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NO-donating aspirin inhibits the growth of leukemic Jurkat cells and modulates β-catenin expression

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Abstract

β-Catenin has been implicated in leukemic cell proliferation. We compared the effects of aspirin (ASA) and the *ortho*, *meta*, and *para* positional isomers of NO-donating aspirin (NO-ASA) on cell growth and β-catenin expression in human Jurkat T leukemic cells. Cell growth inhibition was strong: IC_{50} for *p*-, *o*-, and *m*- were 20 ± 1.6 (mean ± SEM), 15 ± 1.5, and 200 ± 12 μM, respectively, in contrast to that of ASA (3200 ± 375 μM). The *para* isomer of NO-ASA degraded β-catenin in a dose- and time-dependent manner coinciding with increasing expression of activated caspase-3. The caspase inhibitor ZVAD blocked β-catenin cleavage by *p*-NO-ASA and partially reversed cell growth inhibition by *p*-NO-ASA but not that by ASA. A denitrated analog of *p*-NO-ASA did not degrade β-catenin indicating the importance of the NO-donating moiety. Our findings suggest that NO-ASA merits further study as an agent against leukemia.

Keywords: NO-donating aspirin; Aspirin; Leukemia; β-Catenin; Caspases; Cancer; Chemoprevention

Leukemia is the most common form of cancer in children and acute lymphoblastic leukemia (ALL) is the primary cause of cancer-related mortality in children. Developing novel therapeutic approaches is an important goal and insights into the mechanism of leukemia cell proliferation should contribute towards the rational design of effective treatments. The deregulation of the Wnt signaling cascade and its components has been implicated in T cell ALL as well as in B cell chronic lymphocytic leukemia (CLL) [1,2]. The protein β-catenin is a central player of the Wnt signaling pathway that regulates cell–cell adhesion and may promote leukemia cell proliferation [1]. The stabilization and accumulation of β-catenin has a powerful regulatory role in proliferation and differentiation [3,4]. β-Catenin is expressed in T-ALL cells, tumor lines of hematopoietic origin, and primary leukemia cells but is undetectable in normal peripheral blood T cells. Among the leukemic cell lines, β-catenin is expressed in high levels in Jurkat T cells [1,5]. Currently, the role of β-catenin and its regulation in non-adherent cells are not very clear.

Nitric oxide-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs) represent a novel and promising class of cancer chemopreventive compounds. They consist of a traditional NSAID to which a group donating NO has been covalently attached via an aromatic or aliphatic spacer. Emerging data indicate that these compounds combine the chemopreventive properties of traditional NSAIDs against cancer with enhanced safety, efficacy, and potency [6–8]. Nitric oxide-donating aspirin (NO-ASA, which is a traditional ASA molecule bound covalently to $–ONO_2$ via an aromatic spacer) may be effective in colon cancer

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chemoprevention. NO-ASA has been demonstrated to have enhanced potency compared to traditional ASA; and more recently its strong chemopreventive and therapeutic effect in animal models of gastrointestinal cancer has been shown [9,10].

NO-NSAIDs, including NO-ASA, affect profoundly cancer cell renewal and death [11]. We have demonstrated that NO-ASA inhibits the growth of colorectal cancer cells and other cancer cell lines far more potently than ASA [6]. NO-ASA modulates the β-catenin/TCF pathway in human colorectal cancer cell lines by disrupting the interaction of β-catenin and TCF-4 in the nucleus [12,13]. Chemopreventive agents such as traditional NSAIDs and curcumin downregulate β-catenin/TCF signaling. In this study, we explored the effects of NO-ASA in non-adherent cells such as the human Jurkat T-acute lymphoblastic leukemia cells, which are known to express high levels of β-catenin. We examined the effect of NO-ASA on the growth of these cells and on the fate of β-catenin expression, and also examined which part of the NO-ASA molecule likely causes the differential effect that we observed.

Materials and methods

Reagents and cell culture. NO-ASA, *para* isomer (*NCX4040*): 2-((acetyloxy)benzoic acid 4-(nitrooxy methyl)phenyl ester); the *ortho* isomer (NCX 4060): 2-((acetyloxy)benzoic acid 2-(nitrooxy methyl)phenyl ester); and *meta* isomer (NCX 4016): 2-((acetyloxy)benzoic acid 3-(nitrooxy methyl)phenyl ester) were obtained from Nicox, SA, France. ASA was obtained from Sigma. Stock solutions (100 mM) were made in DMSO; final DMSO concentration was adjusted in all media to 1%. Jurkat cells (ATCC TIB-152, Manassas, VA) were grown per ATCC instructions. The inhibitors z-VAD-fmk (ZVAD), z-IETD-fmk (caspase-8 inhibitor), and z-LEHD-fmk (caspase-9 inhibitor) were obtained from Calbiochem and used according to the manufacturer's instructions.

