SMAF-1 Inhibits the APC/β-Catenin Pathway and Shows Properties Similar to Those of the Tumor Suppressor Protein APC

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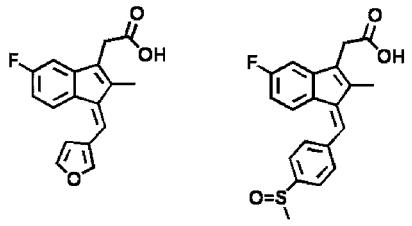
The identification of synthetic molecules with similar or identical functions to those of important proteins is one of the most challenging tasks of chemical biology. Whereas there are many examples for the successful simulation of protein functions by synthetic peptides or by peptidomimetics, only a very few small compounds are known that harbor protein function but are not related to peptides.[1–3] We call these molecules small molecule mimetics of protein function (SMPFs). One possibility to identify a SMPF would be to employ a cell-based screen with the protein in its native environment followed by detailed biochemical characterization.

The Adenomatous Polyposis coli (APC) tumor-suppressor protein plays a central role in the regulation of cell proliferation. The induced APC gene expression leads to apoptosis.[4] One of the intracellular functions of the APC protein is the regulation of the cytoplasmic and the nuclear level of the proto-oncoprotein β-catenin.[5] Thereby APC inhibits β-catenin/TCF-4-induced gene expression. APC, β-catenin, and TCF-4 are key proteins of the Wnt/APC/β-catenin signaling pathway, which runs from the transmembrane Wnt receptor to the TCF-4-activated gene transcription in the nucleus. APC mutations, which are found in the majority of sporadic and also in inherited colorectal tumors, lead to a truncated protein with reduced or no activity. The inactivation of the APC protein leads to a high level of β-catenin and thereby to the induction of target genes with key roles in proliferation. Thus the simulation of the function of the native APC protein might be a strategy in the therapy of tumors caused by APC gene mutations.

Inherited APC gene mutations lead to the cancer predisposition Familial Adenomatous Polyposis coli (FAP). FAP symptoms can be prevented and treated with the nonsteroidal anti-inflammatory drug (NSAID) Sulindac (**2**).[6] Sulindac induces apoptosis of cultured colorectal cancer cells and of cells in adenomatous polyps of FAP patients.[7,8] This effect of Sulindac is independent from its effects on the cyclooxygenase pathway.[9,10] Although the cellular target of Sulindac is not known, its described effects are similar to the effects of the APC protein. It was proposed that Sulindac might be able to simulate APC function.[4] Therefore Sulindac is a suitable starting point for a synthetic library of novel APC mimetics.



1 **2**

Recently, we introduced a library of new Sulindac-related molecules[11] that are able to decrease the proliferation rates of cultured cancer cells. We screened for effects of the compounds on cells transformed with the Wnt-1 proto-oncogene. Several of the 189 molecules inhibited the proliferation of Wnt-transformed cells at significantly lower concentrations than the proliferation of untransformed cells. Based on these findings we analyzed the molecular basis of the Wnt inhibiting effects and especially whether the compounds might be small molecule mimetics of APC function (SMAFs).

In order to substantiate this possibility, we analyzed the effects of one of the most potent compounds, SMAF-1 (**1**), on the survival and proliferation of C57MG/Wnt-1 and SW480 cells, two cell lines harboring an activated APC/β-catenin signaling pathway and an increased intracellular β-catenin level (Figure 1 A). We found effects of the drug on SW480 proliferation at an IC_{50} value of 210 μM, whereas the compound affected C57MG/Wnt-1 only at higher concentrations. Remarkably, the mother compound Sulindac had no effect on the tested cell lines up to 1000 μM (not shown). Next we looked for SMAF-1 effects on cell morphology. We chose C57MG/Wnt-1 cells because of their characteristic morphological features (Figure 1 B).[12,13] After incubation with SMAF-1 or with Sulindac C57MG/Wnt-1 cells showed altered morphology. In contrast to untreated control cells, the treated C57MG/Wnt-1 cells were small and round with large nuclei. These changes indicated a reversion of the Wnt-1-induced transformation. In addition, the morphological changes indicate an activation of apoptosis. In order to confirm an effect of SMAF-1 on apoptosis, we analyzed the presence of phosphatidylserine (PS) on the outer layer of the cell membrane. We looked for Annexin V-positive cells, which can be rated as cells in the early stages of apoptosis.[14] In contrast to untreated cells, C57MG/Wnt-1 cells showed clear Annexin V staining after incubation with SMAF-1 or with the apoptosis-inducing reagent staurosporine as a positive control (Figure 1 C).

