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Induction of p53-independent apoptosis by a novel synthetic hexahydrocannabinol analog is mediated via Sp1-dependent NSAID-activated gene-1 in colon cancer cells

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ABSTRACT

Nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) has received greater attention as a novel molecular target for anti-cancer therapeutics in recent years. We identified a novel synthetic hexahydrocannabinol analog, LYR-8 [(1-((9*S*)-1-hydroxy-6,6,9-trimethyl-6a,7,8,9,10,10a-hexahydro-6*H*-benzo[c]chromen-2-yl)ethanone)], as a potent NAG-1 and apoptosis inducer in a panel of human cancer cells. LYR-8 did not possess any affinity for cannabinoid receptor CB_1 or CB_2, which eliminates the concern about potential psychoactive side effects. LYR-8 dramatically induced NAG-1 expression and apoptosis in HCT116 (wild-type p53) and HT29 (mutant p53) colon cancer cells. The NAG-1 expression by LYR-8 was not blocked by pifithrin-α, a specific p53 inhibitor, which was different from doxorubicin that induced p53-dependent NAG-1 transcriptional activity. The induction of NAG-1 promoter activity by LYR-8 was strongly correlated with increased Sp1 activation as noted in various luc-promoter activities. Furthermore, pretreatment with the specific Sp1 inhibitor mithramycin A completely reversed the LYR-8-induced NAG-1 expression in both HCT116 and HT29 cells. Knockdown of NAG-1 using siRNA significantly reversed LYR-8-induced cell death in both wild-type and mutant p53-expressing colon cancer cells. Furthermore, sensitization with NAG-1 inducer sulindac sulfide synergized LYR-8-induced cell death in both colon cancer cells. These results suggest that induction of NAG-1 via Sp1 activation is a promising therapeutic approach in cancer treatment, and that a novel compound like LYR-8 could be a potent chemotherapeutic agent for colon cancers including p53-mutated cancer.

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1. Introduction

Nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1), a member of the transforming growth factor-β (TGF-β) superfamily, plays significant roles in many cellular processes, including inflammation and cell survival [1–5]. Its anti-tumorigenic and apoptosis-inducing activities have also been demonstrated in cancer cell lines, including colorectal cancer cells, and in NAG-1-overexpressing transgenic mice showing suppressed intestinal neoplasia [3,4]. Accumulating evidence indicates that NAG-1 is induced not only by NSAIDs but also by many anti-tumorigenic and chemopreventive drugs [4,5]. For instance, NAG-1 is up-regulated in human colorectal cancer cells by peroxisome proliferator-activated receptor γ (PPARγ) ligands [5–7], phosphatidylinositol 3-kinase inhibitor [8], and dietary compounds known to have chemopreventive activity, such as resveratrol [9], indole-3-carbinol [10], conjugated linoleic acid [11] and epicatechin gallate [12].

The transcriptional regulation of NAG-1 is complex, as the promoter sequence contains many different *cis*- and *trans*-acting promoter elements. The promoter region of NAG-1 contains binding sites for Egr-1, Sp1, Sp3, p53 and COUP-TF1, indicating that NAG-1 is induced by multiple mechanisms [13,14]. Specifically, NAG-1 is reported as a downstream target of DNA damage-induced and p53-dependent apoptosis [13]. However, it is also reported that the increase in NAG-1 expression by NSAIDs is independent of p53 [3], suggesting that regulation of NAG-1 expression by p53 depends on the stimulus.

Colorectal cancer is one of the leading causes of cancer-related death worldwide. The development of colorectal carcinomas is

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caused by multiple genetic changes, including frequently inactivating mutations of the p53 gene [15]. Thus, development of chemotherapeutic/chemopreventive agents that are capable of inducing p53-independent apoptosis will have potential benefits versus other agents.

Cannabinoids, the active components of the hemp plant *Cannabis sativa*, have been used in medicine for centuries. Recently there has been renewed interest in their therapeutic potentials as they elicit a wide array of effects on the CNS as well as on peripheral tissues [16–18]. Specifically, the anti-cancer activity of cannabinoids has been reported in many different *in vitro* and *in vivo* cancer models [19,20]. Even though cannabinoid and its derivatives have been shown potential effects and promise for cancer treatment, their clinical application is severely limited because of their psychoactivity which is mediated mostly by neuronal CB_1 receptors, primarily found in the brain [21,22]. So, the most obvious alternative possibility is to target specifically peripheral CB_2 receptors. Selective CB_2 agonists that are completely devoid of psychoactivity have been developed, but they have other side effects such as immune suppression [23,24]. Recently, cannabidiol (CBD) has been identified as a nonpsychotropic cannabinoid which exhibits a very weak affinity to both CB receptors [25]. Although CBD has been highlighted for therapeutic value and believed to possess a minor impact on immune function [26], several CBD effects mediated via CB receptors are still under debate [27]. Thus, there is still a demand to develop biologically relevant cannabinoid analogs which do not have a psychoactive activity but retain anti-cancer activities.

The present study investigated the anti-cancer effects of a novel synthetic hexahydrocannabinol analog which is devoid of CB_1 or CB_2 receptor affinity, LYR-8 [(1-((9*S*)-1-hydroxy-6,6,9-trimethyl-6a,7,8,9,10,10a-hexahydro-6*H*-benzo[c]chromen-2-yl)ethanone)], in wild-type and mutant p53-harboring colorectal cancer cells. We show for the first time p53-independent NAG-1 activation by LYR-8 that accompanied apoptosis in colon cancer cells.

2. Materials and methods

2.1. Chemicals and antibodies

Dimethyl sulfoxide (DMSO), protease inhibitor cocktail, propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulindac sulfide, doxorubicin, rosiglitazone and NS-398 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All primers were synthesized and purified by Bioneer Co. (Cheongwon, Korea). The nuclear protein extraction NE-PER kit, the BCA protein assay reagents and the chemiluminescent substrate for horseradish peroxidase were from Pierce Biotechnology (Rockford, IL, USA). The primary antibody specific for NAG-1 was from Millipore Upstate (Billerica, MA, USA). Antibodies against p53, MDM2 (SMP-14), p21, Bax, Bcl-2 and actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Chemical synthesis and treatment of LYR-8

LYR-8 [$C_{18}H_{24}O_3$; 1-(1-Hydroxy-6,6,9-trimethyl-6a,7,8,9,10,10a-hexahydro-6*H*-benzo[*c*]chromen-2-yl)ethanone; molecular weight: 288.39] and other hexahydrocannabinol analogs were synthesized as described previously [28]. The chemical structures are shown in Supplementary Fig. S1.

