differences observed on enzyme inhibition cannot explain the large differences found between CV compounds and doxorubicin at the level of MAPK activation. This implies that additional factors or targets have to be taken into account. An interesting possibility is the role of nonenzymatic reactions between quinoid compounds and

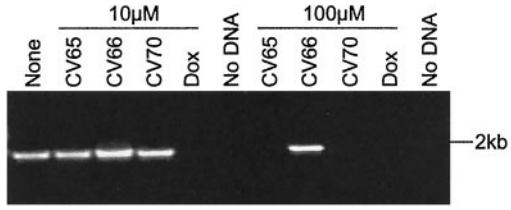


*Fig. 9*. CV65 and CV70 inhibit the amplification of a DNA fragment by Taq polymerase at the concentration of 100 μM but not at 10 μM. PCR reactions were carried out with the RedTaq PCR Reaction Mix (Sigma) containing 1 ng of plasmid MLV-Myc (35) and 500 nM of oligonucleotides 5′-AAGACTCCAGCGCCTTCTCTC-3′ and 5′-GTTTTCCAACTCCGG-GATCTG-3′, which amplify a fragment of about 1900 bp of human c-*MYC* gene in the presence or absence of drugs. The amplified bands in each case are shown.

thiols that have been shown to have profound effects in intracellular ROS levels and cytotoxicity (34).

The matter of how inhibition of stress routes could be related to the apoptotic and cytotoxic effect of CV compounds has not been analyzed in detail. However, it is known that both activation and inhibition of MAPK can be found after stimulation with mitogenic or apoptotic agents, depending on the activation estate of other signaling pathways within cells. We found that CV70 slightly increases the level of EGFR phosphorylation after EGF stimulation in HeLa cells, which would indicate stimulation of a survival route while at the same time clearly inhibiting ERK5 or JNK. It could be that compounds of the type studied here may act on various receptor type molecules at the level of plasma membrane, which, in turn, may activate simultaneously both survival and apoptotic routes that, after prolonged exposure, develop in a dominant (apoptotic) one. In any case, the effect of CV compounds is not attributable to an unspecific effect because the ERK1/2 mitogenic pathway is not inhibited, and only the activation of JNK and ERK5 by some stimuli and not by others was affected.

The *in vivo* data obtained both with ERK5 and JNK, together with the *in vitro* results, offered clues about the site of action of the CV compounds. Thus, because these drugs failed to inhibit *in vitro* JNK and ERK5 activity, the data indicate that the inhibitory action of the drugs *in vivo* must be upstream of JNK, a conclusion also supported by the prevention of ERK5 shifting by these drugs. However, because EGFR activation was not significantly altered by these drugs, their target in the MAPK routes affected must be downstream of the receptors that couple growth factor signaling to the ERK5/JNK routes. It will be interesting to elucidate the identity of the molecules affected by these drugs in the MAPK pathways.

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