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Nitric Oxide–Releasing Hybrid Drugs Target Cellular Processes Through S-Nitrosylation

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Abstract

Nitric oxide (NO)–releasing agents such as JS-K and NO–releasing hybrids such as NO– and NONO–nonsteroidal anti-inflammatory drugs are novel agents with great potential for controlling cancer. Although studied extensively, a key question pertaining to their molecular targets and mechanism of action remains unclear: the role of NO in the overall biological effect of these agents. It has been shown that NO can directly modify sulfhydryl residues of proteins through S-nitrosylation and induce apoptosis. We showed that 3 structurally diverse NO–nonsteroidal anti-inflammatory drugs S-nitrosylated nuclear factor- κ B p65 in vitro and in vivo and also showed that these agents S-nitrosylated caspase-3 in vivo. JS-K reduced nuclear β -catenin and cyclin D1 protein levels without affecting cytosolic β -catenin expression. On the basis of a time course study, S-nitrosylation of nuclear β -catenin was determined to precede its degradation. These data provide a mechanistic role for NO and a rationale for the chemopreventive effects of these novel agents.

Keywords

nitric oxide; NF- κ B; β -catenin; caspase-3; NSAIDs; NO-NSAIDs; NONO-NSAIDs; JS-K; S-nitrosylation; colon cancer; chemoprevention

I. Introduction

Posttranslational modifications of proteins are central molecular mechanisms that mediate signal transductions. One such modification is S-nitrosylation, which involves the addition of nitric oxide (NO) to a cysteine thiol to form an S-nitroso (SNO)-protein.¹ To put this in perspective, S-nitrosylation may be comparable to phosphorylation and ubiquitylation, which are critical cellular regulatory mechanisms.² Two determinants of cellular mass are proliferation and apoptosis. S-nitrosylation by nitrosative stress can regulate cellular homeostasis to maintain the balance between the induction³ and prevention of apoptosis.⁴ The Wnt/ β -catenin and nuclear factor (NF)- κ B are independent pathways involved in the regulation of many physiological and pathological states including development, immune function, inflammation, tumorigenesis, tumor invasion, and metastasis. Transcription factors are highly active in most cancer cells, which make them ideally suited for the development of anticancer drug therapies. Several transcription factors, including TCF/ β -catenin and NF- κ B, are promising targets for cancer therapeutics.⁵ This article reviews published work from my laboratory on S-nitrosylation of NF- κ B, caspase-3, and β -catenin by a number of NO-releasing hybrid compounds both in vitro and in vivo.

IA. NO-Releasing NSAIDs

Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce the risk of and mortality from a number of etiologically different cancers, although the strongest evidence is for colon cancer.⁶ However, regular NSAID use may be associated with serious gastrointestinal and renal side effects, among others.⁷ To improve the safety profile of NSAIDs, numerous strategies have been employed. One approach has been the use of NO, leading to a generation of NO-releasing NSAIDs (NO-NSAIDs, NONO-NSAIDs). The development of NO-NSAIDs was based on the observation that NO could enhance the local gastric mucosal defense mechanisms, thereby decreasing NSAID-induced gastric toxicity.^{8,9} NO-NSAIDs are traditional NSAIDs linked to an NO-releasing group via a chemical spacer. The 3 key structural components of any NO-NSAID are the traditional NSAID moiety; the spacer, which can be either aliphatic (NO-naproxen) or aromatic (NO-aspirin); and the NO-releasing group, which is $-\text{NO}_3$ in all available nitrate esters (Fig. 1).¹⁰ In general, NO-NSAIDs are more potent in inhibiting the growth of various human cancer cell lines compared with their traditional parent NSAID.¹¹ Aromatic spacers generate more potent NSAID derivatives than aliphatic ones,¹² and positional isomerism greatly influences all cell kinetic parameters that are important determinants of cellular mass.¹² NONO-NSAIDs are based on linking an *N*-diazen-1-ium-1,2 diolate functional group to a classical NSAID, which has the potential to generate 2 equivalents of NO.¹³ These agents have potent anti-inflammatory properties and are without any significant gastric toxicity.¹⁴ NONO-aspirin (Fig. 1) possessing *O*²-acetoxymethyl-1-[*N*-(2-hydroxyethyl)-*N*-methylamino] diazen-1-ium-1,2-diolate moiety as the NO donor was used in our studies.¹⁵