50 mM solutions of LYR-8 and other analogs were prepared in DMSO, stored at −20 °C, and then diluted as needed. The compound was stable at the storage as well as cell incubation temperatures (data not shown). For *in vitro* incubations, LYR-8 was directly applied at a final DMSO concentration of 0.1–0.2% (v/v). No significant influence of the vehicle was observed on any of the parameters assessed.

2.3. Cell culture

HCT116 and HT29 cells (human colorectal cancer cells) were obtained from American Type Culture Collection. The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (v/v), 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were maintained at 37 °C in 5% CO_2-humidified atmosphere. The culture medium was replaced every other day. After attaining confluence, the cells were passaged by splitting at a 1:5 ratio.

2.4. RNA isolation and RT-PCR

HCT116 and HT29 cells were homogenized and total RNA was extracted with a spin column using an RNeasy kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Isolated mRNA was reverse transcribed using a Reverse Transcription Kit (Qiagen) and gene expression levels were analyzed by RT-PCR. The primer sequences for NAG-1 and GAPDH were as follows: NAG-1, sense 5′-ACTCCGAAGACTCCAGATTCCGA-3′ and antisense 5′-ATGCACATGGTCACTTGCACCT-3′; and, GAPDH, sense 5′-GGTGAAGGTCGGAGTCAACG-3′ and antisense 5′-CAAAGTTGTCATGGATGACC-3′. The PCR was performed in the presence of 0.5 U Taq DNA polymerase (TaKaRa, Japan) using the specific primers mentioned above. The PCR products were separated in 1.2% agarose gel. The bands of the PCR products were quantified by densitometry, and the levels were expressed as relative mRNA expression to GAPDH.

2.5. Western blotting

Total cell extracts were prepared using RIPA buffer, whereas nuclear protein extracts were prepared using the NE-PER nuclear extraction kit (Pierce). Protein concentrations were measured with the BCA protein assay reagent (Pierce). Equal amounts of total or nuclear extracts were separated on SDS-PAGE and immunoblotted as described previously [29]. The immunoreactive proteins were visualized using an ECL kit (Pierce) and digitally processed using LAS-4000 mini (Fuji, Japan). Membranes were stripped and reprobed with an actin antibody for loading control.

2.6. Cell viability analysis

To analyze induction of cell death after LYR-8 treatment, growing cells were treated with various concentrations of LYR-8 for 48 h in low-serum condition. Cell viability and cell proliferation were assessed using the MTT staining assay [30].

2.7. Flow cytometry analysis

Cellular DNA content and apoptosis were analyzed by flow cytometry as described previously. Briefly, after LYR-8 treatment for the indicated times, the cells were rinsed with PBS, harvested, then fixed by the slow addition of cold 70% ethanol. The fixed cells were washed twice with PBS before staining with PI (1 ml of 20 μg/ml; 1 mg/ml RNase in PBS) for 20 min. Fluorescence was measured and analyzed by a FACSCalibur Flow Cytometer using CellQuest software (Becton Dickinson, San Jose, CA, USA).

2.8. Caspase-3 activity assay

Caspase-3 activity was measured with an ApoAlert caspase colorimetric assay kit (BD Biosciences, CA, USA). Cell lysates were mixed with dithiothreitol (DTT) (10 mM)-rich reaction buffer containing 50 mM DEVD-pNA, a caspase-3 substrate, and incubated for 1 h at 37°C. Enzyme-catalyzed release of pNA was monitored using a microplate reader at 405 nm.

2.9. Plasmid constructs, transient transfection and luciferase assay

NAG-1 luciferase construct (pNAG1086/41) was a kind gift from Prof. Seung Joon Baek and was described previously [8]. NAG-1 promoters (NAG/-514 and NAG/-514-mp53) and Sp1-luc were described previously [31]. p53-luc was purchased from Panomics (Fremont, CA, USA). The reporter plasmid PPRE × 3-TKLuc was obtained from Addgene (Addgene, Cambridge, MA, USA) [32]. Transient transfections were performed using the Lipofectamine procedure according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). Briefly, cells were plated in 24-well plates at a density of 1×10^5 cells/well. After 24 h, cells were transfected with plasmid mixtures containing 0.5 μg of promoter-linked luciferase vector and 0.05 μg of pRL-TK vector for 5 h in OptiMEM media (Invitrogen) according to the manufacturer's protocols. The transfected cells were treated with LYR-8 or the indicated compounds for 24 h. The cells lysates were used for luciferase assay using a dual luciferase reporter assay kit (Promega, Madison, WI, USA), and the emitted light was measured with a luminometer (Turner BioSystems, Sunnyvale, CA, USA).

2.10. Transfection of antisense oligonucleotides

Transfection experiments with antisense oligos were performed with phosphorothioate oligodeoxynucleotides synthesized commercially (Bioneer Co., Cheongwon, Korea). The sequence of p53-antisense oligodeoxynucleotides was 5′-CGGCTCCTCCATGG-CAGT-3′ and the random control antisense oligodeoxynucleotides was 5′-CCGGTGAACGAGCGAGCACA-3′. Oligodeoxynucleotides were delivered the following day after cell seeding as 1:2.5 complexes with the GeneJammer transfection reagent (Stratagene, La Jolla, CA, USA). Based on ratios of efficacy and nonspecific toxicity, an oligonucleotide concentration of 400 nM was used. After 24 h transfection, the cells were treated with DMSO or LYR-8 (10 μM) for 48 h. The efficacy of p53-antisense oligonucleotide was confirmed by immunoblotting and the cell viability was measured by MTT assay.

2.11. Gene silencing using NAG-1 siRNA

HCT116 and HT29 cells were plated and transfected the following day with siRNAs (Dharmacon Research, Lafayette, CO, USA) using DharmaFect-1 and DharmaFect-4 (Dharmacon), respectively. On-target SMART-pool siRNA duplex NAG-1-specific siRNAs (Dharmacon L-019875-00) was used to inhibit NAG-1 expression, and a pool of Non-targeting siRNAs (Dharmacon D-001810-01) was used for control transfections. After 72 h of transfection, the efficiency of siRNA for NAG-1 silencing was assessed by immunoblotting.

2.12. Statistical analysis

The data are presented as means ± S.E.M. Student's *t*-test or one-way ANOVA followed by the Student-Newman-Keuls comparison method was used to calculate *P* values in order to estimate whether the observed differences between experimental results were statistically significant (GraphPad Prism 4.0 software, San Diego, CA, USA). The cell growth inhibition (IC_{50}) values were also determined using GraphPad Prism. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. LYR-8 activates NAG-1 expression in HCT116 and HT29 colon cancer cells

NAG-1, an anti-tumorigenic protein, has been proposed as a novel therapeutic target for colorectal cancers during recent years.

