Protein Kinase C βI Is Implicated in the Regulation of Neuroblastoma Cell Growth and Proliferation

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Abstract
To investigate a putative involvement of protein kinase C (PKC) isoforms in supporting neuroblastoma cell proliferation, SK-N-BE(2) neuroblastoma cells were transfected with expression vectors coding for the C2 and V5 regions from different PKC isoforms. These structures have been suggested to inhibit the activity of their corresponding PKC isoform. The PKC fragments were fused to enhanced green fluorescent protein to facilitate the detection of transfected cells. Expression of the C2 domain from a classical PKC isoform (PKCα), but not of C2 domains from novel PKCβI or PKCγ, suppressed the number of neuroblastoma cells positive for cyclin A and bromodeoxyuridine incorporation. This indicates a role for a classical isoform in regulating proliferation of these cells. Among the V5 fragments from PKCα, PKCβI, and PKCβII, the PKCβI V5 had the most suppressive effect on proliferation markers, and this fragment also displaced PKCβI from the nucleus. Furthermore, a PKCβI-specific inhibitor, LY379196, suppressed the phorbol ester- and serum-supported growth of neuroblastoma cells. There was a marked enhancement by LY379196 of the growth-suppressive and/or cytotoxic effects of paclitaxel and vincristine. These results indicate that PKCβI has a positive effect on the growth and proliferation of neuroblastoma cells and demonstrate that inhibition of PKCβI may be used to enhance the effect of microtubule-interacting anticancer agents on neuroblastoma cells.

Introduction
PKCβ comprises a family of at least 10 related serine/threonine protein kinases. Based on structural properties and cofactor requirements, the family is subgrouped into classical (PKCα, PKCβI, PKCβII, and PKCγ), novel (PKCδ, PKCe, PKCη, and PKCθ), and atypical (PKCd and PKCε) PKCs (1–5). The enzymes of the PKC family have been implicated in the regulation of growth and differentiation in a number of cell types, and several studies suggest that different isoforms may have unique or even opposite effects on cell growth. For instance, in NIH3T3 cells, overexpression of PKCe leads to an increased growth rate, whereas PKCδ has the opposite effects (4), and in R6 embryo fibroblasts, PKCβI overexpression has a positive effect on cell growth, whereas PKCα has a negative effect on cell growth (5). In vascular smooth muscle cells, the two closely related isoforms PKCβII and PKCβI have opposite effects, with PKCβII leading to a decreased doubling time and PKCβI leading to an increased doubling time (6).

In SH-SY5Y neuroblastoma cells, treatment in the presence of serum with a phorbol ester concentration that does not down-regulate PKC leads to neuronal differentiation (7, 8). We have recently shown that PKCe seems to be critical for the induction of neurites that accompanies neuronal differentiation (9). On the other hand, for phorbol ester-mediated increased expression of differentiation marker genes such as neuropeptide Y, a classical PKC isoform is likely to be of importance (10). PKC also seems to be able to support the growth of neuroblastoma cells. The growth of several neuroblastoma cell lines can be supported by phorbol ester in the absence of serum, and inclusion of PKC inhibitors in serum-containing medium leads to a suppressed growth of these cells (10). The identity of the PKC isoform that mediates this effect is currently unknown.

One problem in determining which PKC isoform mediates a certain biological effect is the relative lack of isoform-specific inhibitors. However, in recent years, a picture has emerged suggesting that there may be isoform-specific docking proteins in the cell and that the interaction with these proteins may be essential for the activity of the isoform. RACKs constitute a group of proteins that bind activated PKC, and this interaction is mediated to a large extent by the C2 domain in the PKC molecule (11). In line with these results, expression of isolated C2 domains has been shown to block the translocation and/or activation of PKC isoforms in a specific manner (12–14).

The COOH-terminal V5 region, which comprises approximately 50 amino acids, has also been shown in several studies to mediate isoform-specific interactions or functions. This region is the only difference between the PKCβI and green fluorescent protein; RACK, receptor for activated C-kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; BrdUrd, bromodeoxyuridine; CHO, Chinese hamster ovary; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TRITC, tetramethylrhodamine isothiocyanate.
PKC\(\beta\)II isoforms and is thus likely to determine their unique functions. The V5 region from PKC\(\beta\)II was demonstrated to be the site for the specific interaction of this isoform with F-actin (15) and also to determine the specific function of PKC\(\beta\)II in erythroleukemia K256 cells (16). The corresponding region was shown to be involved in the isoform-specific interaction of PKC\(\alpha\) with the PDZ domain of PICK1 (17) and in the interaction of PKC with a PDZ domain of INAD (18). The latter findings are particularly interesting because PDZ domain-containing proteins may act as scaffolds assembling components of a transduction cascade within one complex (19). There are also results indicating a role for the V5 region in the binding of PKC\(\beta\)II to its RACK (20).

