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Celecoxib-Induced Growth Inhibition in SW480 Colon Cancer Cells Is Associated with Activation of Protein Kinase G

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Abbreviations

NSAIDS, non-steroidal anti-inflammatory drugs; cGMP, guanosine 3',5' monophosphate; PDE, phosphodiesterase; PKG, protein kinase G; JNK1, c-Jun NH2-terminal kinase 1.

Abbreviated title

Celecoxib and Protein Kinase G

Keywords

PKG, NSAID, COX-2, PDE, cGMP.

Abstract

Although it is often assumed that the antitumor effects of non-steroidal anti-inflammatory drugs (NSAIDS) are due to inhibition of cyclooxgenase (COX) activity, specifically COX-2, there is accumulating evidence that COX-2 independent mechanisms can also play an important role. Studies with sulindac sulfone (Aptosyn) and related derivatives have revealed a novel pathway of tumor growth inhibition and apoptosis mediated by activation of the guanosine 3',5' monophosphate (cGMP)-dependent enzyme protein kinase G (PKG). The present study indicates that concentrations of the NSAIDS celecoxib, indomethacin and meclofenamic acid that inhibit growth of SW480 human colon cancer cells inhibit subcellular cGMP-phosphodiesterase (PDE) enzymatic activity and in intact cells induce a 2-3 fold increase in intracellular levels of cGMP. This is associated with phosphorylation of the protein VASP, a marker of PKG activation, activation of JNK1 and a decrease in cellular levels of cyclin D1; effects seen with other agents that cause activation of PKG in these cells. On the other hand even a high concentration of the COX-2 specific inhibitor rofecoxib (500 μM) did not inhibit growth of SW480 cells. Nor did rofecoxib inhibit cGMP-PDE activity or cause other changes related to PKG activation in these cells. Since activation of the PKG pathways by celecoxib, indomethacin and meclofenamic acid in this cell culture system required high concentrations of these compounds, it remains to be determined whether activation of this pathway contributes to the *in vivo* antitumor effects of specific NSAIDS.

Introduction

There is currently considerable interest in the antitumor effects of various non-steroidal anti-inflammatory drugs (NSAIDS). This interest is based on epidemiologic studies and clinical trials. In addition, extensive experimental studies in rodents indicate that specific NSAIDS can inhibit the process of carcinogenesis and/or inhibit the growth of established tumors. In addition, some of these compounds inhibit growth and/or induce apoptosis in cell cultures of various types of human cancer cells (for review see [1-3]). A current paradigm is that these anticancer effects are exerted via inhibition of cyclooxygenase (COX) activity, especially COX-2. However, there is increasing evidence that specific NSAIDS and NSAID-related compounds may exert their antitumor effects, at least in part, through COX-independent mechanisms [3]. Studies with sulindac sulfone (Aptosyn) a metabolite of the NSAID sulindac have revealed a novel mechanism of antitumor activity. This compound does not inhibit COX-1 or -2 activity but, instead, inhibits specific guanosine 3',5' monophosphate (cGMP) phosphodiesterases (PDEs). This causes an increase in intracellular levels of cGMP thus leading to activation of the cGMP-dependent enzyme protein kinase G (PKG). Activation of PKG then leads to a cascade of events that result in growth inhibition and apoptosis [4]. The precise mechanisms responsible for the latter effects are not known with certainty but there is evidence that they include activation of JNK1, induction of $p21^{Cip1}$, a decrease in cellular levels of β-catenin and cyclin D1, and activation of caspase activities [3-8]. Similar molecular effects have been seen with Aptosyn-related derivatives or other compounds that lead to increased cellular levels of cGMP and activation of PKG [3-8]. Therefore, in the present study we examined the possibility that, in addition to its ability to selectively inhibit the activity of COX-2, celecoxib might also inhibit

cGMP-PDE activity and thereby activate the above-described PKG-mediated pathway in SW480 human colon cancer cells. We chose this cell line because it has been used extensively in previous studies on various NSAIDS and on studies with Aptosyn and Aptosyn-related compounds [3-8]. For comparison we included in these studies rofecoxib, another COX-2 specific inhibitor that differs in its structure from celecoxib [9]; meclofenamic acid that selectively inhibits COX-2, and also inhibits lipoxygenase activity [10]; and indomethacin that inhibits both COX-1 and COX-2 [11]. Previous studies have shown that relatively high concentrations of some of these compounds, can inhibit the growth of human cancer cells in cell culture but the precise mechanism was not revealed [3].