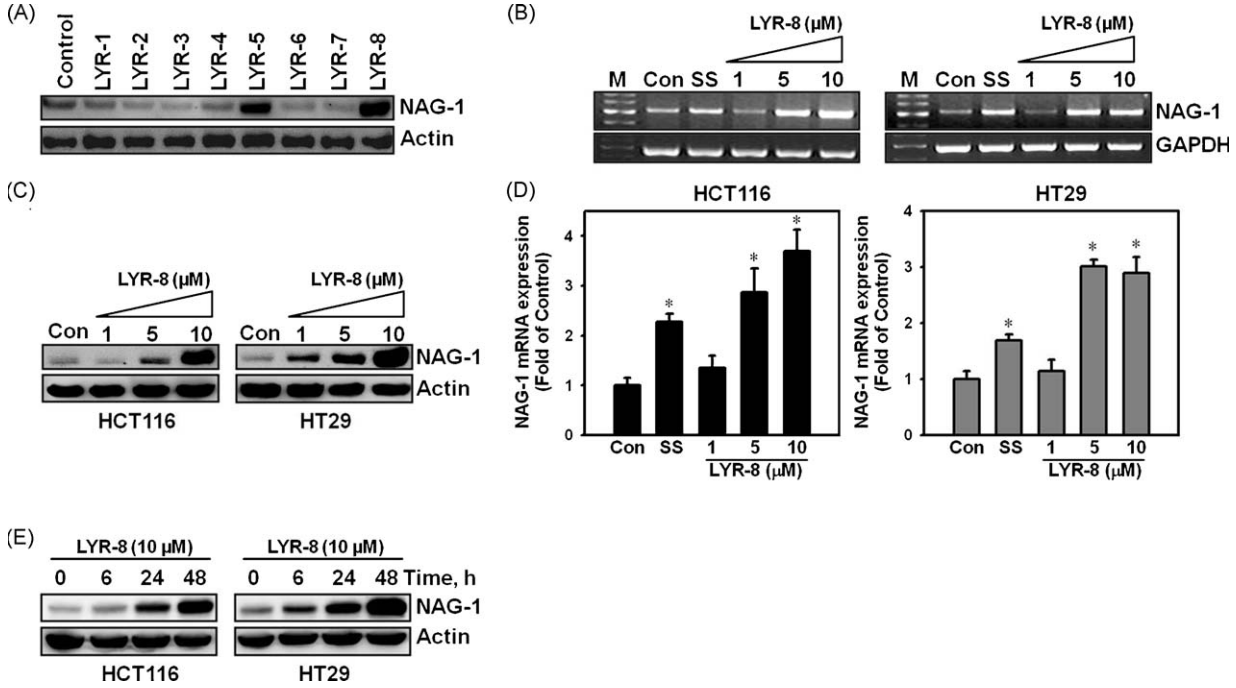

Fig. 1. LYR-8 induces NAG-1 in both HCT116 and HT29 colon cancer cells. (A) HT29 cells were treated with indicated concentrations of LYR analogs or vehicle control (0.1% DMSO), and cell lysates were isolated. Western blot analysis was performed using anti-NAG-1 antibody. Membranes were stripped and reprobed for actin for loading normalization. (B) Induction of NAG-1 mRNA expression in colon cancer cells. Cells treated with sulindac sulfide (10 μM, SS) or LYR-8 (1–10 μM) for 48 h were collected and subjected to RT-PCR analyses for NAG-1 and GAPDH. Bar graphs show relative band density normalized to GAPDH. $^*P < 0.05$ compared to vehicle-treated control group. (C and D) HCT116 and HT29 cells were treated as indicated and Western blot analysis was performed using anti-NAG-1 antibody. Membranes were stripped and reprobed for actin for loading normalization. Representative immunoblot of three independent experiments is shown.

We screened a number of biologically interesting synthetic hexahydrocannabinol analogs for their NAG-1-inducing ability (Supplementary Fig. S1). Among the screened synthetic hexahydrocannabinol analogs, LYR-8 was the most effective in NAG-1 activation in HT29 colon cancer cells (Fig. 1A). As shown in Fig. 1B, the effect of LYR-8 on NAG-1 mRNA induction was concentration-dependent and stronger than that of sulindac sulfide, an NSAID known to induce NAG-1 [33]. The effect of LYR-8-mediated NAG-1 activation was further confirmed by Western blot analysis, which showed that LYR-8 strongly activated NAG-1 expression in a concentration- (Fig. 1C) and time-dependent manner (Fig. 1D). The NAG-1-inducing activity of LYR-8 was similar in both wild-type p53 (HCT116)- and mutant p53 (HT29)-expressing colon cancer cells.

3.2. Cancer cell growth inhibition and apoptosis induction by LYR-8 treatment

NAG-1 is normally suppressed in colon cancer cells and its activation by chemotherapeutic compounds clearly implies its pro-apoptotic and anti-tumorigenic activities [12]. We investigated whether NAG-1 activation by LYR-8 correlated with growth inhibition and apoptosis of cancer cells using several assays: MTT, trypan blue dye exclusion, morphology change, flow cytometry analysis (PI staining) and caspase-3 activity assay. Treatment of colon cancer cells, HCT116 and HT29, with LYR-8 (0–100 μM) for a 48 h period substantially reduced their survival in a concentration-dependent manner, with 13 and 17 μM IC_{50} respectively (Fig. 2A). Our *in vitro* cytotoxicity studies with colon cancer cell lines (HCT116 and HT29) versus non-cancer TCMK-1 (mouse, C2H/Mai, Kidney) cell line indicated that LYR-8 has comparatively little toxicity to normal cells. Based on MTT data, LYR-8 at 10 μM showed little or no cytotoxicity whereas high concentrations of LYR-8 inhibited TCMK-1 cell growth in a concentration-dependent manner, with an IC50 of 30 μM (data not shown). The decreased survival was due to cell death, as determined by the trypan blue assay (Supplementary Fig. S2A). We then examined whether LYR-8-induced cell death was due to apoptosis. Flow cytometric analysis of PI-stained cells showed that LYR-8 (10 μM) time-dependently promoted apoptosis in both HCT116 and HT29 cells (Fig. 2B). Also, membrane blebbing and a decreased number of attached cells on the culture plate were observed (Supplementary Fig. S2B, upper panel). Additionally, nuclear morphology, as assessed by DAPI staining, revealed extensive nuclear condensation and fragmentation (Supplementary Fig. S2B, lower panel). Furthermore, LYR-8-induced caspase-3 activity, a specific marker of apoptosis, in a time-dependent manner in both HCT116 cells and HT29 cells (Fig. 2C). The overall responses of both cell lines to LYR-8 were comparable in terms of growth inhibition and apoptosis induction.