Cell growth inhibition assay. The growth inhibitory effect of NO-ASA on Jurkat T cells was measured using a colorimetric MTT assay kit (Roche). Briefly, cells were plated in 96-well plates at a density of 13.7×10^3 cells/well and, following overnight incubation, NO-ASA was added to the culture medium. Viable cells were quantified with MTT substrate according to the manufacturer's instructions. Growth inhibition was expressed as percentage of the corresponding control.

Western blot analysis. After treatment with test drug, cells were harvested and lysed in buffer containing 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, and 0.5% IGEPAL, 10% glycerol in the presence of proteinase inhibitors. Proteins were fractionated by SDS–PAGE and transferred to Immobilon P membrane. Primary mouse monoclonal antibodies were against the following at the dilutions indicated: β-catenin, 1:1000 (BD-Transduction Laboratories); and α-actin, 1:1000 (Santa Cruz Biotechnology), caspase-3, 1:500 (Sigma). Secondary antibodies conjugated to horseradish peroxidase (1:4000) were from Sigma. Immunoreactive protein was detected using ECL chemiluminescence (Amersham).

Statistics. Data are presented as means ± SEM for at least three different sets of plates and treatment groups. Statistical comparison among the groups was performed using a one-way analysis of variance followed by the least significant difference method.

Results

NO-donating aspirin strongly inhibits growth of human leukemic of Jurkat cell line

We examined the effect of NO-ASA on Jurkat T cell growth. NO-ASA strongly inhibits cell growth as measured by MTT assay in these cells in a concentration-dependent manner. Compared to ASA, all three isomers of NO-ASA inhibited cell growth strongly; the IC_{50} of *p*-NO-ASA for cell growth inhibition at 24 h is 20 ± 1.6 μM [this and all subsequent values are means ± SEM] in contrast to that of ASA, which is 3200 ± 375 μM. The IC_{50}s for *ortho* and *meta* NO-ASA isomers were 15 ± 1.5 and 200 ± 12 μM, respectively (Fig. 1). Thus, all three positional isomers are more potent growth inhibitors of human leukemic Jurkat cell line than traditional ASA. Based on the IC_{50}s for cell growth inhibition, showing the *p*- and *o*-isomers to be very similar, we concentrated our studies in comparing the *p*- and *m*-isomers to the parent compound, ASA.

The para isomer of NO-ASA degrades β-catenin

We examined whether NO-ASA can affect the expression levels of β-catenin which is present in large amounts in these cells, thus facilitating such a study. Growing Jurkat cells were treated with *p*-NO-ASA, ASA, or vehicle at the concentrations shown in Figs. 2A and B, for 24 h followed by protein extraction and detection of β-catenin by immunoblotting. The *para* isomer of NO-ASA degraded β-catenin in a concentration-dependent manner between 0 and 20 μM; the β-catenin levels were drastically reduced at 50 μM NO-ASA, a concentration much higher than the IC_{50} for cell growth (Fig. 2A). Various concentrations of ASA up to 5 mM had no effect on β-catenin levels (Fig. 2B). Similarly, *m*-NO-ASA up to 300 μM had no effect on β-catenin levels (data not shown). Fig. 2C compares the exact fate of β-catenin at the IC_{50} values for all three compounds, *p*-NO-ASA (20 μM), *m*-NO-ASA (200 μM) or ASA (3.2 mM) for 24 h. In all these studies, only *p*-NO-ASA (20 μM) degraded β-catenin to a smaller size fragment which migrated faster at approximately 85 kDa and was recognized by the antibody. This phenomenon can also be seen in Fig. 2A in a concentration-dependent manner. There was no major change in the levels of α-actin at these concentrations. A time course study of the cleavage of β-catenin by *p*-NO-ASA at 20 μM showed that it occurred as early as 3 h and became quite prominent at 18 h (Fig. 2D).