If SMAF-1 mimics the functions of the APC tumor suppressor, then it is assumed to reduce the intracellular β-catenin level and to inhibit β-catenin/TCF-4-induced gene expression. Treatment with Sulindac or SMAF-1 led to a significant reduc-

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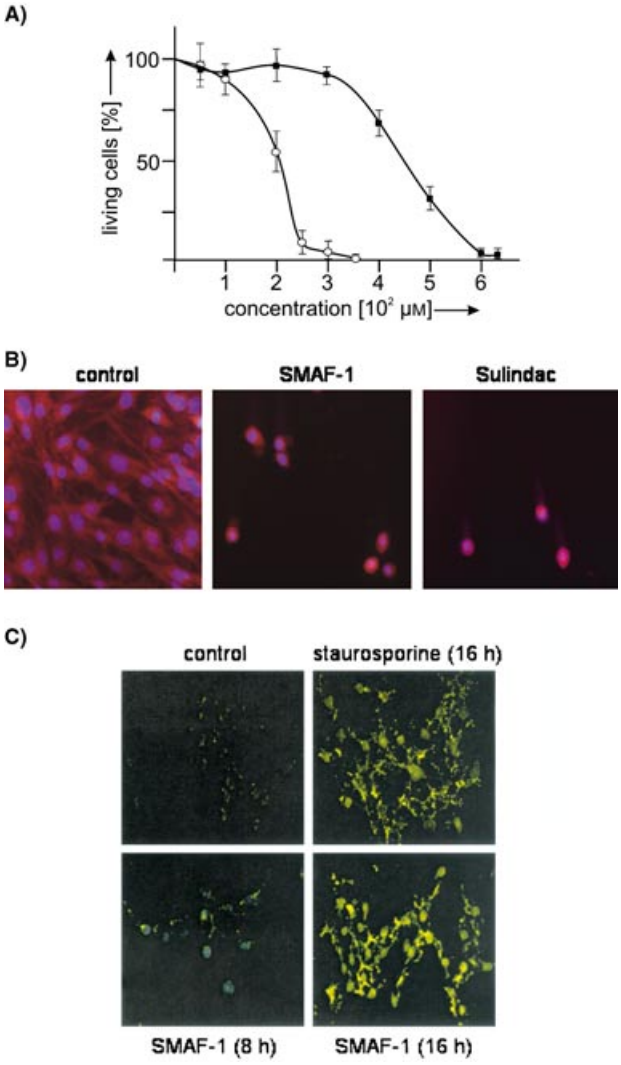


Figure 1. *A) Concentration-dependent effects of SMAF-1 on the proliferation of C57MG/Wnt-1 (■) and SW480 cells (○) measured by a conventional proliferation assay after 24 h incubation. B) Effects of SMAF-1 or Sulindac on the morphology of C57MG/Wnt-1 cells. For better visualization cells were double-stained for actin (red) and DNA (blue). Untreated cells are shown for control. C) Effect of SMAF-1 on apoptosis-like membrane asymmetry of C57MG/Wnt-1 cells. Membrane asymmetry was visualized by Annexin V staining. Untreated and staurosporine-treated C57MG/Wnt-1 cells are shown as control.*