Although cannabinoid action is mostly mediated through CB receptors, we found that the growth-inhibiting activity of LYR-8 was not mediated through CB receptors. Pretreatment with the respective CB_1 and CB_2 antagonists, AM281 and AM630, failed to block the growth inhibitory effect of LYR-8 in HT29 cells (Fig. 2D). In competitive receptor binding assays, LYR-8 did not exhibit any affinity for either of CB receptors (Supplementary Fig. S3).

3.3. p53 is partially involved in LYR-8-induced cell death of HCT116 cells with wild-type p53 but not required for NAG-1 activation

We further explored the signaling molecules involved in LYR-8-induced growth inhibition and apoptosis. As HCT116 and HT29

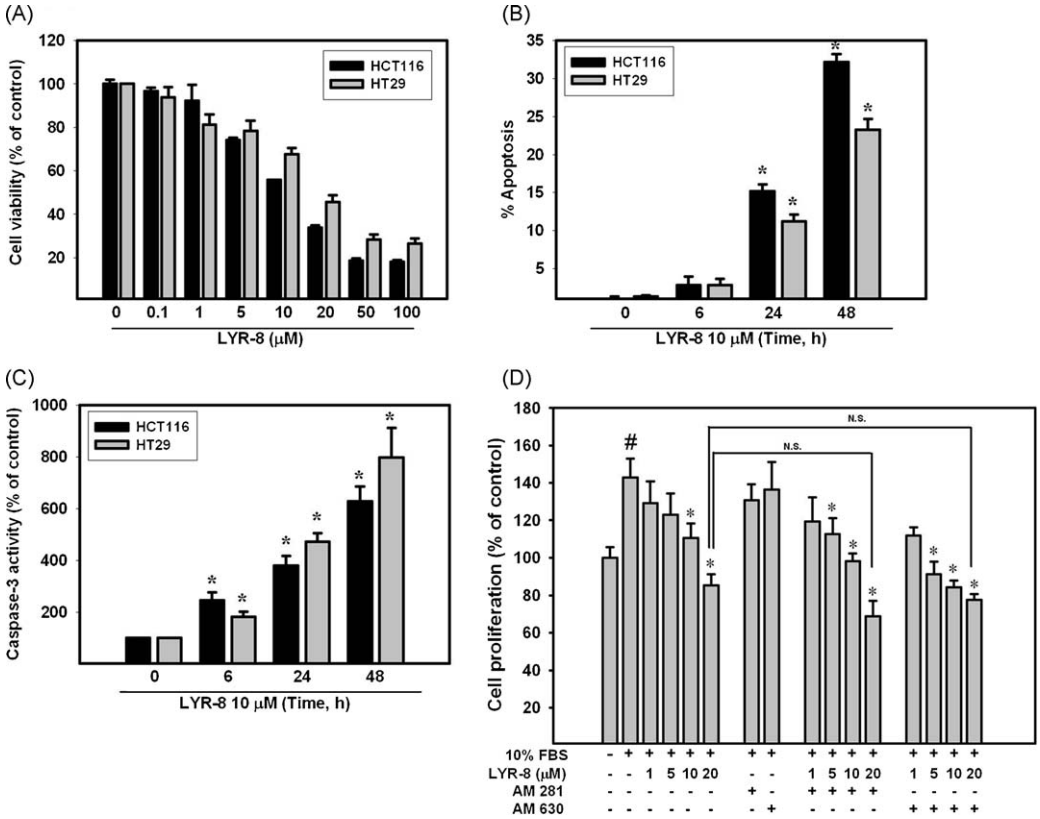

Fig. 2. Apoptosis-inducing effect of LYR-8 on colon cancer cells. (A) HCT116 and HT29 cells treated for 48 h with or without the indicated concentration of LYR-8 were analyzed for viability by MTT assay. (B) Cells treated as above were stained with PI, and apoptosis was measured by fluorescence-activated cell sorting (FACS) analysis. *$P < 0.05$ versus control (0 h). (C) Caspase-3 activity was determined using a caspase-3 assay kit as described in Materials and Methods. Bar graphs show the means ± S.E.M. of three different experiments. *$P < 0.05$ versus control (0 h). (D) HT29 cells were pretreated with 5 μM of specific CB_1 and CB_2 receptor antagonists, AM281 and AM630 respectively, 1 h before LYR-8 treatment. After 48 h, cell proliferation was measured by MTT assay. Bar graphs show the means ± S.E.M. of nine measurements. *$P < 0.05$ versus FBS-induced group.