The –ONO_2 group of p-NO-ASA is critical for the cleavage of β-catenin

Pharmacologically, the –ONO_2 moiety is considered pivotal for the novel properties of NO-ASA when

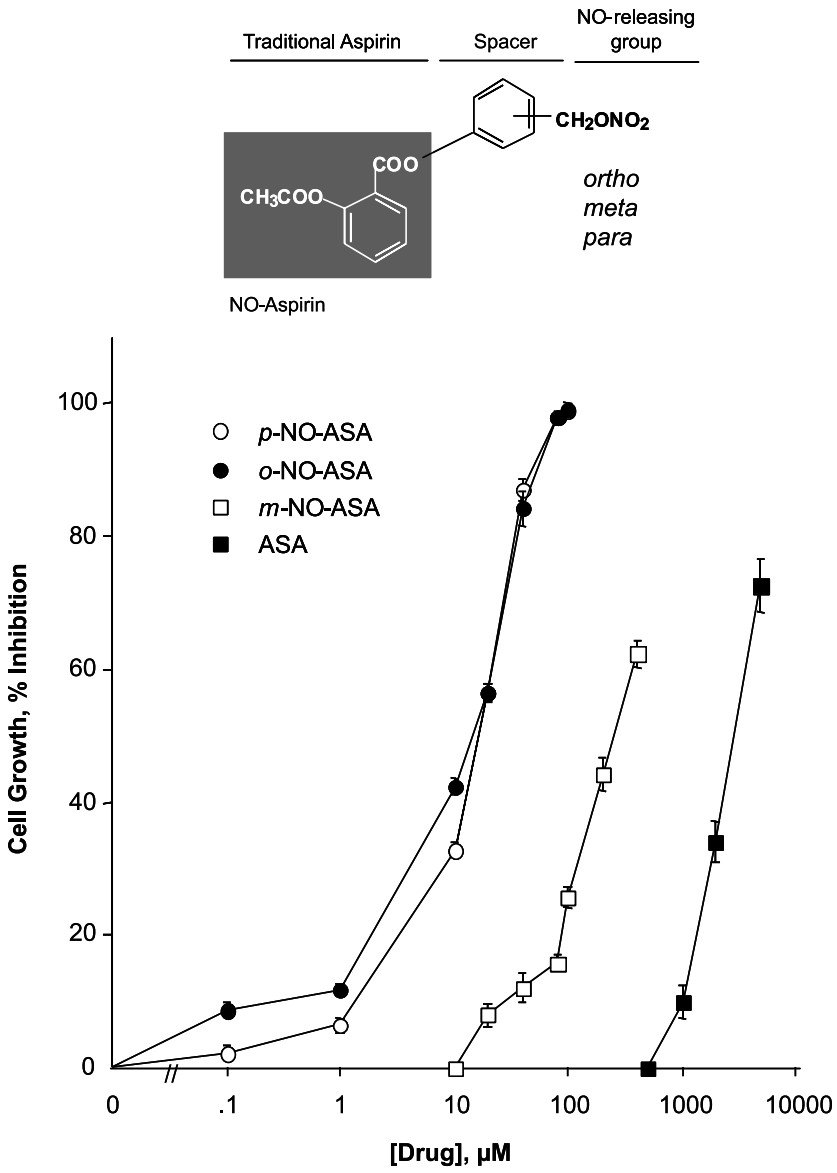

Fig. 1. Effect of positional isomers of NO-ASA and ASA on Jurkat T cell growth. Cells were treated with indicated concentrations of *p*-, *m*-, *o*-NO-ASA or ASA for 24 h and assayed for growth as described in Materials and methods. Values represent means ± SEM of three representative experiments performed in triplicate.

compared to those of ASA and our previous studies have underscored its importance [6,12]. We investigated the contribution of the key structural components of the NO-ASA molecule to the cleavage of β-catenin. We used two structural analogs of *p*-NO-ASA, one in which the $-ONO_2$ group had been replaced by an –OH group (denitrated analog) and another in which the CH_3COO- was replaced by an –OH (deacetylated analog). The CH_3COO- group is the critical part of ASA for inactivation of COX.