IB. JS-K, a Novel NO Donor of the Diazeniumdiolate Class

JS-K, (*O*²-(2,4-Dinitrophenyl) 1-[(4-ethoxy-carbonyl) piperazin-1-yl]diazene-1-ium-1,2-diolate), is a member of the arylated diazeniumdiolate class of nitric oxide prodrugs (Fig. 1) that has shown promise as an anticancer drug.¹⁶ It has potent antileukemia activity both in vitro and in vivo using a xenograft model¹⁶ as well as being effective against renal, prostate, and brain cancer cells.^{17,18}

II. RESULTS AND DISCUSSION

The possibility that NO released from any NO-NSAID or JS-K could modulate different molecular targets important to cellular homeostasis/toxicity has not been fully understood. In this review I address the role of NO using JS-K and structurally diverse NO-NSAIDs and look at *S*-nitrosylation of NF- κ B p65, caspase-3, and β -catenin as possible modified targets that are of biological importance.

IIA. NO-NSAIDs Inhibit Growth of Colon Cancer Cells, Reduce NF- κ B Protein Levels and Increase Caspase-3 Enzyme Activity

Aspirin and *m*-NO-aspirin inhibited the growth of human colon HT-29 cancer cells in a concentration-dependent manner (Fig. 2). The IC_{50} s for cell growth inhibition at 24 h was $192 \pm 6 \mu\text{M}$, $95 \pm 5 \mu\text{M}$, and $2800 \pm 210 \mu\text{M}$ for *m*-NO-aspirin, NO-naproxen, and naproxen, respectively. An IC_{50} for aspirin could not be determined because the value was $>5000 \mu\text{M}$.¹⁵ This represents an increase in potency of >26 -fold for *m*-NO-aspirin and ~ 29 -fold for NO-naproxen over their corresponding parent NSAID.

NF- κ B is an antiapoptotic transcription factor that regulates numerous genes active in the inflammatory process; its primary transcriptional-activating form is the p50-p65 heterodimer. NF- κ B requires a reducing environment to bind DNA, and this redox sensitivity is conferred by a single cysteine within the DNA binding site.¹⁹ NO can both stimulate and inhibit NF- κ B activity. Activation results from stimulation of p21ras,

indicating the NO effect to be upstream of NF- κ B,²⁰ whereas inhibition is through *S*-nitrosylation.^{19,21} In studies from my laboratory, both *m*-NO-aspirin and NO-naproxen also reduced NF- κ B p65 protein levels (Fig. 3A and 3B). For *m*-NO-aspirin this reduction was significant at 200 μ M, its IC₅₀ for cell growth inhibition.¹⁵

Caspase activation is a relatively early event in apoptosis. Using aspirin, *m*-NO-aspirin, naproxen, and NO-naproxen at their respective IC₅₀ for cell growth inhibition, we showed a time-dependent increase in caspase-3 activity (Table 1). The exception was aspirin; we used 3000 μ M for comparison to naproxen. The caspase-3 inhibitor, z-VAD-FMK, inhibited the enzyme activity at all time points, suggesting that the caspase activation by these compounds was specific.¹⁵

IIB. NO-NSAIDs *S*-Nitrosylate NF- κ B p65 In Vitro and In Vivo

We showed that the NF- κ B p65 monomer to be a target of *S*-nitrosylation by *m*-NO-aspirin and NO-naproxen in vitro, and by *m*-NO-aspirin, NONO-aspirin, and NO-naproxen in vivo. This links the nuclear accumulation of *S*-NO-NF- κ B p65 to reductions in levels of NF- κ B protein.¹⁵ Although the molecular mechanism(s) of this regulation was not delineated, our results strongly suggest that when NF- κ B p65 is *S*-nitrosylated, it accumulates and, therefore, it is no longer available for activation or DNA binding; thus, decreases observed in its protein levels are reported here.