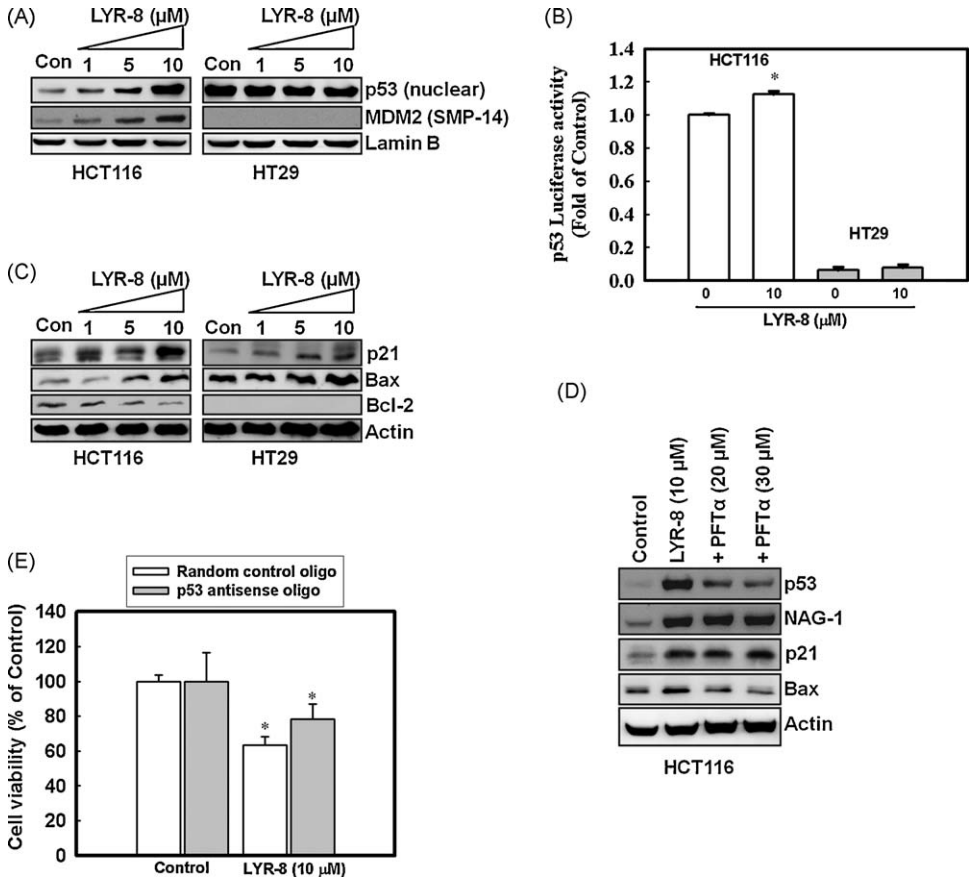

Fig. 3. LYR-8 activates expression of apoptotic molecules in a p53-independent manner. (A) Colon cancer cells treated with the indicated concentrations of LYR-8 for 48 h were collected and subjected to Western blot analyses for p53, MDM-2, or Lamin B. Representative immunoblot from three independent experiments are shown. (B) Wild-type (HCT116) and mutant (HT29) p53-expressing colon cancer cells were transfected with p53-luc along with pRL-TK as transfection control. Cells were then grown in the presence or absence of LYR-8 for 24 h. Luciferase activity was measured and expressed as relative luciferase units (RLU) (firefly luciferase/*Renilla* luciferase). Bar graphs show the means ± S.E.M. of three different experiments. $^{*}P < 0.05$ versus vehicle-treated control (HCT116). (C) Western blot analysis of p21, Bax, Bcl-2, or actin. Data are representative of three independent experiments. (D) HCT116 cells were pretreated with the indicated concentrations of pifithrin-α for 12 h and then cultured for another 48 h in the presence or absence of LYR-8. Western blot analyses were performed for p53, NAG-1, p21, or Bax. (E) HCT116 cells expressing wild-type p53 were either transfected with 400 nM p53-antisense or non-target oligonucleotide using GeneJammer transfection reagent. After 24 h, transfected cells were added with fresh medium (1% FBS) with or without LYR-8 and further cultured for 48 h. Cell viability was determined by MTT assay. $^{*}P < 0.05$ versus vehicle-treated control.

cells differ in p53 status, the basal p53 promoter activity was much lower in HT29 cells than in HCT116 cells, which were similar to p53-negative SKOV3 ovarian cancer cells (Supplementary Fig. S4). Western blot analysis of nuclear fraction showed that LYR-8-induced upregulation of p53 and MDM2 proteins in a concentration-dependent manner in HCT116 cells (Fig. 3A). However, in HT29 cells with mutated p53, the basal level of p53 was very high and was not altered by LYR-8 treatment. Similarly, p53 promoter activity remained very low after LYR-8 stimulation (Fig. 3B). MDM2, found in negligible amounts, also remained undetectable after LYR-8 treatment (Fig. 3A). Despite the robust upregulation of p53 and MDM2 proteins, only minimal induction of p53 promoter activity could be detected in the luciferase assay after LYR-8 treatment in HCT116 cells (Fig. 3B). Based on the above differences, we questioned whether induction of apoptosis-related proteins by LYR-8 in the two cell lines was different. Interestingly, LYR-8 significantly induced p21 and Bax expression regardless of p53 status. On the other hand, LYR-8 caused downregulation of the anti-apoptotic protein Bcl-2 in HCT116 cells, whereas no Bcl-2 protein was detected by western blot analysis of the total lysate of HT29 cells (Fig. 3C).

To further delineate the role of p53 in LYR-8-stimulated NAG-1 induction in HCT116 cells, we employed a specific p53 inhibitor, pifithrin-α. As seen in Fig. 3D, although significant attenuation of p53 expression was observed in the pifithrin-α-pretreated group, no significant effect on LYR-8-induced NAG-1 expression was observed. p21 expression remained high after pifithrin-α pretreatment, whereas LYR-8-induced Bax expression was blocked by pifithrin-α pretreatment (Fig. 3D). Since these observations indicate that LYR-8-induced p53 activation is responsible for Bax expression, we further examined the role of increased p53 in LYR-8-induced apoptosis of HCT116 cells. Although partial attenuation of LYR-8-induced cell death was observed in p53-antisense-oligonucleotide transfected groups, substantial cell death was sustained (Fig. 3E).

3.4. LYR-8 activates NAG-1 promoter activity

Several *cis*- and *trans*-acting elements are located in the NAG-1 promoter region. Involvement of various transcription factors including p53, ATF3, EGR-1 and Sp1 is suggested in NAG-1 upregulation by anti-cancer drugs. In addition, putative binding sites for PPRE (PPAR response element) and ARE (anti-oxidant response element) are found in the NAG-1 promoter region [34]. Therefore, we assessed NAG-1 promoter activity using various luciferase constructs to elucidate the molecular mechanism by which LYR-8 mediates NAG-1 activation at the transcriptional level. First, we employed pNAG1086/41, a plasmid construct containing four putative ATF3 binding sites [10]. Treatment with sulindac sulfide, but not LYR-8, transactivated the NAG-1 promoter