The aim of this study was to elucidate which PKC isoform is important for neuroblastoma cell proliferation. This was done primarily by using an approach in which the above-mentioned domains of different PKC isoforms were introduced into the cells to inhibit endogenous PKC isoforms. The effects of this expression on proliferation markers were then analyzed.

Results

The C2 domain has been suggested to act as a PKC isoform-specific inhibitor when introduced into cells (11). cDNAs coding for C2 domains from the PKC isoforms that are consistently expressed in neuroblastoma cells (PKC\(\alpha\), PKC\(\beta\), PKC\(\delta\), and PKC\(\epsilon\)) were amplified with PCR and introduced into the pEYFP-N1 expression vector, which generated a PKC C2-EGFP fusion protein when transfected into mammalian cells. PKC\(\alpha\), PKC\(\delta\), and PKC\(\epsilon\) C2-EGFP fusions were readily expressed when introduced into SK-N-BE(2) cells (Fig. 1), whereas the corresponding fusion construct for PKC\(\beta\) generated essentially no fusion protein (data not shown).

The effect of expression of the PKC\(\alpha\), PKC\(\delta\), and PKC\(\epsilon\) C2 constructs was evaluated for a possible influence on neuroblastoma cell proliferation using SK-N-BE(2) cells. Because the transfection efficiency of these cells is generally between 2% and 5% (9), the expression of EGFP was used to identify transfected cells. After transfection, the cells were incubated with medium containing 10% FCS for 40 h, and then the percentage of transfected cells staining positive for two proliferation markers, incorporation of BrdUrd and cyclin A expression, was assessed (Fig. 2A). This experiment revealed that the C2 domain from PKC\(\alpha\) suppressed the number of SK-N-BE(2) cells positive for the two proliferation parameters by 35% by expression of the PKC\(\alpha\) C2 domain. These effects were statistically significant. The effects of C2 domains from PKC\(\delta\) and PKC\(\epsilon\) were minor (8–15% inhibition) and not significant, indicating that the inhibition is specific for the PKC\(\alpha\) C2 domain. The fact that the results were similar for both BrdUrd incorporation and cyclin A staining indicates that either marker can be used to analyze proliferation of neuroblastoma cells.

We have previously found that TPA can support the growth of neuroblastoma cells (10). To investigate whether the PKC C2 domains have the same effects on TPA-treated neuroblastoma cells as they have on serum-supported cells, the incorporation of BrdUrd was analyzed after incubation with 16 nM TPA in SHTE medium (B). BrdUrd incorporation in A and B and detection of cyclin A with immunofluorescence in A were used to estimate proliferating activity of transfected cells. Data are expressed as the percentage of transfected cells positive for either BrdUrd or cyclin A and are the mean ± SE of four (A) or three (B) separate experiments. Controls (-) are cells expressing EGFP only. Approximately 200 transfected cells identified by the presence of EGFP (A) or an average of around 100 transfected cells (B) were scored on each coverslip. Levels of significance: \(P < 0.05\) (+) and \(P < 0.01\) (++) compared with control cells (Student’s t test).
only minor nonsignificant effects of the PKCδ (16% inhibition) and PKCe (6% inhibition) C2 domains.

The selective suppression of neuroblastoma cell proliferation markers by the C2 domain from PKCα suggests that this isoform may be of importance in regulating the proliferation of these cells. However, C2 domains of classical isoforms have a large degree of homology, and this, together with the fact that it was not possible to test the C2 domain of PKCβ, precludes the drawing of definite conclusions.

We have previously demonstrated that mRNA for classical PKC isoforms PKCα, PKCβII, and PKCδII but not PKCγ is present in SK-N-BE(2) neuroblastoma cells (10). We also demonstrated the presence of PKCα and PKCβII protein, but due to antibody cross-reactivity, we could not determine whether PKCβII protein is present in neuroblastoma cells. To clarify this issue, several antibodies were examined for cross-reactivity with PKCα or PKCβII and also showed that PKCβII protein is expressed in SK-N-BE(2) cells (Fig. 3A and B). Hence, of the classical isoforms, these cells express PKCα, PKCβII, and PKCδII, and any of these may have been influenced by the PKCα C2 domain.