Materials and Methods

Cell cultures and cell proliferation assays

SW480 human colon cancer cells were maintained in DMEM medium with 10% fetal bovine serum. For cell proliferation assays, the cells were plated in triplicate at a density of $2x10^4$ cells per well in 6-well (35mm-diameter) plates with 2 ml of DMEM medium containing 10% FBS. One day later, the cells were refed with fresh medium containing either celecoxib (0, 1, 5, 10, 25, 50, 75, 100 or 200 μM), rofecoxib (0, 1, 10, 25, 50, 100, 250 or 500 μM), indomethacin (0, 10, 50, 100, 250, 500, 750 or 1000 μM), or meclofenamic acid (0, 1, 10, 25, 50, 100, 250 or 500 μM). Two days later the number of attached cells per well was counted using a Coulter counter and the extent of inhibition of cell proliferation was determined. IC_{50} values (concentration that caused 50% inhibition of growth) were calculated from a graph using SigmaPlot software.

cGMP-PDE assays

SW480 cells were grown to confluence in DMEM medium containing 10% FBS. The cells were harvested using Pancreatin, homogenized in 8 mM Tris-Ac (pH 7.4) containing 5 mM MgAc, 0.1 mM EDTA, 0.8% Triton-100 and protease inhibitors, and the extracts were centrifuged at 100,000 x g. The supernatant fraction was then used to determine cyclic GMP-PDE activity with 0.25 μM cGMP as the substrate, as described previously [4,12]. Increasing concentrations of celecoxib (0.01 to 1000 μM), rofecoxib (0.01 to 200 μM), indomethacin (0.1 to 1000 μM), and meclofenamic acid 0.01 to 1000 μM) were added to the assay system and their EC_{50} values (concentration that caused 50% inhibition of enzyme activity) were calculated from a graph using Sigma Plot software [4].

cGMP assays

SW480 cells were plated in triplicate at a density of $5x10^3$ cells per well in 96-well plates with 100 μl of DMEM medium containing 10% FBS. One day later, the cells were refed with fresh medium containing either DMSO (0.1%), celecoxib (50 μM), rofecoxib (100 μM), indomethacin (500 μM), or meclofenamic acid (250 μM). After exposure of the cells to these compounds for either one or 24 hours extracts were prepared and the intracellular levels of cGMP were determined using a cGMP colorimetric competitive ELISA kit (R&D Systems), as previously described [7].

JNK1 assays

SW480 cells were treated with DMSO (0.1%), celecoxib (50 μM), rofecoxib (100 μM), indomethacin (500 μM) or meclofenamic acid (250 μM) for one hour and lysed in a lysis buffer (20 mM Tris HCl (pH7.5), 0.5 % NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 20 mM β-glycerophosphate, 25 % glycerol). JNK1 was then immunoprecipitated with an anti-JNK1 antibody (Santa Cruz) for 2 hours, and assayed for *in vitro* kinase activity with GST-c-Jun(1-79) (New England Biolab) as the substrate, in a kinase reaction buffer containing 20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 20 μM ATP, 20 mM β-glycerophosphate, and 1 μCi [γ-^{32}P]ATP, with incubation at 37^o for 20 min. The reaction mixture was then subjected to SDS-PAGE, followed by autoradiography, as previously described [5,6].

Western blot analysis

Cellular proteins were extracted by cell lysis in RIPA buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 2 mM EDTA, 2 mM EGTA, 1 mM DTT) that contained protease inhibitors (20 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM PMSF) and phosphatase inhibitors (1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate). One hundred μg of protein in total cell extracts were subjected to SDS-PAGE. Proteins were then transferred to an Immobilon-P (Millipore) membrane at 60V for 3 hours at 4^{o}C. The membranes were subsequently blocked with 5% dry milk in TBS-T (20 mM Tris HCl, pH 7.6, 137 mM NaCl, 0.05% Tween-20), and then immunoblotted with the indicated antibody. The immunoblots were visualized with the Enhanced Chemiluminescence (ECL) Western Blotting System (Amersham).

The monoclonal antibody to VASP was purchased from Transduction Laboratories, the polyclonal antibody to cyclin D1 from UBI, and the monoclonal antibody to actin from Sigma.

Results

NSAIDS inhibit the proliferation of SW480 cells.