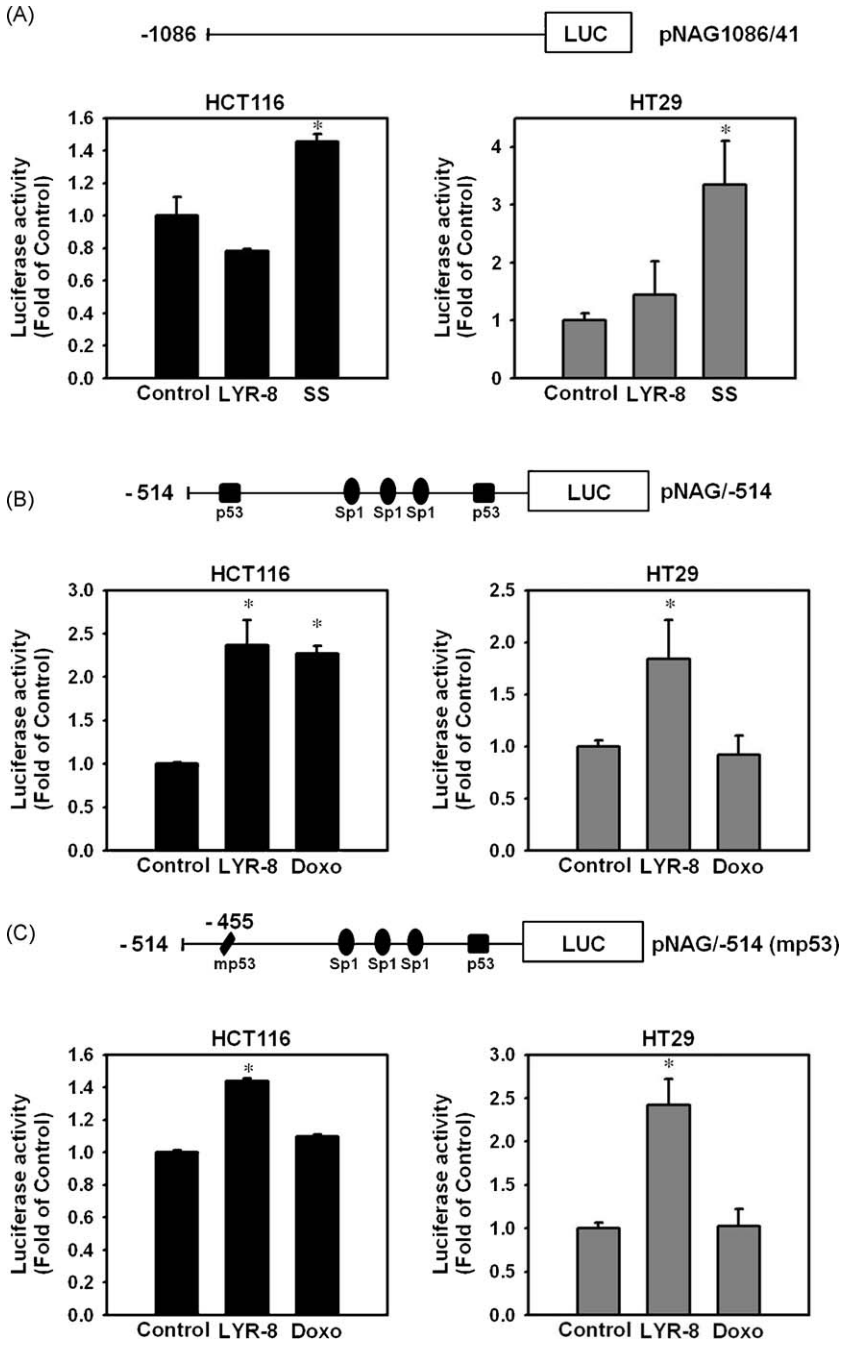

Fig. 4. LYR-8 activates NAG-1 promoter activity irrespective of p53 status in cancer cells. (A–C) HCT116 and HT29 cells were co-transfected with NAG-1 promoters, pNAG1086/41 (A), pNAG/-514 (B), or pNAG/-514-mp53 (C) and pRL-TK using Lipofectamine transfection reagent followed by treatment with 10 μM LYR-8 for 24 h. sulindac sulfide (10 μM, SS) and doxorubicin (0.5 μM, Doxo) were used as positive controls for comparison. Data are expressed as relative luciferase units (RLU) (*firefly* luciferase/*Renilla* luciferase) and bar graphs show the means ± S.E.M. of three experiments. $^{*}P < 0.05$ versus vehicle-treated control.

compared to the vehicle-treated control (Fig. 4A). Since ATF3 was not a target of LYR-8 treatment, we next transfected the cells with pNAG/-514 construct containing binding sites for Sp1 and p53, and treated with LYR-8 or doxorubicin, which is a well known DNA-damaging agent that induced p53-dependent transcriptional activity. LYR-8 significantly increased pNAG/-514 promoter activity in both cell lines, while doxorubicin induced pNAG/-514 promoter activity only in HCT116 cells expressing wild-type p53 (Fig. 4B). Since the region spanning −514 to +90 contains three Sp1 and two p53 binding sites, we performed the same experiment with modified pNAG/-514 plasmid containing a mutated p53 site [31] to further clarify the p53-independent NAG-1 activation. As shown in Fig. 4C, LYR-8 still significantly increased the NAG-1 activation, whereas doxorubicin did not induce NAG-1 activation after one of the p53 binding sites in the promoter region of pNAG/-514 was mutated.

3.5. Sp1 binding site is important for NAG-1 activation in colon cancer cells

Because LYR-8 increased NAG-1 promoter activities in a p53-independent manner in constructs containing mutated p53 and Sp1 binding sites, we conducted additional experiments using the Sp1 reporter vector [31]. As shown in Fig. 5A, LYR-8 increased luciferase activity in both cell lines transfected with this Sp1 reporter. LYR-8 also induced PPARγ activation in HCT116 cells but