The COOH-terminal V5 region of the PKC molecule is highly variable between the isoforms and is the only difference between PKCβII and PKCδII. This region is therefore a putative site for isoform-specific protein interactions, and expression of an isolated V5 region may perhaps block the corresponding endogenous isoform. Expression vectors coding for the V5 domain of the classical isoforms expressed in neuroblastoma cells (PKCα, PKCβII and PKCδII) fused to the COOH terminus of EGFP were constructed, and the generation of proteins of anticipated size was shown with Western blot after transfecting the constructs into SK-N-BE(2) cells (Fig. 1). The PKC5V-EGFP fusion proteins migrated as doublets, perhaps reflecting different states of phosphorylation of the two characterized phosphorylation sites in this region (21). SK-N-BE(2) cells were transfected with the vectors, and the number of transfected cells expressing cyclin A was analyzed (Fig. 3C). The V5 region from PKCβII significantly suppressed the number of cyclin A-positive cells with 47%, whereas the V5 regions from PKCα and PKCδII had only minor effects on this parameter. This result highlights PKCβII as the PKC isoform of importance for neuroblastoma cell proliferation.

If the V5 domains act as inhibitors of PKC activity, it is likely that they localize to the same intracellular sites as the endogenous PKC isoform that they inhibit. The EGFP-PKC5V fusion proteins all displayed a similar intracellular distribution (Fig. 4, A–C). There was a prominent localization to the nucleus of all fusion proteins, but in most cells, a presence in the cytosol could also be seen. Analysis of the intracellular distribution of the corresponding endogenous PKC isoforms (Fig. 4, D–F) revealed that PKCα and PKCβII immunoreactivity was essentially localized to the cytosol, with no staining in the nucleus. In contrast, PKCδ was found primarily in the nucleus. To investigate whether the most effective V5 fragment, PKCβII V5, influences the nuclear localization of PKCβII, cells were transfected with expression vector coding for the EGFP-PKCβII V5 fusion protein. Endogenous PKCβI was visualized with an antibody that reacts with the PKCβ regulatory domain and thus detects both PKCβ isoforms (Fig. 4, G–I). The staining with this antibody was generally weaker than that with the other PKCβ antibodies, revealing PKCβI immunoreactivity only in the nucleus and no reactivity corresponding to the PKCβII localization to the cytosol. In many cells overexpressing EGFP-PKCβII V5, there was a marked reduction in nuclear staining with the PKCβ antibody. Because PKCβI is the isoform found in the nucleus, it suggests that the PKCβII V5 fragment indeed displaces PKCβI, implying that this isoform is inhibited by the PKCβII V5 fragment.

The fact that expression of PKCβII V5 suppresses neuroblastoma cell proliferation raises the possibility that overexpression of PKCβII may augment proliferation of these cells. Expression vectors with cDNA for EGFP under the control of a SV40 promoter and PKCβII or PKCβIII under the control of a cytomegalovirus promoter were transfected into SK-N-BE(2) cells. With these vectors, EGFP and PKC will be expressed as separate proteins, and the fluorescence of EGFP can be used to identify transfected cells. The generation of PKCβII and PKCδII from the respective plasmid was confirmed by Western blot analysis of CHO cells transfected with the vectors (Fig. 3A). The expression vectors coding for PKCβII and PKCδII were transfected into SK-N-BE(2) cells, and 40 h later, the incorporation of BrdUrd in transfected cells was assessed (Fig. 5). Overexpression of neither PKCβ isoform had any marked effect on the percentage of cells...
staining positive for BrdUrd, which may indicate that the amount of PKC\(\beta\) molecules is not rate-limiting for neuroblastoma cell proliferation.

Recently, inhibitors that are specific for the PKC\(\beta\) isoforms have been described (22). To further establish that the PKC\(\beta\) isoforms are of importance for proliferation of neuroblastoma cells, the effect of such an inhibitor, LY379196, on the proliferation and growth was investigated. To quantify the proliferation under these circumstances, it was not necessary to analyze cell division activity at the single cell level, as it was with the transfection experiments. Therefore, incorporation of \(^{3}H\)thymidine could be used as a measure of proliferation.