We used the biotin switch assay in which proteins are specifically biotin-labeled on *S*-nitrosylated cysteines.²² In this protocol, nitrosothiols are selectively reduced with ascorbate to reform the thiol, which then is reacted with the thiol-modifying reagent biotin-HPDP. Thus, in the absence of ascorbate there would not be a signal for *S*-nitrosylated proteins, and in the presence of ascorbate we observed the *S*-nitrosylated proteins. In whole HT-29 colon cancer cell lysates, we observed that a group of proteins were *S*-nitrosylated after treatment with *m*-NO-aspirin, or NO-naproxen, and the NO-donor SNAP was used as a positive control (Fig. 4A).¹⁵

To determine whether NF- κ B p65 protein can be modified by *S*-nitrosylation in situ, we did a time-course experiment; we treated HT-29 cells with *m*-NO-aspirin and NO-naproxen for 0, 3, 8, and 24 hours followed by quantification of the *S*-nitrosylated NF- κ B (*S*-NO-NF- κ B p65). We observed that *m*-NO-aspirin caused a significant increase in *S*-NO-NF- κ B p65 over the 0–24 hour time period (Fig. 4B); however, with NO-naproxen, the increase in *S*-NO-NF- κ B p65 was less prominent. To show specificity of NO, we pretreated the cells for 1 hour with carboxy-PTIO, an NO scavenger, before adding the NO-NSAIDs. Figure 4C shows that the increase in *S*-NO-NF- κ B p65 protein levels was abrogated when the cells were pretreated with carboxy-PTIO and then treated with *m*-NO-aspirin or NO-naproxen, suggesting that oxidative modification of a cysteine residue was most likely affected by *S*-NO.

We also showed that structurally diverse NO-NSAIDs *S*-nitrosylated NF- κ B p65 and caspase-3 proteins in vivo. We used an acute model of ulcerogenesis of the stomach: rats starved for 48 hours were treated with aspirin, *m*-NO-aspirin, NONO-aspirin, naproxen, and NO-naproxen for 6 hours, after which their stomachs were removed and analyzed.²³ Our data showed that a group of proteins were *S*-nitrosylated by all 3 NO-releasing agents (Fig. 5A). Aspirin and naproxen did not *S*-nitrosylate any proteins beyond what was seen in the background. All 3 NO-NSAIDs *S*-nitrosylated the NF- κ B p65 protein of the stomach tissues (Fig. 5B). It is noteworthy that these agents *S*-nitrosylated caspase-3 (Fig. 5B). *S*-nitrosylation of caspases is known to inhibit apoptotic activity.²⁴ However, we showed that NO-NSAIDs increase caspase-3 enzyme activity (Table 1) and at the same time form *S*-NO-caspase-3. This apparent discrepancy may be explained in 2 ways. First, we do not know

what percentage of caspase-3 protein is *S*-nitrosylated; if the degree of *S*-nitrosylation is low, then it is unlikely that it would have a major effect. Second, it has been shown recently that SNO-caspase transnitrosylates (transfers its NO group) to another protein, X-inhibitory apoptosis protein, forming SNO-X-inhibitory apoptosis protein, promoting cell death.²⁵

Overall, we have shown that structurally diverse NO-NSAIDs can *S*-nitrosylate NF- κ B both in vitro and in vivo, leading to a reduction in NF- κ B protein levels. These results provide the first direct evidence for the role of NO released from NO-NSAIDs in apoptosis and cell growth inhibition.

IIC. JS-K Inhibits the Growth of Human Leukemia Jurkat T Cells and Strongly Inhibits β -Catenin/TCF Transcriptional Activity

Acute lymphoblastic leukemia is the most common malignant disease in children and accounts for 70% of all childhood leukemias.²⁶ The protein β -catenin is intimately involved in the Wnt signaling pathway that regulates cell-cell adhesion and may promote leukemia cell proliferation.²⁷ Elevated levels of genes targeted by β -catenin, such as *cyclin D1*, promote genetic instability, leading to tumorigenesis.²⁸ It previously had been shown that JS-K induced differentiation and apoptosis in human myeloid leukemia HL-60 cells.²⁹ However, HL-60 cells poorly express β -catenin; therefore, to better understand the role of NO on this pathway, we decided to evaluate the effects of JS-K in *acute lymphoblastic leukemia* Jurkat T cells, which express high levels of β -catenin.³⁰