Jurkat cells were treated for 24 h with equimolar concentrations (20 μM) of *p*-NO-ASA or its denitrated, or the deacetylated analogs. In contrast to *p*-NO-ASA, the denitrated analog was unable to degrade β-catenin and there were no changes in the β-catenin levels in comparison to vehicle control (Fig. 2E). The deacetylated derivative was capable of cleaving β-catenin similar to *p*-NO-ASA. This strongly suggests that the CH_3COO- group does not appear to have a role in cleaving β-catenin. The levels of α-actin protein were constant. Thus, $-ONO_2$ may contribute significantly to β-catenin cleavage.

p-NO-ASA-induced cleavage of β-catenin is caspase-dependent

To determine whether caspases play a role in the cleavage of β-catenin by *p*-NO-ASA in Jurkat cells, we studied the effect of a pan-caspase inhibitor z-VAD-FMK

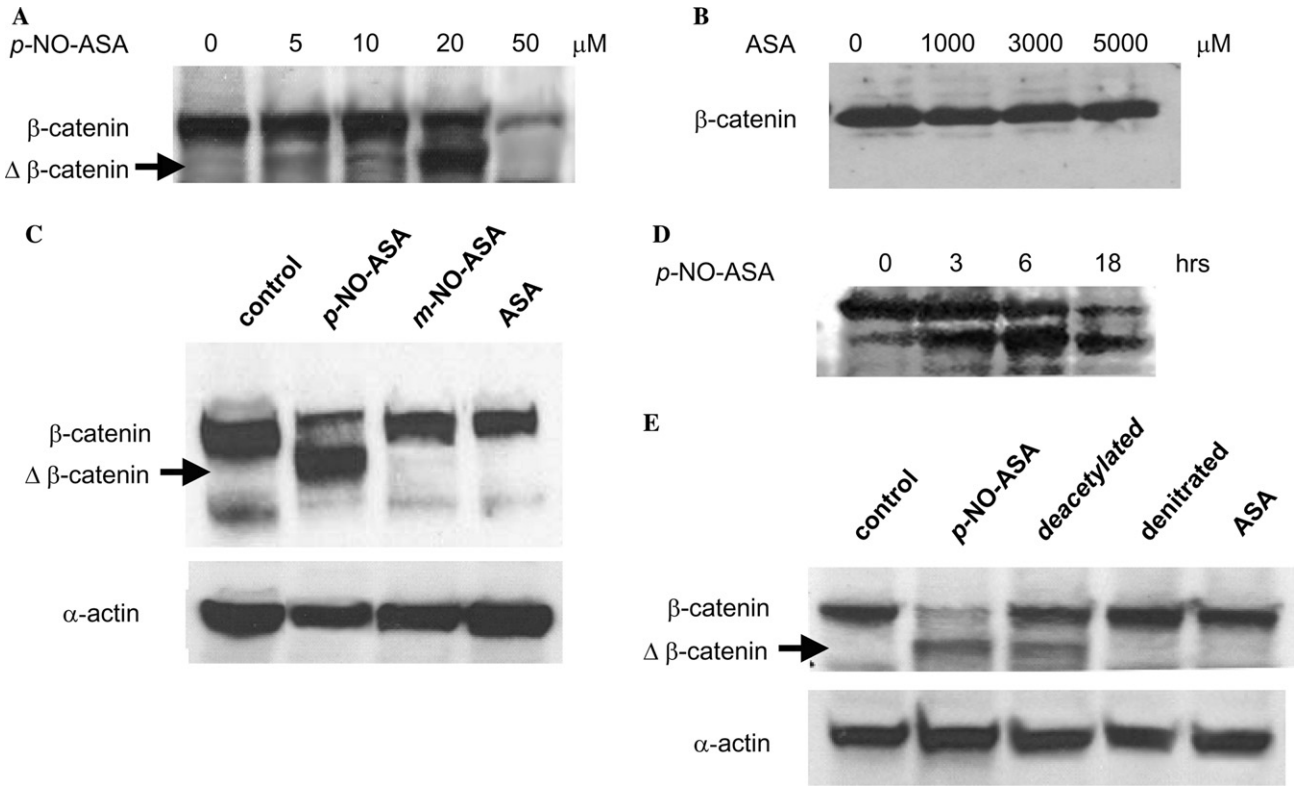

Fig. 2. The *para* isomer of NO-ASA degrades β-catenin and the $-ONO_2$ moiety is critical for this effect. Jurkat cells treated with increasing concentrations of *p*-NO-ASA for 24 h were analyzed for total β-catenin expression by immunoblot of lysates. (A) Concentration-dependent degradation of β-catenin, Δβ-catenin representing a cleaved form of the protein; increasing concentrations of ASA had no effect on total β-catenin levels (B). Cells treated with ASA, *p*-, or *m*-NO-ASA at the respective IC_{50} for growth were compared for cleavage of β-catenin (C). Time-dependent cleavage of β-catenin was observed by 20 μM *p*-NO-ASA (D). The levels of β-catenin were examined by treatment of cells with *p*-NO-ASA and corresponding structural analogs (denitrated or deacetylated analog, 20 μM each) or ASA (3.2 mM). Blots were reprobed for α-actin (E).