In our initial studies we examined the effects of celecoxib, rofecoxib, indomethacin and meclofenamic acid on the proliferation of SW480 human colon cancer cells. The cells were treated with increasing concentrations of celecoxib (0, 1, 5, 10, 25, 50, 75, 100 or 200 μM), rofecoxib (0, 1, 10, 25, 50, 100, 250, or 500 μM), indomethacin (0, 10, 50, 100, 250, 500, 750 or 1000 μM), or meclofenamic acid (0,1, 10, 25, 50, 100, 250 or 500 μM) for 2 days and the numbers of attached cells per well were counted. The IC_{50} values (i.e., concentration that produced 50% inhibition of growth) for celecoxib, indomethacin and meclofenamic acid were 46, 533 and 163 μM, respectively. Although it is a potent inhibitor of COX-2 [9], rofecoxib did not inhibit the growth of SW480 cells even when tested at a concentration as high as 500 μM (Table 1). Previous studies also found that celecoxib is much more active than rofecoxib in inhibiting the growth of cultures of human cancer cells [13] or transformed rodent cells [14], even though both compounds have similar potencies with respect to inhibiting COX-2 activity [9,13,14].

NSAIDs inhibit subcellular cGMP-PDE activity

PDEs catalyze the hydrolysis of cAMP or cGMP to the biologically less active 5'-nucleoside monophosphates [12,14]. The sulindac metabolite sulindac sulfone (Aptosyn) inhibits the cGMP specific PDEs 2 and 5, thus leading to sustained increases in cellular levels of

cGMP, and, thereby, activation of PKG [4]. Therefore we examined possible effects of the above NSAIDS on cGMP-PDE enzymatic activity of a supernatant fraction obtained from SW480 cells. Celecoxib and indomethacin inhibited the hydrolysis of cGMP with EC_{50} values of 67 and 697 μM, respectively (Table 1). These values are slightly higher than the IC_{50} values for growth inhibition obtained with these two compounds (Table 1), which suggest that celecoxib and indomethacin may have some targets other than PDE. On the other hand, meclofenamic acid had an EC_{50} value of 601 μM (Table 1) which is 3 times higher than its IC_{50} for growth inhibition (Table 1). This suggests that the growth inhibitory effect of this drug may be mediated by additional targets [10]. It is of interest that in similar cGMP PDE assays the EC_{50} value for the well studied cGMP-PDE inhibitor Aptosyn is 113 μM [4]. Thus, celecoxib is a more potent inhibitor of this enzyme activity than Aptosyn. At the same time, we should stress that celecoxib, indomethacin and meclofenamic acid inhibit the subcellular activity of COX-2 at about 1 μM [2,9,10,14,15]. Rofecoxib did not display an inhibitory effect on cGMP-PDE enzymatic activity even when tested at 1000 μM (Table 1). This lack of a cGMP-PDE inhibitory effect of rofecoxib correlates with its lack of a growth inhibitory effect on SW480 cells (Table 1).

NSAIDs cause an increase in cellular levels of cGMP.

To examine possible effects of these NSAIDS on cGMP-PDE activity in intact SW480 cells, we assayed intracellular levels of cGMP. SW480 cells were treated for one hour with either DMSO (0.1%), celecoxib (50 μM), rofecoxib (100 μM), indomethacin (500 μM), or meclofenamic acid (250 μM). Extracts were then prepared and intracellular levels of cGMP were determined by a colorimetric competitive ELISA. As shown in Fig. 1, at 1 hour celecoxib, indomethacin and meclofenamic acid caused about a two-fold increase in cellular levels of

cGMP. These results are similar to those obtained with other cGMP PDE inhibitors or guanylyl cyclase activators [4,7]. However, rofecoxib did not have a detectable effect on the cellular level of cGMP. Similar results were obtained when the cells were treated with these drugs for 24 hours (data now shown).

NSAIDs induce phosphorylation of VASP.

As described in the Introduction, an increase in intracellular levels of cGMP in SW480 cells causes activation of PKG [4,7]. Our laboratory discovered that in SW480 cells this leads to rapid phosphorylation of the PKG substrate vasodilator-stimulated phosphoprotein (VASP), and demonstrated that phosphorylated VASP (VASP-P) provides a convenient biomarker of PKG activation in these cells [7]. Therefore, we investigated the effects of the above four compounds on the phosphorylation status of VASP. SW480 cells were treated with either DMSO (0.1%), celecoxib (50 μM), rofecoxib (100 μM), indomethacin (500 μM), or meclofenamic acid (250 μM) for 0, 1 or 2 days and then protein extracts were collected and examined by Western blot assays using a VASP specific antibody. In these assays the unphosphorylated VASP protein can be detected as a 46kDa protein whereas VASP-P can be readily detected as a slower migrating 50kDa band [7]. We found that when the cells were treated with only the DMSO solvent VASP-P was not detected but when they were treated with celecoxib, indomethacin, or meclofenamic acid VASP-P was detected at 24 hours and was even more abundant at 48 hours. However, rofecoxib did not induce detectable phosphorylation of VASP (Fig. 2).

NSAIDs activate JNK1.