SK-N-BE(2) cells that had been cultured in serum-free medium or medium supplemented with 16 \(\mu\)M TPA or 10% FCS were exposed to \(^{3}H\)thymidine, and after a 3-h incubation period, the incorporation was analyzed (Fig. 6A). The incorporation of \(^{3}H\)thymidine by cells that had been grown in serum-free medium or in medium supplemented with 16 \(\mu\)M TPA was suppressed by LY379196 in a concentration-dependent manner. Culture in the presence of 100 \(\mu\)M LY379196 led to a 28% suppression for cells grown in serum-free medium. There was a tendency toward a similar effect on TPA- and serum-supported cells, but the effect was not as pronounced. The concentration of LY379196 needed for this effect is in accordance with an effect on PKC\(\beta\) isoforms because the IC\(_{50}\) of this compound is 50 \(\mu\)M for PKC\(\beta\)I and 30 \(\mu\)M for PKC\(\beta\)II.4

To investigate whether the suppressed proliferation rate by LY379196 results in a suppression of the growth of neuroblastoma cells, a MTT assay was used to estimate the amount of viable SK-N-BE(2) cells. This was measured after 3 days of culture in the presence of increasing concentrations of LY379196 (Fig. 6B). SK-N-BE(2) neuroblastoma cells were cultured in either regular growth medium (i.e., in the presence of 10% serum) or SHTE medium containing 16 \(\mu\)M TPA. For this parameter, there was a concentration-dependent effect of LY379196 for both culture conditions. Inclusion of 100 \(\mu\)M LY379196 in the medium caused a decrease in the amount of viable cells of 16% for cells cultured with serum and 30% for cells cultured with TPA.

By itself, the PKC\(\beta\) inhibitor did not completely block proliferation, and there was still a significant growth of neuro-

Fig. 4. Intracellular distribution of overexpressed PKC\(\alpha\), PKC\(\beta\), and PKC\(\beta\)II V5 domains and corresponding endogenous PKC isoforms. A–C, SK-N-BE(2) cells were transfected with vectors coding for fusion proteins between EGFP and V5 domains from PKC\(\alpha\) (A), PKC\(\beta\)I (B), and PKC\(\beta\)II (C). Images demonstrate the localization of the V5-EGFP proteins visualized by the fluorescence of EGFP. D–F, immunofluorescence analysis of endogenous PKC\(\alpha\) (D), PKC\(\beta\)I (E), and PKC\(\beta\)II (F) in SK-N-BE(2) cells. The immunoreactivity was visualized with TRITC-conjugated secondary antibodies. G–I, SK-N-BE(2) cells were transfected with vector coding for the EGFP-PKC\(\beta\)I V5 fusion protein, and 16 h after transfection, the cells were analyzed with immunofluorescence using an anti-PKC\(\beta\) antibody, which is not directed against the V5 region, as primary antibody. The same microscopic field depicts (G) the EGFP-PKC\(\beta\)I V5 protein visualized by the fluorescence of EGFP, (H) immunoreactivity of the anti-PKC\(\beta\) antibody visualized by a secondary TRITC-conjugated antibody, and (I) a phase-contrast image.
blastoma cells in the presence of LY379196. This raises the possibility that it is necessary to influence other mechanisms of importance for growth to detect a substantial effect of the PKCβ inhibitor. LY379196 was therefore combined with increasing concentrations of the anticancer agents etoposide, paclitaxel, or vincristine. The effect of the drug combinations on the number of viable SK-N-BE(2) cells after culture for 3 days in regular growth medium was analyzed with a MTT assay. For etoposide, no substantial enhancement of the cytotoxic/growth-suppressive effect was observed when 100 nM LY379196 was included in the medium (data not shown). On the other hand, when paclitaxel or vincristine was combined with 100 nM LY379196, the potency of these drugs for reduction of viable cell number was enhanced (Fig. 7). For paclitaxel, the concentration that induced half maximal effect was reduced from 540 nM (range, 500–580 nM, two experiments) to 130 nM (range, 110–140 nM) in the presence of LY379196. The corresponding effect on vincristine was a reduction from 18 nM (range, 18–19 nM, two experiments) to 8 nM (range, 5–13 nM).