JS-K strongly inhibited cell growth in a concentration and time-dependent manner (Fig. 6A). The IC₅₀s for cell growth inhibition were 14 ± 0.7 and 9 ± 1.2 μ M at 24 and 48 hours, respectively.³¹ JS-K also reduced the transcriptional activity of β -catenin in a concentration-dependent fashion (Fig. 6B); the IC₅₀ for this inhibition was 5.2 ± 0.6 μ M.³¹ This finding raised the possibility that JS-K could downregulate expression of β -catenin. To address this, cytoplasmic and nuclear proteins were isolated from cells treated for 24 hours with various concentrations of JS-K. JS-K had no appreciable effect on cytosolic β -catenin protein level or on the total cellular level; however, it reduced nuclear β -catenin protein levels in a concentration-dependent manner (Fig. 7A). This reduction was 27 ± 2.9 , 67 ± 2 , and $92 \pm 1\%$ at 20, 50, and 100 μ M, respectively.³¹ Of the several genes whose transcription is regulated by β -catenin/TCF-4, *cyclin D1* plays an important role in carcinogenesis.³² Therefore, we evaluated whether the decrease in the expression of nuclear β -catenin affected *cyclin D1* levels. Our results indicated that at 50 μ M, JS-K reduced *cyclin D1* levels significantly (Fig. 7B). Using the NO scavenger carboxy-PTIO, we also showed that the decrease in nuclear β -catenin expression was NO-dependent (Fig. 7C). As with the NO-NSAIDs, we used the biotin switch assay to assess whether JS-K causes *S*-nitrosylation of proteins. As before, in the absence of ascorbate, no visible bands were observed (Fig. 8A, lane 1); however, in the presence of ascorbate total lysates from the untreated cells showed basal levels of *S*-nitrosylation of some proteins (Fig. 8A, lane 2).³¹ Increasing concentrations of JS-K *S*-nitrosylated several cellular proteins in a concentration-dependent manner (Fig. 8A, lanes 3–5). Nuclear extracts from cells treated with JS-K showed increased total *S*-NO content of the nuclear proteins in a concentration-dependent manner, suggesting an overall increased *S*-nitrosylation in the nucleus (Fig. 8B).

IID. JS-K Reduces Nuclear Expression of β -Catenin Through *S*-Nitrosylation

Treatment of Jurkat cells for 24 hours with different concentrations of JS-K followed by detection of *S*-nitrosylated nuclear β -catenin (*S*-NO- β -catenin) protein showed a concentration-dependent degradation of nuclear β -catenin (Fig. 9A). To determine whether *S*-nitrosylation of nuclear β -catenin preceded the degradation of the protein, we performed a time-course analysis for both *S*-nitrosylation of the nuclear β -catenin and its degradation.

This analysis showed that β -catenin was *S*-nitrosylated in a time-dependent manner from 0 to 6 hours, followed by a decrease at 24 hours (Fig. 9B). A comparison of the *S*-nitrosylated nuclear β -catenin to the total nuclear β -catenin from the same extracts showed that β -catenin protein levels were unchanged from 0 to 3 hours and then degraded at 24 hours (Fig. 9C), whereas *S*-nitrosylation occurred earlier at 0 to 6 hours (Fig. 9B). On the basis of these findings, it may be concluded that *S*-nitrosylation of nuclear β -catenin precedes its degradation.³¹ Experiments performed in the presence of carboxy-PTIO reversed the effects of JS-K, confirming that the levels of nuclear *S*-NO- β -catenin were NO-dependent (Fig. 9D).

III. CONCLUSIONS AND FUTURE DIRECTIONS

A major need in chemoprevention is the development of effective and safe agents. In general, NSAIDs do not meet these criteria; hence, the development of NO- and NONO-NSAIDs. Clearly, NO-releasing agents such as JS-K and NO-releasing hybrids will have a profound effect on the direction of our efforts to harness cancer either directly or by providing a platform to develop better agents.

S-nitrosylation affects many different proteins that have a central role in cellular function or dysfunction. Therefore, understanding *S*-nitrosylation in cancer can spearhead efforts to develop new *S*-nitrosylation-related therapeutic agents that will target proteins of interest. To that end, once we have a better understanding of the pathways affected by this ubiquitous process, targeted *S*-nitrosylation and denitrosylation are certain to have a major role cancer treatment.

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ABBREVIATIONS

Carboxy-PTIO	2-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
JS-K	O ² -(2,4-Dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazene-1,2-diolate
<i>m</i>-NO-aspirin	2-(acetyloxy)-3-[(nitrooxy) methyl] phenyl ester
NF	nuclear factor
NO	nitric oxide
NO-naproxen	2-(S)-(6-methoxy-2naphthyl)-propionic acid 4-nitrooxybutyl ester
NONO-aspirin	O ² -(acetylsalicyloyloxymethyl)-1-(pyrrolidin-1-yl) diazene-1-ium-1, 2-diolate
NSAIDs	nonsteroidal anti-inflammatory drugs
SNAP	<i>S</i> -nitroso- <i>N</i> -acetyl-penicillamine
SNO	<i>S</i> -nitroso