We previously reported that Aptosyn, and the Aptosyn related compounds OSI-248 and OSI-461 that are potent inhibitors of cGMP-PDEs, cause rapid activation of a novel PKG-

MEKK1-SEK1-JNK1 pathway in SW480 cells [5,6]. In addition, we found that a dominant-negative mutant of JNK1 inhibited the induction of apoptosis by CP248, thus providing evidence that activation of JNK1 plays a critical role in the growth inhibitory effects of this class of compounds [5,6]. Therefore, we examined whether celecoxib, rofecoxib, indomethacin or meclofenamic acid also caused activation of JNK1 kinase activity. SW480 cells were treated with either DMSO (0.1%), celecoxib (50 μM) rofecoxib (100 μM), indomethacin (500 μM), or meclofenamic acid (250 μM) for one hour and then protein extracts were collected for JNK1 assays (Fig. 3). We found that celecoxib, indomethacin and meclofenamic acid caused strong activation of JNK1. However, rofecoxib did not activate JNK1 when tested at either 100 μM (Fig. 3) or 200 μM (data not shown). Like Aptosyn, celecoxib and indomethacin also caused, within 6 hours, an increase in cellular levels of c-Jun (data not shown).

NSAIDS induce downregulation of cyclin D1.

Previous studies indicate that treatment of SW480 cells with Aptosyn or related compounds causes a decrease in cellular levels of cyclin D1, which is probably related to a decrease in cellular levels of β-catenin [4,5]. Therefore, we examined whether the four compounds cause downregulation of the expression of cyclin D1 in SW480 cells. The cells were treated with either DMSO (0.1%), celecoxib (50 μM), rofecoxib (100 μM), indomethacin (500 μM), or meclofenamic acid (250 μM), for 0, 1, 2 or 3 days and then protein extracts were collected and examined by Western blot assays using a cyclin D1 antibody (Fig. 4). Celecoxib, indomethacin and meclofenamic acid induced marked downregulation of cyclin D1 within one day. Rofecoxib did not induce downregulation of cyclin D1 even after treatment for 3 days (Fig. 4).

Discussion

The present studies indicate that when the NSAIDs celecoxib, indomethacin and meclofenamic acid were tested at concentrations in the range that inhibit the growth of SW480 human colon cancer cells they inhibited subcellular cGMP PDE enzymatic activity (Table 1). In intact SW480 cells similar concentrations of these three compounds induced a 2-3 fold increase in intracellular levels of cGMP (Fig. 1). This was associated with phosphorylation of the protein VASP (Fig. 2), presumably through activation of PKG. Treatment with these three compounds also caused activation of JNK1 and a decrease in cellular levels of cyclin D1. All of these effects have been previously described with various agents that cause activation of PKG in SW480 cells [4-7]. On the other hand even when tested at relatively high concentrations the COX-2 specific compound rofecoxib did not inhibit the growth of SW480 cells, nor did it cause any of the above-described changes related to inhibition of cGMP-PDE and activation of PKG (Tables 1 and Figs. 1-4). Taken together, these findings suggest that the growth inhibition induced by celecoxib, indomethacin and meclofenamic acid in SW480 cells seen in the present study is mediated, at least in part, via activation of PKG.

A hypothetical scheme based on the present results in shown in Fig. 5. NSAIDs such as celecoxib, indomethacin and meclofenamic acid inhibit cGMP-specific phosphodiesterase 2 and 5, thus causing increased cellular levels of cGMP. This leads to activation of PKG. Activation of PKG may causes several downstream effects such as activation of MEKK1-SEK1-JNK1 pathway, phosphorylation of VASP and inhibition of β-catenin pathway. Activation of JNK1 pathway and inhibition of β-catenin pathway were reported to be important in various apoptotic signaling pathways.

Our findings are consistent with previous cell culture studies with celecoxib indicating that growth inhibition or induction of apoptosis by celecoxib in various types of cancer cell lines appears to occur via a COX-2 independent mechanism, although the precise mechanism was not identified. Thus, inhibition of growth and/or induction of apoptosis in human colon cancer cell lines [15] or a rat cholangiocarcinoma cell line [16,17] required higher concentrations of celecoxib 20 – 50μM) than those required to inhibit the production of PGE_2, the product of COX-2. Furthermore, these concentrations of celecoxib inhibited the growth of HCT-15 colon cancer cells even though these cells do not express COX-1 or COX-2 [15]. Also, the SW480 cells used in this study do not express detectable amounts of Cox-2 (unpublished studies). In addition, Han et al. [17] found that inhibition of the growth of human cholangiocarcinoma cells by 50 μM celecoxib was not reversed by the COX-2 product PGE_2. Nor did antisense depletion of COX-2 in these cells mimic the effects of celecoxib.