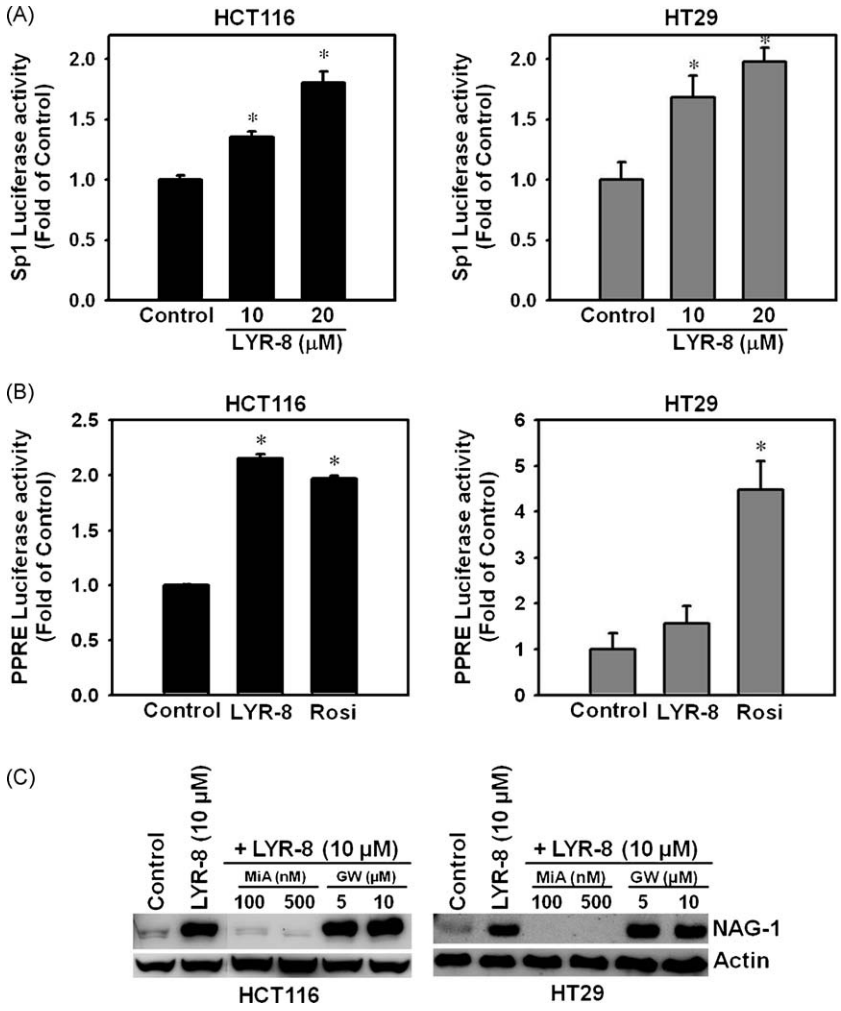

Fig. 5. Sp1 activation but not PPRE is important for LYR-8-induced NAG-1 expression. Sp1-luc (A) or PPRE-luc (B) along with pRL-TK was introduced into both HCT116 (left) and HT29 (right) cells. Cells were then grown in the presence or absence of LYR-8 for 24 h. rosiglitazone (10 μM, Rosi) was used as a positive control for PPRE induction. Luciferase activity was measured and expressed as relative luciferase units (RLU) (*firefly* luciferase/*Renilla* luciferase). Bar graphs show the means ± S.E.M. of three experiments. $^{*}P < 0.05$ versus vehicle-treated control. (C) HCT116 and HT29 cells were pretreated with indicated concentrations of mithramycin A or GW9662 for 1 h and then treated with LYR-8 for 48 h. Western blot analyses were performed for NAG-1 and actin. Representative immunoblots of three independent experiments are shown.

not in HT29 cells (Fig. 5B). Next, we used the Sp1 inhibitor mithramycin A and the PPARγ antagonist GW9662 to assess the regulatory role of these transcription factors in LYR-8-induced NAG-1 expression. Western blot analysis showed that pretreatment with mithramycin A completely abrogated NAG-1 expression in both cell lines, whereas GW9662 pretreatment failed to produce any changes (Fig. 5C).

3.6. LYR-8-mediated NAG-1 activation, at least in part, leads colon cancer cell death

To clearly understand the role of NAG-1 in LYR-8-induced cell death and apoptosis, we employed target siRNA against NAG-1. LYR-8-induced robust activation of NAG-1 in both cell lines; this LYR-8-induced activation was not altered by non-target siRNA but was completely suppressed by NAG-1 siRNA (Fig. 6A). Knockdown of NAG-1 did not alter the p53 but markedly decreased p21 induction by LYR-8, showing a possible link between NAG-1 activation, cell death and p21. Furthermore, NAG-1-specific knockdown significantly attenuated cell death induced by LYR-8 in both cell lines (Fig. 6B). However, the recovery of cell death was partial in all tested concentrations (0–50 μM) (data not shown). Conversely, the importance of NAG-1 in LYR-8-induced cell death was confirmed by the result that sensitization with NAG-1 inducer sulindac sulfide synergized LYR-8-induced cell death in both colon cancer cells (Fig. 6C).

4. Discussion

In the present study, we identified LYR-8, a hexahydrocannabinol analog, as a novel and potent NAG-1 inducer. LYR-8-induced NAG-1 expression in colon cancer cells occurred irrespective of p53 status and was Sp-1-dependent, which led to apoptosis in both wild-type and mutant p53-expressing colon cancer cells. These results are in agreement with those obtained in comparative studies showing that p53-independent NAG-1 activation may be useful as a potential therapeutic target for the apoptosis of colon and other types of cancer cells [10,35,36].

LYR-8 and other analogs are structurally related to the cannabinoids (Supplementary Fig. S1), which exhibited significant growth inhibitory activity in a panel of human cancer cell lines in our preliminary studies. It is widely reported that most of the effects of cannabinoids are mediated by the activation of specific receptors, CB_1 or CB_2 [37]. Despite the promise of the cannabinoid system as a target for cancer treatment, the clinical use of cannabinoid-related compounds have largely been limited due to undesired CB_1-mediated psychoactive effects. In contrast to conventional cannabinoids, LYR-8 did not have binding affinity

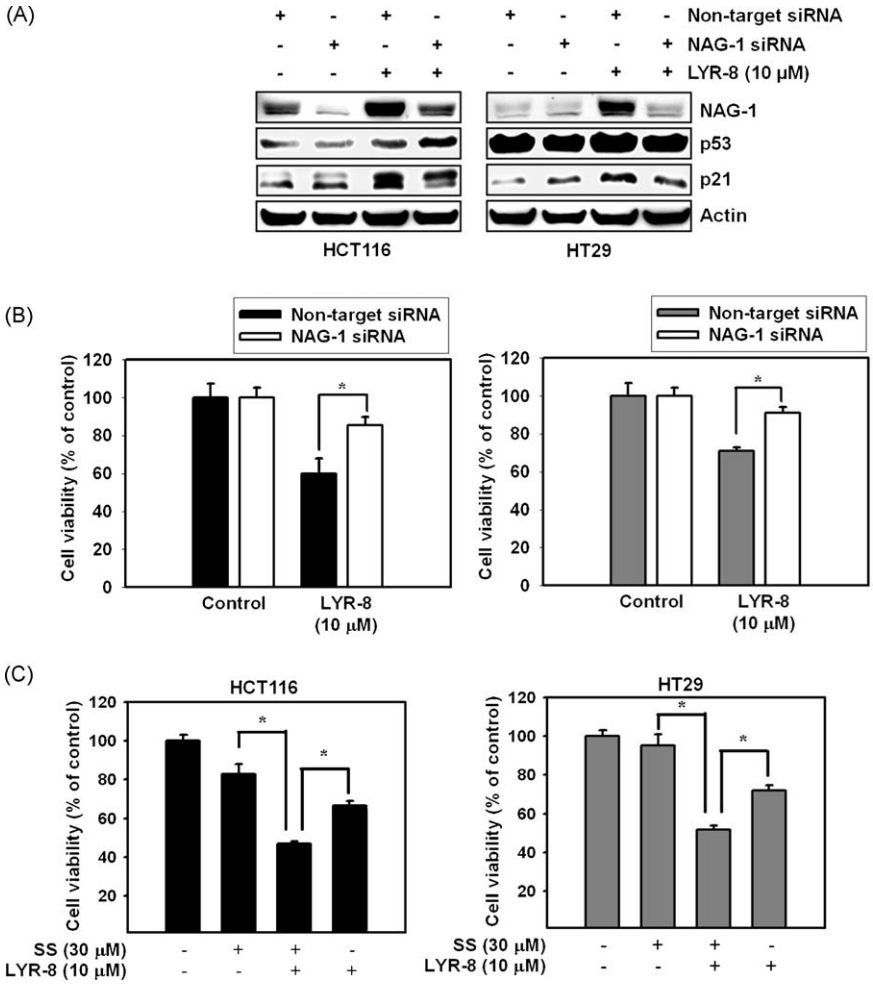

Fig. 6. Effect of NAG-1 modulation on LYR-8-induced cell death in HCT116 and HT29 cells. (A) The cells were transfected with NAG-1 siRNA (100 nM) using Dharmafect transfection reagent. After 48 h, Western blot analyses for NAG-1, p53 and p21 were performed in HCT 116 (left) and HT29 (right) cells. (B) NAG-1 knockdown cells, HCT 116 (left) and HT29 (right), were treated with 10 μM LYR-8 for 48 h and their viability was measured by MTT assay. The data represent the means ± S.E.M. of nine measurements. $^{*}P < 0.05$ versus non-target siRNA-treated group. (C) HCT116 (left) and HT29 (right) cells were treated with LYR-8 or sulindac sulfide (SS) or both for 48 h. Cell viability was measured by MTT assay. Bar graphs show the means ± S.E.M. of nine measurements. $^{*}P < 0.05$ versus LYR-8 or SS alone treatments.