References

1. Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ, Loscalzo J. *S*-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proc Natl Acad Sci U S A*. 1992; 89:444–448. [PubMed: 1346070]

2. Lopez-Otin C, Hunter T. The regulatory crosstalk between kinases and proteases in cancer. *Nat Rev Cancer*. 2010; 10:278–292. [PubMed: 20300104]
3. Son K, Kim YM. In vivo cisplatin-exposed macrophages increase immunostimulant-induced nitric oxide synthesis for tumor cell killing. *Cancer Res*. 1995; 55:5524–5527. [PubMed: 7585626]
4. Li J, Billiar TR, Talanian RV, Kim YM. Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. *Biochem Biophys Res Commun*. 1997; 240:419–424. [PubMed: 9388494]
5. Darnell JE Jr. Transcription factors as targets for cancer therapy. *Nat Rev Cancer*. 2002; 2:740–749. [PubMed: 12360277]
6. Thun MJ, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. *J Natl Cancer Inst*. 2002; 94:252–266. [PubMed: 11854387]
7. Bjorkman DJ. Current status of nonsteroidal anti-inflammatory drug (NSAID) use in the United States: risk factors and frequency of complications. *Am J Med*. 1999; 107:3S–8S. discussion 8S–10S. [PubMed: 10628588]
8. del Soldato P, Sorrentino R, Pinto A. NO-aspirins: a class of new anti-inflammatory and antithrombotic agents. *Trends Pharmacol Sci*. 1999; 20:319–323. [PubMed: 10431210]
9. Wallace JL, del Soldato P. The therapeutic potential of NO-NSAIDs. *Fundam Clin Pharmacol*. 2003; 17:11–20. [PubMed: 12588626]
10. Kashfi K. Anti-inflammatory agents as cancer therapeutics. *Adv Pharmacol*. 2009; 57:31–89. [PubMed: 20230759]
11. Kashfi K, Ryan Y, Qiao LL, Williams JL, Chen J, Del Soldato P, Traganos F, Rigas B. Nitric oxide-donating nonsteroidal antiinflammatory drugs inhibit the growth of various cultured human cancer cells: evidence of a tissue type-independent effect. *J Pharmacol Exp Ther*. 2002; 303:1273–1282. [PubMed: 12438552]
12. Kashfi K, Borgo S, Williams JL, Chen J, Gao J, Glekas A, Benedini F, Del Soldato P, Rigas B. Positional isomerism markedly affects the growth inhibition of colon cancer cells by nitric oxide-donating aspirin in vitro and in vivo. *J Pharmacol Exp Ther*. 2005; 312:978–988. [PubMed: 15528453]
13. Velazquez CA, Praveen Rao PN, Citro ML, Keefer LK, Knaus EE. O₂-acetoxymethyl-protected diazeniumdiolate-based NSAIDs (NONO-NSAIDs): synthesis, nitric oxide release, and biological evaluation studies. *Bioorg Med Chem*. 2007; 15:4767–4774. [PubMed: 17509888]
14. Velazquez C, Praveen Rao PN, Knaus EE. Novel nonsteroidal antiinflammatory drugs possessing a nitric oxide donor diazen-1-ium-1,2-diolate moiety: design, synthesis, biological evaluation, and nitric oxide release studies. *J Med Chem*. 2005; 48:4061–4067. [PubMed: 15943479]
15. Chattopadhyay M, Goswami S, Rodes DB, Kodala R, Velazquez CA, Boring D, Crowell JA, Kashfi K. NO-releasing NSAIDs suppress NF- κ B signaling in vitro and in vivo through S-nitrosylation. *Cancer Lett*. 2010; 298:204–211. [PubMed: 20674154]
16. Shami PJ, Saavedra JE, Wang LY, Bonifant CL, Diwan BA, Singh SV, Gu Y, Fox SD, Buzard GS, Citro ML, Waterhouse DJ, Davies KM, Ji X, Keefer LK. JS-K, a glutathione/glutathione S-transferase-activated nitric oxide donor of the diazeniumdiolate class with potent antineoplastic activity. *Mol Cancer Ther*. 2003; 2:409–417. [PubMed: 12700285]
17. Shami PJ, Saavedra JE, Bonifant CL, Chu J, Udupi V, Malaviya S, Carr BI, Kar S, Wang M, Jia L, Ji X, Keefer LK. Antitumor activity of JS-K [O₂-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl) piperazin-1-yl]diazen-1-ium-1,2-diolate] and related O₂-aryl diazeniumdiolates in vitro and in vivo. *J Med Chem*. 2006; 49:4356–4366. [PubMed: 16821795]
18. Chakrapani H, Kalathur RC, Maciag AE, Citro ML, Ji X, Keefer LK, Saavedra JE. Synthesis, mechanistic studies, and anti-proliferative activity of glutathione/glutathione S-transferase-activated nitric oxide prodrugs. *Bioorg Med Chem*. 2008; 16:9764–9771. [PubMed: 18930407]
19. Marshall HE, Stamler JS. Inhibition of NF- κ B by S-nitrosylation. *Biochemistry*. 2001; 40:1688–1693. [PubMed: 11327828]
20. Lander HM, Hajjar DP, Hempstead BL, Mirza UA, Chait BT, Campbell S, Quilliam LA. A molecular redox switch on p21(ras). Structural basis for the nitric oxide-p21(ras) interaction. *J Biol Chem*. 1997; 272:4323–4326. [PubMed: 9020151]