A number of COX-2 independent targets have been implicated in the action of specific NSAIDS. These include cGMP PDEs, IKKβ (IκB kinase β), 3-phosphoinositide independent kinase 1 (PDK1), ribosomal S6 kinase 2 (RSK2), Ras, collagenase type XI, and PPAR-α, -γ and δ (for review see ref. 3). The ability of celecoxib to directly inhibit PDK1 in a subcellular assay is of interest since this occurs at a relatively low concentration (3.5 μM) of the drug and this effect would be expected to prevent activation of the survival factor Akt [18]. Indeed, suppression of Akt activation was seen in HT29 colon cancer cells treated with celecoxib, but this occurred at a concentration of 100 μM [18]. Therefore it is not known with certainty whether PDK1 is a direct *in vivo* target of celecoxib.

We should stress that the present studies are confined to cell cultures of a colon cancer cell line and the effects observed required high concentrations of celecoxib, indomethacin and

meclofenamic acid. Therefore, the *in vivo* significance of our findings remains to be determined. Although there is limited pharmacokinetic data on these compounds in humans, the concentrations we used probably exceed blood levels in humans. Thus the blood level for celecoxib in humans is about 2- 5 μM [15]. However, we treated the cells for only 1 to 3 days. It is possible that during chronic administration to patients these compounds may be concentrated in tumor tissue and also exert cumulative effects. There is evidence that colonic epithelial cells may be exposed to sulindac sulfide concentrations that are 20-fold higher than those in the serum [3]. Furthermore, it has been suggested that NSAIDs may be concentrated in the mildly acidic extracellular environment of tumor tissue and further concentrated within tumor cells [3]. Recent clinical studies indicate that celecoxib caused a reduction in polyp growth in patients with familial polyposis coli. However, this effect required a relatively high dose, i.e. 400 mg b.i.d., but was not significant at the recommended anti-inflammatory dose of 100 mg b.i.d. [19] These findings raise the possibility that the *in vivo* antiproliferative effects of celecoxib and other specific NSAIDS may involve, in addition to COX-2, targets with a lower affinity than COX-2, including cGMP PDEs. Therefore, it would be of interest to examine tumor tissue from experimental animals and humans treated with celecoxib or other NSAIDS for VASP phosphorylation and other biomarkers of PKG activation.

Because of previously published studies and the present data it is of interest to examine structure-function relationships amongst various NSAIDS and NSAID-related compounds with respect to their relative abilities to inhibit COX-1, COX-2 and cGMP PDE activity. Sulindac sulfide and indomethacin can inhibit both COX-1 and COX-2 [3] as well as cGMP PDEs ([3]and Table 1), whereas sulindac sulfone (Aptosyn) does not inhibit COX-1 or COX-2 [3] but does inhibit cGMP PDEs [3,4]. Celecoxib [3] and meclofenamic acid [10] specifically inhibit COX-2

and not COX-1, and at relatively high concentrations they both inhibit cGMP-PDE activity (Table 1). Rofecoxib is rather unique since, although like celecoxib it specifically inhibits COX-2 [9], even at relatively high concentrations it does not inhibit cGMP-PDE activity (Table 1). Presumably this reflects the unique structure of rofecoxib [9]. Molecular modeling studies [20] may rationalize these findings and also aid in the design of novel compounds that provide either a more specific or a multiprong attack on cancer cells.

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Figure legends

Fig. 1. Treatment of SW480 cells with specific NDAIDS increases cellular levels of cGMP

SW480 cells were plated in triplicate at a density of $5x10^3$ cells per well in 96-well plates with 100 μl of DMEM medium containing 10% FBS. One day later, the cells were refed with fresh medium containing either DMSO (0.1%), celecoxib (50 μM), rofecoxib (100 μM), indomethacin (500 μM), or meclofenamic acid (250 μM). One hour later intracellular levels of cGMP were measured using a cGMP colorimetric competitive ELISA kit (R&D Systems). This figure indicates mean values for triplicate assays and S.D's. Similar results were obtained in two additional studies.

Fig. 2. Induction of VASP phosphorylation by specific NSAIDS

SW480 cells were treated with either DMSO (0.1%), celecoxib (50 μM), rofecoxib (100 μM), indomethacin (500 μM), or meclofenamic acid (250 μM) for 0, 1 or 2 days. Protein extracts were then collected and Western blot assays were performed with an anti-VASP antibody. The slower migrating band represents the phosphorylated VASP protein (P-VASP). Similar results were obtained in a repeat experiment.