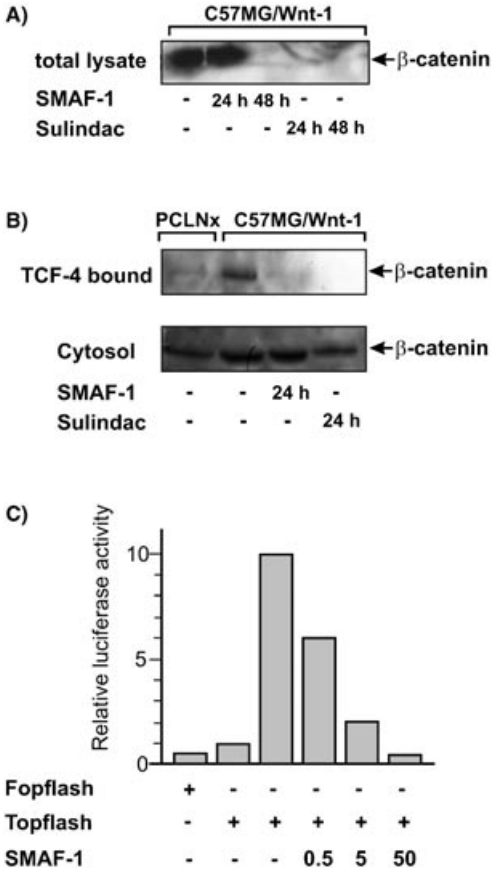
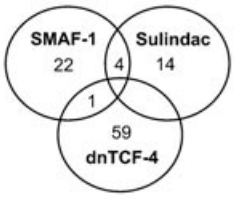


Figure 2. *A) Effect of SMAF-1 or Sulindac on the total β-catenin levels from C57MG/Wnt-1 cells. Levels of β-catenin were analyzed by Western blotting of total lysates. In each lane equal amounts of 10 µg total protein were loaded. B) Effect of SMAF-1 or Sulindac on the level of TCF-4-bound β-catenin. Analysis was performed by Western blot of immunoprecipitated TCF-bound β-catenin. The levels of cytosolic β-catenin are shown for comparison. PCLNx designates untreated C57MG/PCLNx control cells. C) Effect of SMAF-1 on the gene-expression activation of β-catenin. Analysis was performed by reporter-gene assays, as described in the Experimental Section. Columns indicate the relative luciferase activity. The micromolar SMAF-1 concentrations in the assay are shown.*

tion of the total β-catenin level in C57MG/Wnt-1 cells (Figure 2A). In addition to its transcription-activating function in the Wnt pathway, β-catenin can bind to the cytosolic domain of E-cadherin.[15] Therefore the reduction of β-catenin in the total lysates might be due to a decrease of the cytosolic or of the nuclear pool of β-catenin. In order to differentiate between these two pools, we analyzed the fractionated cytosolic pool and the TCF-4-bound pool of β-catenin (Figure 2B). Remarkably, we found a significant reduction in the TCF-4-bound form of β-catenin, whereas the level of the cytosolic β-catenin remained unchanged. These results indicate that the SMAF-1-induced decrease of the intracellular β-catenin level was mainly due to the decrease of the TCF-4-bound and transcription-activating form of β-catenin. In a reporter-gene assay, we found that SMAF-1 was able to reduce the β-catenin/TCF-4-induced promoter activation in a concentration-dependent manner (Figure 2C).

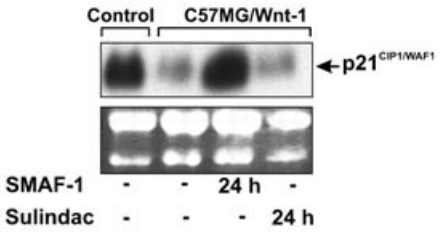
In order to get hints about analogies and differences between SMAF-1, Sulindac, and APC function on a more general level of gene expression, we compared the expression patterns in SMAF-1- or Sulindac-treated C57MG/Wnt-1 cells with the pattern of untreated C57MG/Wnt-1 cells by microarray hybridization. (The complete results of the differential expression analysis of SMAF-1- and Sulindac-treated cells are available upon request.) Differences in expression levels were regarded as significant when they exceeded the factor of 2.5. In summary, 27 genes showed increased expression levels in the presence of SMAF-1, whereas 18 genes were induced by Sulindac (Figure 3A). The increased levels of the four genes, which were changed in the presence of both compounds, were confirmed by Northern blot (not shown). Three of these, cytochrome P450, alcohol dehydrogenase, and glutathione S-transferase alpha-1, are involved in the metabolism of xenobiotics. The induction of these compound-metabolizing enzymes can be