Discussion
The aim of this study was to elucidate which PKC isoform is involved in regulating the proliferation of neuroblastoma cells and to investigate whether inhibition of this isoform could be used to attenuate the growth of these cells. To selectively block individual PKC isoforms, we used the fact that the introduction of RACK-interacting C2 domains has been shown to block activation and translocation of specific PKC isoforms (12–14). C2 domains from different PKC isoforms were expressed in neuroblastoma cells, and the number of transfected cells positive for proliferation markers was estimated. This demonstrated that only a C2 domain from a classical isoform, PKCα, caused a substantial decrease in the amount of cells displaying markers representing proliferation activity. Significantly, the same pattern was observed for both BrdUrd incorporation and expression of cyclin A, clearly indicating that it is an effect on the amount of cells going through the cell cycle. It also demonstrates that either of these markers may be used to estimate the proliferation activity in these cells.

These results could suggest that PKCα has a positive effect on the proliferation of neuroblastoma cells. However, the C2 domains of PKCα and PKCβ are highly homologous, and because the PKCβ C2 construct could not be expressed, the effect of the PKCα C2 domain is not sufficient to conclude that PKCα is the isoform involved in proliferation. Neuroblastoma cells express PKCα and PKCβII (10) and
PKCβ (Fig. 3B), and it is possible that any of these is the target for inhibition by the PKCa C2 domain.

In an attempt to distinguish the activities of these isoforms, we used the COOH-terminal V5 region, which comprises approximately 50 amino acids, and hypothesized that this fragment would function as an inhibitor of the respective isoform. The basis for the hypothesis is the fact that the V5 region is the only difference between the two PKCβ isoforms and is thus likely to be involved in the specific effects of these isoforms. The V5 region has also been demonstrated to be involved in protein-protein interactions and PKC functions (15, 17, 18). When the effects of the expression of V5 domains were analyzed in this study, it was evident that the V5 region from PKCβI, as opposed to V5 fragments from PKCa or PKCβII, caused a substantial suppression of the number of cyclin A-positive cells. This highlights PKCβ as a PKC isoform of relevance for neuroblastoma cell proliferation and adds to those studies that have suggested that introduction of the V5 fragment may be a fruitful approach for intervening with the function of certain isoforms.

However, it is not clear whether the V5 regions will function as isoform-specific inhibitors in all cellular settings. The isoform-selective effects of the C2 domains have been demonstrated by their capacity to specifically block the translocation of the corresponding isoform to membranes or other intracellular sites. The limited transfection efficiency of neuroblastoma cells only allows analysis of subcellular localization at the single cell level. The localization pattern of PKCa and PKCβII, which was rather diffuse in the cytosol, has not allowed us to determine whether the V5-EGFP fusion protein influences the localization or the activity of these isoforms. Despite the lack of effects of the PKCa and PKCβII V5 fragments on the proliferation parameters, the possibility that the activities of these isoforms are also of importance for neuroblastoma cell proliferation cannot be excluded. All PKCV5-EGFP fusion proteins displayed a high density in the nucleus, but a substantial amount of fluorescence could also be noted in the cytosol. Endogenous PKCβII was also shown to be present primarily in the nucleus, in contrast to PKCa and PKCβII, which were expressed in the cytosol. The PKCβ immunoreactivity in the nucleus, most likely representing PKCβI, was frequently displaced in cells expressing PKCβI V5, which indicates that the effect of PKCβI V5 on proliferation is indeed due to interference with PKCβI function.

The hypothesis that a PKCβ isoform is of importance for optimal proliferation activity of neuroblastoma cells is further supported by the effects of the PKCβ inhibitor LY379196. Although the suppression of proliferation and growth by this compound was not as great as the effects of the PKCβI V5 region, the effect of LY379196 was obtained at concentrations that inhibit only the PKCa isoforms. The IC_{50} of LY379196 for PKCβI has been shown to be 50 nM, and the corresponding values for PKCa, PKCaI, and PKCa are 600 nM, 700 nM, and 5 μM. Because concentrations higher than 100 nM may partially inhibit other PKC isoforms, higher concentrations were not used to exclude partial inhibition of other PKC isoforms. The maximal concentration used in our experiments (100 nM) was only two times the IC_{50} for PKCβI (50 nM) that would lead to a 60–70% inhibition of PKCβI, assuming Michaelis-Menten kinetics. The potentially remaining 30–40% PKCβI activity may explain the relatively smaller effect of LY379196 compared with the PKCβI V5 fragment.

The discrepancy between the two methods of inhibition could also be due to the fact that LY379196 will inhibit both PKCβ isoforms, whereas the PKCβI V5 fragment may block only PKCβI. There are indeed reports indicating that PKCβI will augment and PKCβII will suppress proliferation in the same cell (6). It is also possible that the BrdUrd assay with 30 min of labeling is more sensitive than the thymidine incorporation assay, in which there is a 3-h labeling period.