Fig. 3. Activation of JNK1 by specific NSAIDs

SW480 cells were treated with DMSO (0.1%), celecoxib (50 μM), rofecoxib (100 μM), indomethacin (500 μM) or meclofenamic acid (250 μM) for one hour. The cells were lysed and then JNK1 was immunoprecipitated with an anti-JNK1 antibody (Santa Cruz), and assayed for *in*

vitro kinase activity using GST-c-Jun(1-79) as the substrate, as previously described [5]. Similar results were obtained in two additional studies.

Fig. 4. Downregulation of cyclin D1 by specific NSAIDs

SW480 cells were treated with either DMSO (0.1%), celecoxib (50 μM), rofecoxib (100 μM), indomethacin (500 μM), or meclofenamic acid (250 μM) for 0, 1 or 2 days. Protein extracts were then collected and Western blot assays were performed with an anti-cyclin D1 antibody. Similar results were obtained in a repeat experiment.

Fig. 5. Hypothetical scheme of the apoptotic signal transduction pathway activated by NSAIDs

NSAIDs such as celecoxib, indomethacin and meclofenamic acid inhibit cGMP-specific phosphodiesterase 2 and 5, thus causing increased cellular levels of cGMP. This leads to activation of PKG. Activation of PKG may causes several downstream effects such as activation of MEKK1-SEK1-JNK1 pathway, phosphorylation of VASP and inhibition of β-catenin pathway.

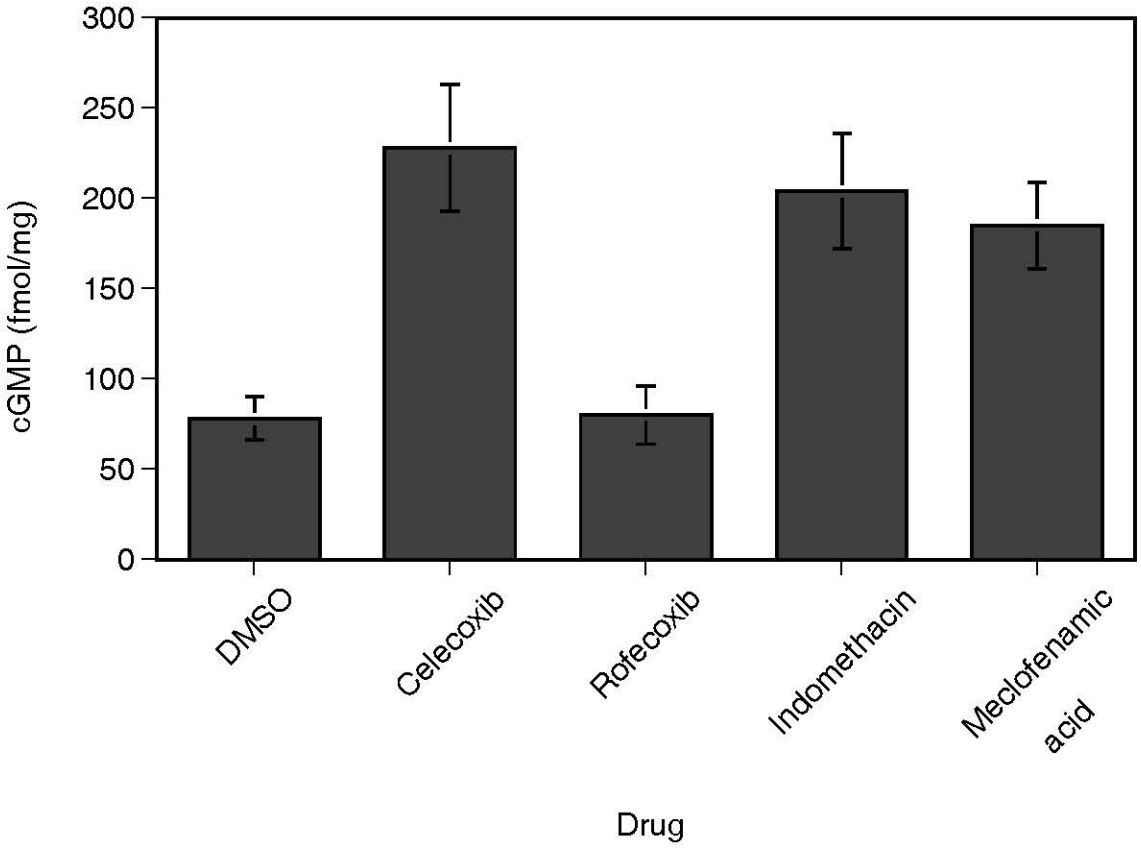
300
250
200
150
100
50
0
cGMP (fmol/mg)
DMSO
Celecoxib
Rofecoxib
Indomethacin
Meclofenamic acid
Drug