21. Kelleher ZT, Matsumoto A, Stamler JS, Marshall HE. NOS2 regulation of NF-kappaB by S-nitrosylation of p65. *J Biol Chem.* 2007; 282:30667–30672. [PubMed: 17720813]
22. Jaffrey SR, Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE.* 2001; 2001:pl1. [PubMed: 11752655]
23. Chattopadhyay M, Velazquez CA, Pruski A, Nia KV, Abdelatif K, Keefer LK, Kashfi K. Comparison between NO-ASA and NONO-ASA as safe anti-inflammatory, analgesic, antipyretic, anti-oxidant, prodrugs. *J Pharmacol Exp Ther.* 2010; 335:443–450. [PubMed: 20679133]
24. Maejima Y, Adachi S, Morikawa K, Ito H, Isobe M. Nitric oxide inhibits myocardial apoptosis by preventing caspase-3 activity via S-nitrosylation. *J Mol Cell Cardiol.* 2005; 38:163–174. [PubMed: 15623433]
25. Nakamura T, Wang L, Wong CC, Scott FL, Eckelman BP, Han X, Tzitzilonis C, Meng F, Gu Z, Holland EA, Clemente AT, Okamoto S, Salvesen GS, Riek R, Yates JR 3rd, Lipton SA. Transnitrosylation of XIAP regulates caspase-dependent neuronal cell death. *Mol Cell.* 2010; 39:184–195. [PubMed: 20670888]
26. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin.* 2009; 59:225–249. [PubMed: 19474385]
27. Chung EJ, Hwang SG, Nguyen P, Lee S, Kim JS, Kim JW, Henkart PA, Bottaro DP, Soon L, Bnvini P, Lee SJ, Karp JE, Oh HJ, Rubin JS, Trepel JB. Regulation of leukemic cell adhesion, proliferation, and survival by beta-catenin. *Blood.* 2002; 100:982–990. [PubMed: 12130512]
28. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A.* 1999; 96:5522–5527. [PubMed: 10318916]
29. Shami PJ, Moore JO, Gockerman JP, Hathorn JW, Misukonis MA, Weinberg JB. Nitric oxide modulation of the growth and differentiation of freshly isolated acute non-lymphocytic leukemia cells. *Leuk Res.* 1995; 19:527–533. [PubMed: 7658698]
30. Tsutsui J, Moriyama M, Arima N, Ohtsubo H, Tanaka H, Ozawa M. Expression of cadherin-catenin complexes in human leukemia cell lines. *J Biochem.* 1996; 120:1034–1039. [PubMed: 8982873]
31. Nath N, Chattopadhyay M, Pospishil L, Cieciura LZ, Goswami S, Kodala R, Saavedra JE, Keefer LK, Kashfi K. JS-K, a nitric oxide-releasing prodrug, modulates ss-catenin/TCF signaling in leukemic Jurkat cells: evidence of an S-nitrosylated mechanism. *Biochem Pharmacol.* 2010; 80:1641–1649. [PubMed: 20797387]
32. Lin SY, Xia W, Wang JC, Kwong KY, Spohn B, Wen Y, Pestell RG, Hung MC. Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc Natl Acad Sci U S A.* 2000; 97:4262–4266. [PubMed: 10759547]

FIGURE 1.