A)



Gene	Fold Change SMAF-1	Sulindac	dnTCF-4
Cytochrome	4.3	4.4	–
Alcohol dehydrogenase 2 complex	4.5	3.2	–
Placental proliferin 2	4.9	2.6	–
Glutathione S-transferase alpha-1	27	16.5	–
$p21^{CIP1/WAF1}$	6.2	1.5	3.3

B)



C)

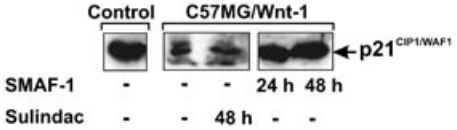


Figure 3. *A) Summary of the differential gene expression analysis by microarray hybridization. ESTs or hypothetic genes were not included. Numbers of genes are shown with at least 2.5-fold-increased expression after incubation with SMAF-1 (left circle), Sulindac (right circle), or dnTCF-4 (bottom circle). Gene expression data of dnTCF-4 transfected cells are from ref. [18]. The table lists the genes that were induced in treated cells together with the factors of their induction compared to untreated cells. B) Effects of SMAF-1 or Sulindac on the transcription level of $p21^{CIP1/WAF1}$. Expression of $p21^{CIP1/WAF1}$ was analyzed by Northern blotting in C57MG/PCLNx control cells and in compound-treated and untreated C57MG/Wnt-1 cells. To show equal loading, the ethidium bromide-stained agarose gel is shown. C) The effects of SMAF-1 or Sulindac on the $p21^{CIP1/WAF1}$ protein level. Analysis was performed by Western blotting of cellular lysates containing equal amounts of cellular protein.*

rated as a response of the cell to treatment with the xenobiotic compounds.[16] The fourth gene codes for proliferin 2, which can either stimulate or inhibit various stages of angiogenesis.[17] Next we compared the differential expression pattern obtained with SMAF-1 and Sulindac with the pattern induced by the expression of dominant negative TCF-4 (dnTCF-4), which was published recently.[18] Since APC inhibits β-catenin/TCF-4-induced transcription, the consequences of dnTCF-4 expression should be comparable to the effects of APC activation. The tumor-suppressor gene $p21^{CIP1/WAF1}$ was induced by both SMAF-1 and by dnTCF-4 (Figure 3A). Microarray data showed a 6.2-fold increase in the $p21^{CIP1/WAF1}$ expression level by SMAF-1 incubation and a 3.3-fold increase by dnTCF-4. The expression of $p21^{CIP1/WAF1}$, which is inhibited by the direct β-catenin/TCF-4 target Myc, is important for G1 arrest and cell differentiation.[18] The central role of $p21^{CIP1/WAF1}$ makes it a key element of the effects induced by SMAF-1. It is noteworthy that we detected only a 1.5-fold increase in the gene transcription after Sulindac treatment. Recent reports showed that Sulindac unfolds its antiproliferative activity by the induction of $p21^{CIP1/WAF1}$, and that $p21^{CIP1/WAF1}$ is essential for tumor inhibition by Sulindac.[19,20] Northern blot analysis confirmed our results (Figure 3B). SMAF-1 restored the level of $p21^{CIP1/WAF1}$ to a similar level as that detected in C57MG/PCLNx control cells, whereas Sulindac had almost no effect on the $p21^{CIP1/WAF1}$ transcription level. We also proved by Western blot analysis the SMAF-1-induced restoration of the $p21^{CIP1/WAF1}$ expression on the protein level (Figure 3C). Keeping in mind the important role of $p21^{CIP1/WAF1}$ in tumor suppression, the effects of SMAF-1 on $p21^{CIP1/WAF1}$ expression indicate the potential of compounds like SMAF-1 in antitumor therapy.