Figure 1

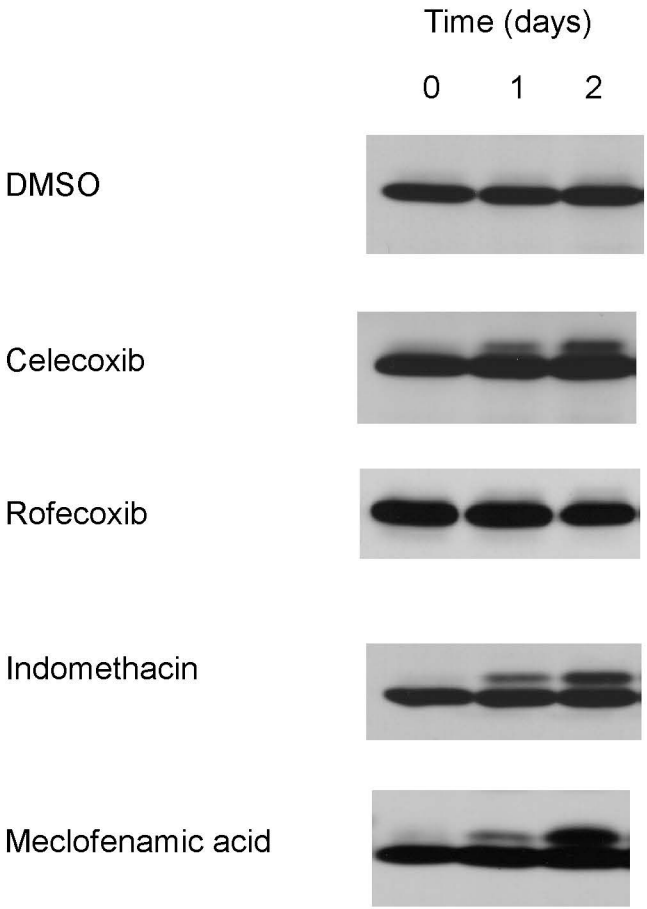
Time (days)
0
1
2
DMSO
Celecoxib
Rofecoxib
Indomethacin
Meclofenamic acid

Figure 2

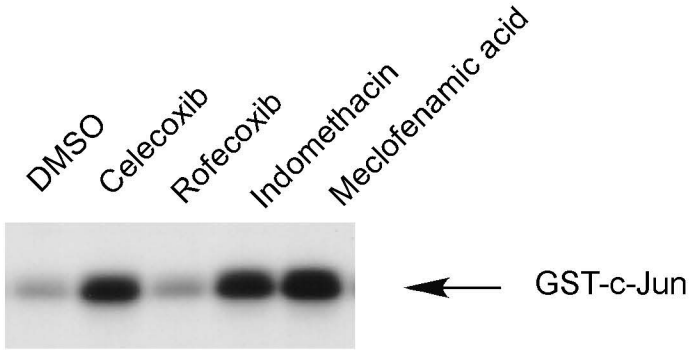
DMSO
Celecoxib
Rofecoxib
Indomethacin
Meclofenamic acid
GST-c-Jun

Figure 3

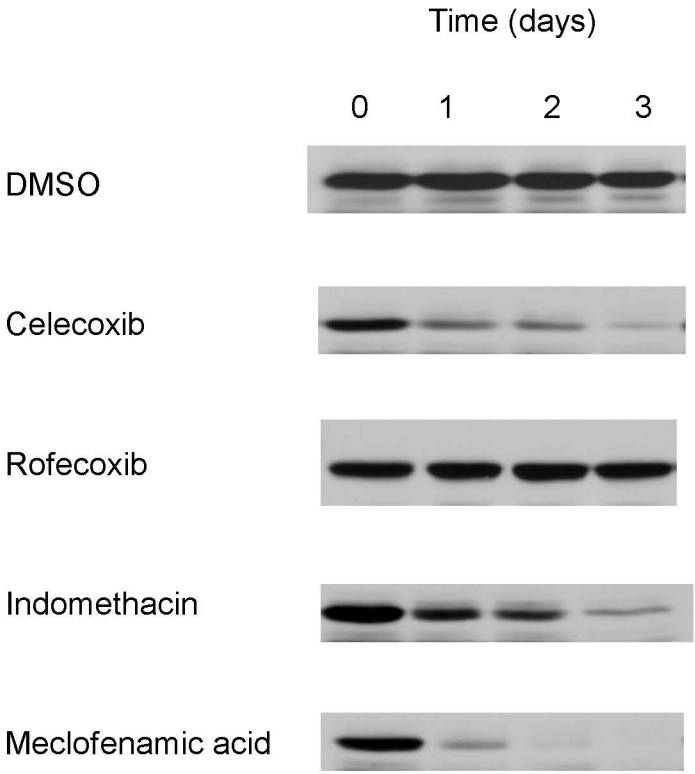
Time (days)
0
1
2
3
DMSO
Celecoxib
Rofecoxib
Indomethacin
Meclofenamic acid