(ZVAD). Jurkat cells were pretreated for 30 min with 100 μM ZVAD followed by addition of *p*-NO-ASA (20 μM) for 24 h. Immunoblotting for β-catenin revealed that ZVAD can completely block its degradation (Fig. 3A, lanes 2 and 4). ZVAD was also capable of blocking β-catenin degradation by the deacetylated derivative (Fig. 3B). The initiator caspases, caspase-8 and caspase-9, are known to be Fas receptor-mediated and mitochondria-mediated, respectively. Either of these caspases can activate the downstream effector caspase-3. The inhibitors z-IETD-fmk (caspase-8 inhibitor) or z-LEHD-fmk (caspase-9 inhibitor), 30 μM each, were able to block *p*-NO-ASA associated degradation of β-catenin (Fig. 3C). An examination of the time course for caspase activation using an anti-caspase-3 antibody, which recognizes both the procaspase-3 and the activated caspase-3, showed that at 3 h, *p*-NO-ASA induced the activated form of caspase-3 (Fig. 3D). This time point is also in coherence with the appearance of cleaved β-catenin fragments.

Growth inhibition by p-NO-ASA is reversed by ZVAD

p-NO-ASA and ASA both inhibit the growth of Jurkat cells, yet their molecular targets appear to be different. Caspase activation may be a key feature of *p*-NO-ASA unlike ASA. We examined whether blocking caspase activation could indeed block the inhibition of growth that we observed at the IC_{50} concentrations of *p*-NO-ASA or ASA. Jurkat cells were pretreated with ZVAD (100 μM) for 30 min followed by *p*-NO-ASA (20 μM) or ASA (3.2 mM) or vehicle (control) for 24 h. Growth inhibition was measured by the MTT assay. Whereas cells were approximately 50% growth inhibited in the absence of ZVAD, this inhibition was partially reversed by ZVAD for *p*-NO-ASA but not for ASA (Fig. 4). This effect was also concentration-dependent, since 50 μM ZVAD also partially reversed the inhibitory effects of *p*-NO-ASA but to a lesser extent (data not shown). The inhibitory effect of ASA (3.2 mM) was only marginally reversed by 100 μM ZVAD (Fig. 4).

Discussion

These studies highlight three significant points with respect to the mechanism of action of NO-ASA that merit deeper insight. First, the previously reported