at either CB receptor (Supplementary Fig. S3). In addition, LYR-8-induced inhibition of cell proliferation in HT29 cells was not blocked by the CB_1 and CB_2 antagonists, AM281 and AM630, confirming that LYR-8 action was not mediated through CB receptors (Fig. 2D). In agreement with previous studies [38,39], determination of the structure-activity relationship of synthetic LYR analogs showed that the alkyl group on the C-3 aromatic position seems to be important for receptor affinity. The presence of a short carbon side chain on C-3 (methyl group in LYR-7; Δ^9-THC has a pentyl group) resulted in very weak CB receptor affinity (data not shown), while complete elimination (no carbon side chain on C-3 in LYR-8) failed to bind with either CB receptor (Supplementary Fig. S3). Since LYR-8 does not have CB_1 binding affinity and CB_1 receptor-activation is largely responsible for the psychoactive effects of cannabinoids, in agreement with Blazquez et al. [40], we propose that improved non-psychoactive cannabinoid analogs such as LYR-8 could be potential anti-cancer agents.

In the present study, we found that LYR-8 concentration-dependently increased the expression of p53 along with increased p21 and the Bax/Bcl-2 ratio in cells with wild-type p53 status. Interestingly, we found a similar degree of apoptosis and increased p21 and Bax expression by LYR in the mutant p53-expressing HT29 cells. Thus, colon cancer cells of different p53 status showed similar responses to LYR-8 treatment, including NAG-1 activation. Although pifithrin-α blocked LYR-8-induced Bax expression, it had little or no effect on cell survival inhibition and NAG-1 over-expression. The current study thus suggests that LYR-8-mediated NAG-1 and p21 expression, as well as suppression of colon cancer cell survival, is predominantly p53-independent. Although both NAG-1 and p21 were initially identified as p53-dependent genes, our results are consistent with many other findings suggesting that a variety of transcription factors can bind to the promoters and activate these genes in a p53-independent manner [10,40–42]. In many cases, the p53 status of colorectal cancer largely influences the effectiveness of anti-cancer chemotherapy, since p53 gene alteration in cancer cells plays an important role in drug resistance, which leads to unpredictable outcome and low efficacy [43,44]. Using isogenic variants of colorectal cancer cells with different p53 status, Aneja and colleagues [45] explained the role of p53 in the sensitivity of colon cancer cells to noscapine-induced apoptosis. Lack of p53-dependent apoptosis is a common phenomenon in various cancers harboring non-functional p53. In such cases, a novel anti-cancer agent that is capable of inducing p53-independent NAG-1 activation and apoptosis, such as LYR-8, may be of great advantage.

Diverse natural and synthetic compounds with a wide range of chemical structures induce the expression of NAG-1, suggesting that multiple mechanisms may be responsible for its enhanced expression. Our results from luciferase assays indicated that the Sp1 site is an important regulator for NAG-1 induction by LYR-8.

This result was consistent with previous findings that Sp1 binding site at the transcriptional level is required for p53-independent NAG-1 activation [36]. To test whether Sp1 activation is indeed responsible for LYR-8-induced NAG-1 overexpression in colon cancer cells, endogenous Sp1 was inhibited and the resulting effects on endogenous NAG-1 levels were analyzed. Sp1 activity was inhibited by treating cells with mithramycin A, an aureolic antiobiotic that has been shown to selectively inhibit Sp1 activation through inhibition of GC-rich DNA-binding proteins. Mithramycin A completely abrogated LYR-8-induced NAG-1 protein expression, which means that Sp1 activation is required for NAG-1 expression. Notably, it has been well documented that either Sp1 or Egr-1 can bind to a Sp1 binding site in the NAG-1 promoter region [31]. Given that both Sp1 and Egr-1 nuclear activator proteins are involved in NAG-1 activation and apoptosis of colon cancer cells [8,26], LYR-8 and other hexahydrocannabinols could be useful for the prevention and treatment of colon cancers.

Several studies using *in vitro* and *in vivo* models of colon cancer have demonstrated that NAG-1 is a potent tumor suppressor gene [46]. For instance, anti-tumorigenic compounds, such as resveratrol and several NSAIDs [9], diallyl disulfide [47], genistein [48], and troglitazone [5], induce NAG-1 expression in colorectal cancer cells. Furthermore, Baek et al. [4] reported that transgenic mice (NAG-$1^{Tg+/BL6}$) expressing human NAG-1 are resistant to chemically and genetically induced polyp formation. They found an approximately 50% reduction in intestinal polyps after azoxymethane treatment of NAG-$1^{Tg+/BL6}$ mice and 40% inhibition of polyp formation in the intestine by crossing NAG-$1^{Tg+/BL6}$ mice with Apc^{Min+} mice. Similarly, celecoxib treatment in mice inhibited tumor growth and induced NAG-1 expression [46]. Consistent with these reports, we found NAG-1 was a common target for both wild-type and mutant p53-expressing colon cancer cells. When NAG-1 levels were reduced by transfecting siRNAs specific to NAG-1 and the cells were treated with LYR-8, colon cancer cells showed marked increases in cell viability compared to those cells transfected with non-target siRNA. The forced inhibition of NAG-1 also inhibited p21 levels in both cell lines. Furthermore, a synergistic inhibition of cell viability by NAG-1 inducer sulindac sulfide and LYR-8 indicated the role of NAG-1 in colon cancer cells. Overall, our results suggest that NAG-1, at least in part, is an important downstream target in LYR-8-induced growth inhibition of colon cancer cells irrespective of p53 mutation.

In summary, we demonstrated for the first time that hexahydrocannabinols activate NAG-1 and induce apoptosis independent of p53 but via Sp1 activation. As p53 is often mutated in colon cancer cells and a lack of functional p53 is associated with an increased risk of cancer development, our findings suggest that LYR-8-like agents may have great pharmacological value by preferentially inducing the death of cancer cells regardless of p53 status.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.03.008.

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