Here we have identified a new SMPF, a small molecule that is able to mimic functions of a distinct protein. Based on the well-established effects of Sulindac on APC-negative tumors and on our findings described herein, we conclude that SMAF-1 has a target located within the APC/β-catenin pathway and upstream of β-catenin. Our results support the hypothesis that SMAF-1 and NSAIDs like Sulindac inhibit the APC/β-catenin pathway by executing major functions of the APC protein, even in cells with an activated APC/β-catenin pathway. These functions include i) an inhibitory effect on the proliferation of cells with activated APC/β-catenin pathway, ii) induction of apoptosis, iii) decrease of the intracellular β-catenin level, iv) inhibition of β-catenin-induced activation of the TCF-4 promoter, v) inhibition of β-catenin/TCF-4-induced gene expression, and vi) the expression regulation of a gene playing a key role in proliferation control. Future in vivo studies will show the potential of compounds like SMAF-1 as antitumor compounds in the treatment of tumors with mutationally inactivated APC.

Experimental Section

Compounds and cells: SMAF-1 ((*Z*)-5-fluoro-2-methyl-1-(3′-furylmethylene)-3-indene acetic acid) was synthesized by common organic-chemical methods as described previously.[11] All other reagents were purchased from Sigma. SW480 cells, which lack functional APC, were purchased from ATCC. C57MG/Wnt-1 and C57MG/PCLNx cells were kindly provided by Jan Kitajewski (Columbia University, New York).[12] C57MG/Wnt-1 and C57MG/PCLNx cells were derived from C57MG cells by transfection with the murine Wnt-1 oncogene or the empty vector PCLNx, respectively. If not otherwise mentioned, cells were incubated with 100 μM SMAF-1 or Sulindac for 24 h before analysis.

Cellular assays for apoptosis: The morphology of apoptotic cells was visualized by fluorescence microscopy. Cells were double-stained for nuclear DNA and for the cytoskeletal protein actin.[21] The loss of plasma-membrane asymmetry is an early event in apoptosis that results in the exposure of phosphatidylserine (PS) at the outer plasma-membrane leaflet.[14] As Annexin V interacts specifically with PS, detection of the bound Annexin V can be used as a method to screen for cells in the early phases of apoptosis.[22] Cells treated with the apoptosis-inducing kinase-inhibitor staurosporine (1 μM) were used as control.[23] PS was detected by incu-

bating cells with biotinylated Annexin V (Roche) and with Cy3-labelled streptavidin.

Analysis of total and TCF-4-bound β-catenin: For the analysis of total β-catenin, cell lysates were calibrated to equal protein concentrations with PBS and analyzed by Western blotting. For the analysis of cytosolic and TCF-bound β-catenin, total lysates were centrifuged, and the supernatants, defined as cytosols, were calibrated to equal protein concentrations. The pelleted nuclei were lysed and calibrated to equal protein concentrations. TCF-4 was immunoprecipitated by standard procedures, and the coimmunoprecipitated β-catenin was detected by Western blotting.

Gene-expression analysis and reporter-gene assay: Differential expression patterns from C57MG/Wnt-1 cells after incubation with SMAF-1 or Sulindac (100 μM) were analyzed by array hybridization with the murine whole-genome array U74 by Affymetrix. The major part of all known murine genes and 6000 murine ESTs are represented on this chip. Gene-chip-array analysis and data evaluation were performed by following established protocols. Northern blot analysis was used to confirm the differential transcription of all genes selected from the array analysis. The β-catenin/TCF-4 reporter gene assay was performed as described.[24,25]

Keywords: antitumor agents · biological chemistry · biomimetics · NSAIDs · proteins

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