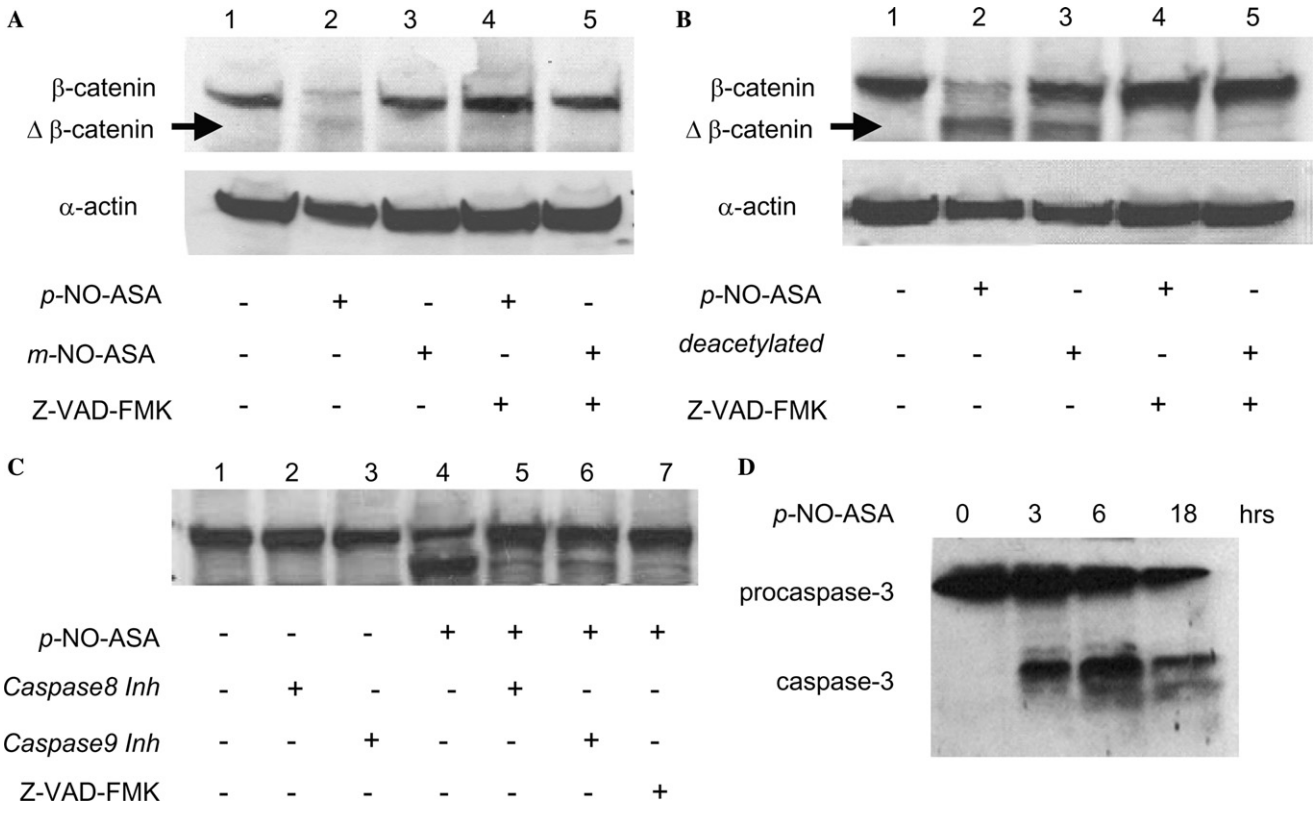

Fig. 3. *p*-NO-ASA mediated cleavage of β-catenin is reversed by ZVAD. Jurkat cells were pretreated with 100 μM ZVAD-FMK for 30 min or untreated followed by addition of 20 μM *p*-NO-ASA or 200 μM *m*-NO-ASA (A) or 20 μM deacetylated analog of *p*-NO-ASA (B) for 24 h; β-catenin levels were detected by blotting. Pretreatment with caspase-8 inhibitor or caspase-9 inhibitor (50 μM) blocked *p*-NO-ASA mediated cleavage of β-catenin (C) shown in lanes 5 and 6, respectively. Procaspase-3 and activated caspase-3 were detected by immunoblotting in a time course study with 20 μM *p*-NO-ASA (D).

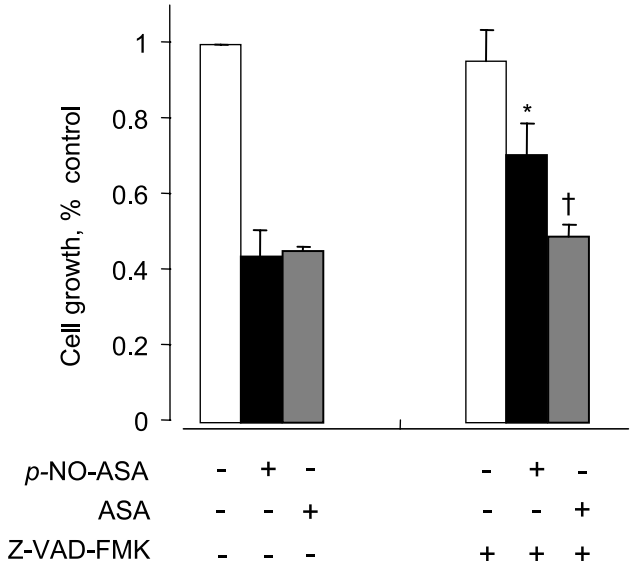

Fig. 4. *p*-NO-ASA mediated cell growth inhibition is reversed by ZVAD. Jurkat cells, pretreated with 100 μM ZVAD-FMK for 30 min or untreated, were followed by either 20 μM *p*-NO-ASA or 3.2 mM ASA (respective IC_{50} concentrations) and subsequently analyzed for growth as described in Materials and methods. Values represent means ± SEM of three representative experiments performed in triplicate. $^{*}P < 0.05$ and $^{\dagger}P > 0.05$ compared to absence of ZVAD.

radically different potency between traditional ASA and NO-ASA is given more credence by our findings. *p*-NO-ASA is approximately 160- to 180-fold more potent than ASA in inhibiting the growth of Jurkat cells. Second, cleavage of β-catenin seems to be an important mechanism by which NO-ASA exerts its effect on Jurkat cells; however, there are distinct mechanistic differences between positional isomers of NO-ASA regarding the degradation of β-catenin. Third, the importance of the $-ONO_2$ moiety is underscored by our structure–activity relationship study.