Chemical structures of *m*-NO-aspirin, NONO-aspirin, NO-naproxen, and JS-K. For *m*-NO-aspirin, NONO-aspirin, and NO-naproxen, the parent nonsteroidal anti-inflammatory drug is shown in the shaded boxes.

FIGURE 2.

Effect of *m*-NO-aspirin and NO-naproxen on HT-29 human colon cancer cell growth. Cells were treated with various concentrations of *m*-NO-aspirin (ASA) (A) and NO-naproxen (B) and their corresponding traditional nonsteroidal anti-inflammatory drugs (NSAIDs). Cell numbers were determined at 24 hours, from which IC₅₀s for growth inhibition were determined. Results are means ± SEM of these different experiments with triplicate plates.

* $P < 0.01$ compared with the respective NSAID. Adapted from Chattopadhyay et al¹⁵ with permission from Elsevier.

FIGURE 3.

Nitric oxide (NO)–nonsteroidal anti-inflammatory drugs reduce nuclear factor (NF)– κ B p65 protein levels. HT-29 cells treated with increasing concentrations of *m*-NO-aspirin (*A*) and NO-naproxen (*B*) for 24 hours were analyzed for NF– κ B p65 protein levels by immunoblots. Results are mean $\pm$ SEM of 3 independent experiments, * P < 0.05 compared with the respective untreated cells. Reproduced from Chattopadhyay et al¹⁵ with permission from Elsevier.

FIGURE 4.

Nitric oxide (NO)-nonsteroidal anti-inflammatory drugs (NSAIDs) *S*-nitrosylate nuclear factor (NF)- κ B p65 in vitro. HT-29 cells were treated with *m*-NO-aspirin (*m*-NO-ASA), NO-naproxen, or SNAP (positive control, 500 μ M) at their respective IC₅₀s for cell growth inhibition and then evaluated for *S*-nitrosylation using an anti-biotin antibody. **A:** In the absence of ascorbate, there is a loss of biotinylation, resulting in no visible bands (control cells lane 1). In the presence of ascorbate, there is endogenous *S*-nitrosylation (lane 2). *m*-NO-ASA, NO-naproxen, and SNAP *S*-nitrosylated several cellular proteins (lanes 3–5). **B:** *m*-NO-ASA (200 μ M) increases *S*-nitrosylated NF- κ B p65 in a time-dependent manner. **C:** Pretreatment with carboxy-PTIO abrogated the effects of NO-NSAIDs for *S*-NO-NF- κ B p65 expression. The bar graphs represent mean $\pm$ SEM for 3 different blots. * P < 0.05 versus controls at time 0. Reproduced from Chattopadhyay et al¹⁵ with permission from Elsevier.

FIGURE 5.

Nitric oxide (NO)–nonsteroidal anti-inflammatory drugs (NSAIDs) induce *S*-nitrosylation of nuclear factor (NF)- κ B p65 and caspase-3 in vivo. Rats were treated with vehicle, aspirin (ASA), *m*-NO-ASA, NONO-ASA, naproxen, or NO-naproxen in an acute model of stomach ulcerogenesis. *A*: In the presence of ascorbate, only the NO-NSAIDs show a strong *S*-nitrosylated signal. *B*: NO-NSAIDs cause *S*-nitrosylation of NF- κ B p65 and caspase-3 in rat stomach tissues. Results are representative of 2 different preparations. Adapted from Chattopadhyay et al¹⁵ with permission from Elsevier.

FIGURE 6.

Inhibition of Jurkat T cell growth and inhibition of transcription factor (TCF) 4 responsive reporter gene by JS-K. In Fig. 6A, cells were treated with various concentrations of JS-K for 24 and 48 hours, cell numbers were then determined and IC₅₀s for growth inhibition calculated. Results represent means $\pm$ SEM of three different experiments performed in triplicate. In Fig. 6B, Jurkat T cells, co-transfected with luciferase reporter plasmids (TOP or FOP) and the pSV- β gal were treated for 24 hours with various concentrations of JS-K. Relative TCF activity (fold) of treated cells is shown (dimethyl sulfoxide [DMSO] control was set as 1); values are mean $\pm$ SEM of 3 different experiments performed in triplicate. * P < 0.05 compared with control cells treated with DMSO. Adapted from Nath et al³¹ with permission from Elsevier.

FIGURE 7.