PKC has been shown to be involved in the regulation of proliferation in several cell systems, but the isoform of importance may vary depending on cell type. Overexpression of PKCa was shown to enhance smooth muscle cell proliferation (23), whereas decreased PKCa levels led to increased proliferation of CaCo-2 cells (24). Furthermore, overexpression of PKCa in 3Y1 cells was shown to suppress the activity of the transcription factor E2F that is positively linked to cell cycle progression (25). A specific effect of PKCβI on proliferation and growth has been shown for rat fibroblasts (5) and vascular smooth muscle cells (6). For both these cell types, overexpression of other classical isoforms, PKCa in fibroblasts and PKCβI in smooth muscle cells, resulted in a suppression of growth and proliferation. A PKCβI isoform was shown to be positively involved in endothelial cell growth (26), and PKCβII has been demonstrated to enhance the proliferation of colonic epithelium (27). In fibroblasts, PKCa was shown to enhance the growth rate, whereas PKCaI suppressed the growth rate (4). Taken together, these data demonstrate that there are cell type-specific effects of different PKC isoforms on proliferation. This may reflect that PKC has no direct general effect on the cell division machinery but rather influences proliferation by modifying cell-specific pathways controlling the cell division. The present study highlights PKCβI as a PKC isoform with positive effects on neuroblastoma cell proliferation and growth.

When the PKCβ inhibitor LY379196 was combined with paclitaxel or vincristine, a substantial enhancement of their effect was observed. This effect was obtained using 100 nM LY379196, a concentration specific for PKCβ but not sufficient to obtain maximal inhibition of PKCβI. These data are in line with the finding that PKCβ inhibition sensitized Lewis lung carcinoma cells to paclitaxel (28) and suggest that PKCβ inhibition could also be used to increase the sensitivity of neuroblastoma cells to microtubule-interacting anticancer drugs.

In conclusion, this study provides indications that PKCβI is of importance for optimal neuroblastoma cell proliferation and growth. It suggests that introduction of a PKCβI V5 fragment into the cells is one way to intervene with this effect. It also shows that inhibition of a PKCβ isoform, together with treatment with microtubule-interacting anticancer agents, may be a fruitful approach to attenuate the growth of neuroblastoma cells.
Table 1  PKC C2 and V5 constructs used in this study

<table>
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<tr>
<th>Construct</th>
<th>PKC isoform, amino acids</th>
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| αC2E      | PKCα, 155–312            | F: CGCAGATCTGAGACAGCATGAGGGAGGACGATTT  
|           |                          | R: AACGTCGACCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTA |
mouse IgG (Jackson Laboratories) for 60 min. The coverslips were mounted, and for cyclin A experiments, transfected cells were scored for cyclin A positivity.

\[ [\text{H}]\text{Thymidine Incorporation.} \] SK-N-BE(2) cells were seeded at a density of 8000 cells/well in 96-well culture plates. The indicated compounds, TPA (Sigma) and LY379196 (kindly provided by Dr. D. K. Ways, Eli Lilly, Indianapolis, IN), had been added in 50 μl of medium before the addition of cells in the same volume. Two days after seeding, 20 μCi of \text{[H]}thymidine were added to each well, and after incubation for 3 h, incorporated \text{[H]}thymidine was recovered with a cell harvester (Skatron). The radioactivity was measured with scintillation counting.

**Analysis of Cell Growth/Viability.** SK-N-BE(2) neuroblastoma cells were seeded at a density of 3000 cells/well in 96-well culture plates. The indicated compounds had been added in 50 μl of medium before the addition of cells in the same volume. After 3 days, the amount of viable cells was analyzed measuring the conversion of the tetrazolium salt MTT to formazan according to the supplier’s protocol (Promega). Paclitaxel (Sigma) was solubilized in DMSO, and vincristine sulfate (Sigma) was solubilized in water. For paclitaxel, control cells were incubated with 0.4% DMSO. For calculations of the concentration that elicited a half-maximal effect, a nonlinear curve fit was performed with the equation \( y = A_0 + (A_1 - A_0)/(1 + x/x_0) \) where \( x_0 \) corresponds to the half-maximal concentration.

**Acknowledgments**

Dr. D. K. Ways is gratefully acknowledged for providing LY379196 and for fruitful suggestions and comments.

**References**