That NO-ASA is dramatically more potent than traditional ASA in inhibiting the growth of cancer cells is consistent with our previous findings on cells from solid tumors [10]. For example, NO-ASA displayed the same property in a variety of human cancer cell lines (e.g., colon, pancreas, prostate, etc.) and was approximately between 1000- and 5000-fold more potent than traditional ASA in inhibiting the growth of colon cancer cells [6]. That a similar effect is shown in leukemic cells further strengthens the conclusion that this is likely a universal property of NO-ASA [14,15].

Second, cleavage of β-catenin is likely an important mechanism by which NO-ASA exerts its effect on cancer

cells. That this effect is biologically relevant is underscored by the fact that a caspase inhibitor reversed the growth inhibitory effect of *p*-NO-ASA. Our data indicate that *p*-NO-SA (20 μM) activates caspase-3 while the *meta* isomer does not have this effect. This finding is in excellent agreement with a recent report showing that the *ortho* isomer of NO-ASA increased caspase-3 expression in prostate cancer epithelial cell lines and in primary cultures of prostatic stroma [16]. Thus, it appears that caspase-3 activation is an effect shared by *para* and *ortho* isomers of NO-ASA but not by the *meta* isomer. In this as in other studies, there is a clear difference between the biological activity of *o*- and *p*-NO-ASA on the one hand and the *m*-NO-ASA on the other [12]. Our data confirm this difference.

The two positional isomers modulate β-catenin expression differently: the *para* isomer degrades β-catenin in a time- and concentration-dependent fashion, whereas the *meta* isomer does not. The interesting finding is that both inhibit the growth of Jurkat T cells. In our study, at their IC_{50} concentrations, 20 μM for *p*-NO-ASA and 200 μM for *m*-NO-ASA, they inhibited cell growth (each by 50%, and cells were still viable as observed by trypan blue staining—data not shown) but β-catenin was cleaved only by *p*-NO-ASA. It is conceivable that cleavage of β-catenin may not be required for cell growth inhibition. Instead, cell growth inhibition through the Wnt pathway may occur through disruption of the interaction of β-catenin and TCF-4 in the nucleus, a mechanism that we have described for colon cancer cells [12]. If this is the case, β-catenin cleavage may play an accessory role potentiating cell growth inhibition, and this could explain the higher potency of *p*-NO-ASA compared to *m*-NO-ASA.

Caspase-3-mediated cleavage of β-catenin is not unique to NO-ASA and has been described for other chemopreventive agents. For example, in human colon cancer lines curcumin induced a strong cleavage pattern consisting of several lower molecular weight fragments of β-catenin. The sequence of events that included activation of caspase-3 followed by cell death was thought to explain the cell growth inhibitory effect of curcumin [17]. Cleavage of β-catenin by caspase-3 activation has been described with NSAIDs such as indomethacin, sulindac, and aspirin [18,19].

Finally, the NO-donating moiety of *p*-NO-ASA is critical for the observed effects. This is shown clearly by our structure–activity relationship study in which the denitrated derivative failed to cleave β-catenin. In contrast, the deacetylated analog of NO-ASA had an effect on β-catenin very similar to that of *p*-NO-ASA. These findings are consistent with other observations on the biological activity of these two analogs of NO-ASA [12].

In conclusion, NO-ASA is a potent inhibitor of the growth of Jurkat T cells suggesting its therapeutic potential in leukemias. Mechanistically, our studies indicate that NO-ASA inhibits the growth of Jurkat T cells, at least in part, by activating caspase-3, which in turn degrades β-catenin leading ultimately to cell growth inhibition. This mechanism is restricted to the *p*-isomer of NO-ASA, whereas the less potent *m*-isomer employs a different and as yet not fully clarified mechanism. Our findings also underline the dramatic pharmacological differences between NO-ASA and traditional ASA. Both the intriguing mechanistic questions raised by our data and the potential for a therapeutic application to leukemias of this novel class of compounds merit further evaluation.

Acknowledgments

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