Figure 4

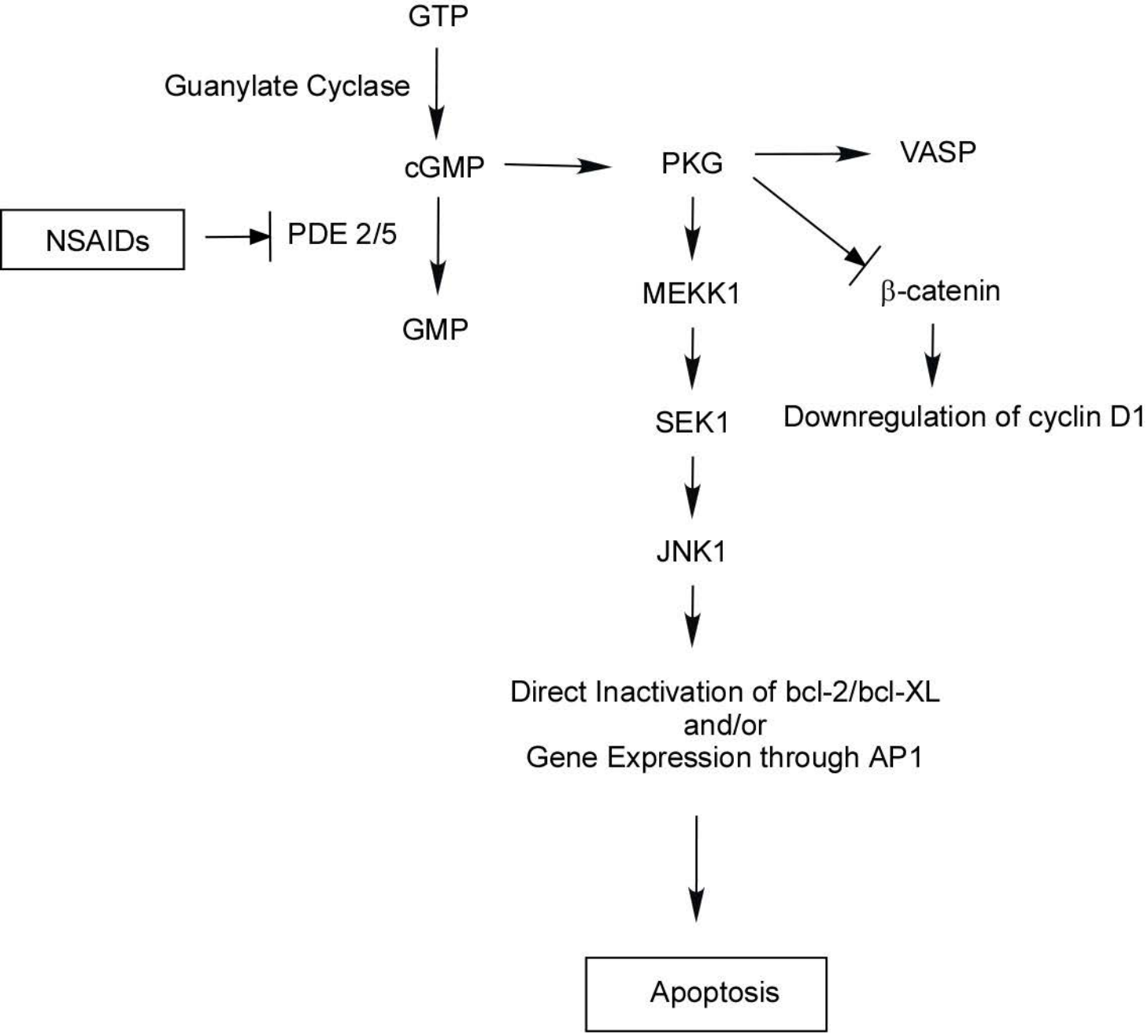
GTP
Guanylate Cyclase
cGMP
PKG
VASP
NSAIDs
PDE 2/5
GMP
MEKK1
β-catenin
SEK1
Downregulation of cyclin D1
JNK1
Direct Inactivation of bcl-2/bcl-XL
and/or
Gene Expression through AP1
Apoptosis

Figure 5