JS-K reduces levels of nuclear β -catenin and cyclin D1. Jurkat T cells treated for 24 hours with increasing concentrations of JS-K were analyzed for total, cytosolic, and nuclear β -catenin expression by immunoblot of lysates (*A*, left side). Densitometry evaluation of levels of nuclear β -catenin and β -actin performed from 3 such immunoblots are shown in *A*, on the right side. $*P < 0.01$ compared with untreated controls. The protein levels of cyclin D1 also were reduced by JS-K (*B*, left) and as shown for 3 immunoblot evaluations in *B*, right. $^{\dagger}P < 0.05$ compared with untreated controls. *C* shows that the reduction in nuclear β -catenin by JS-K was mediated by nitric oxide. Reproduced from Nath et al³¹ with permission from Elsevier.

FIGURE 8.

JS-K induces *S*-nitrosylation of nuclear proteins detected by the biotin switch assay (BSA). *A*: In the absence of ascorbate, the BSA leads to loss of biotinylation, resulting in no visible bands in the control samples (lane 1). In the presence of ascorbate, total lysates from the untreated cells showed basal levels of *S*-nitrosylation (*S*-NO) of some proteins (lane 2). Increasing concentrations of JS-K (lanes 3–5) *S*-nitrosylated several cellular proteins in a dose-dependent manner. *B*: Increasing concentrations of JS-K increased the total *S*-nitrosylated content of the nuclear proteins in a dose-dependent manner, at 50- μM JS-K, the percentage increase was $180 \pm 12\%$ compared with control cells (no JS-K was seen in presence of ascorbate). Results are representative of 2 different experiments. Reproduced from Nath et al³¹ with permission from Elsevier.

FIGURE 9.

JS-K degrades nuclear β -catenin via release of nitric oxide. JS-K degraded *S*-nitrosylated (*S*-NO) nuclear β -catenin in a concentration-dependent manner (*A*). The cytoplasmic levels of *S*-nitrosylated α -actin (Fig. 8*A*, lower panel) were used as an internal control for the biotin switch assay and were not altered by JS-K. Nuclear β -catenin was *S*-nitrosylated between 0 and 6 hours (*B*), whereas total nuclear β -catenin protein levels were degraded at 24 hours (*C*). Pretreatment with carboxy-PTIO reversed the effects of JS-K (*D*). Results are representative of 2 different experiments $\pm$ range. Reproduced from Nath et al³¹ with permission from Elsevier.

TABLE 1

Time Dependence of Caspase-3 Enzyme Activity in HT-29 Human Colon Cancer Cells

Time (hr)	ASA 3000 μM	<i>m</i> -NO-ASA 200 μM	Naproxen 3000 μM	NO-Naproxen 100 μM
0	5.5 $\pm$ 1.0 (5.2 $\pm$ 0.5)	3.1 $\pm$ 0.5 (3.3 $\pm$ 0.3)	5.1 $\pm$ 1.3 (5.4 $\pm$ 0.4)	4.1 $\pm$ 0.8 (4.3 $\pm$ 0.6)
3	6.2 $\pm$ 1.2 (5.5 $\pm$ 0.2)	6.8 $\pm$ 0.3* (2.1 $\pm$ 0.5)	5.8 $\pm$ 0.3 (5.3 $\pm$ 0.5)	8.0 $\pm$ 0.4* (5.2 $\pm$ 0.7)
8	11.0 $\pm$ 0.6* (4.8 $\pm$ 0.5)	15.2 $\pm$ 0.4* (3.0 $\pm$ 0.6)	9.5 $\pm$ 0.5* (4.7 $\pm$ 0.4)	19.6 $\pm$ 0.6 [†] (4.9 $\pm$ 0.5)
24	25.8 $\pm$ 0.8 [†] (4.5 $\pm$ 0.4)	24.3 $\pm$ 1.1 [†] (2.9 $\pm$ 0.4)	23.2 $\pm$ 0.3 [†] (5.0 $\pm$ 0.8)	26.8 $\pm$ 0.3 [†] (3.6 $\pm$ 0.3)

Caspase-3 enzymatic activity was determined as a function of time using drug concentrations at their respective IC₅₀ for cell growth inhibition.

Aspirin at 3000 μM was used for comparison with naproxen. Activity in parentheses is when the cells were pretreated with the caspase-3 inhibitor, z-VAD-FMK. Results are mean $\pm$ SEM for 3 different cell preparations done in triplicate.

* $P < 0.05$ and

[†] $P < 0.01$ compared with untreated control cells (0 time point). Reproduced from Chattopadhyay et al¹⁵ with permission from Elsevier.