Chitosan-based nanoparticles with damnacanthal suppress CRM1 expression

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Abstract. Cancer is one of the leading causes of mortality worldwide. Phytochemicals may be promising anticancer agents given their various chemical structures and diverse biological activities. Damnacanthal (DAM) is a major bioactive component of Noni, which has been investigated previously as a cancer-preventive or chemotherapeutic agent. DAM has also been reported to exhibit anti-proliferative activity in several cancer types. In the present study, it was identified that DAM downregulates chromosome maintenance protein 1 (CRM1) expression in human cancer cells. The application of chitosan-based nanoparticles (NPs) with DAM also induced CRM1 downregulation, which suggests that chitosan-based NPs may be effective vehicles for delivery of phytochemicals such as DAM. It was also identified that DAM increased the levels of the tumor suppressor non-steroidal anti-inflammatory drugs-activated gene 1 in the nucleus, thereby leading to enhanced anticancer effects. The results of the present study indicate that DAM and its nanoformulation may be a candidate anticancer drug.

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Introduction

Cancer is one of the most common causes of mortality worldwide. Although various therapies for cancer have been developed, further research is required to decrease the mortality caused by cancer.

Phytochemicals are present in dietary plant-based products, and several phytochemicals have been identified to inhibit tumorigenesis in experimental animals and/or *in vitro* assays (1). Therefore, chemoprevention using phytochemicals may be a way to decrease cancer-associated mortality. The phytochemical damnacanthal (3-hydroxy-1-methoxyanthraquinone-2-aldehyde; DAM) is an anthraquinone compound, primarily present in plants of the Rubiaceae family. It has been reported to have anticancer and cancer-preventive activities, acting via various molecular targets, including induction of non-steroidal anti-inflammatory drugs-activated gene 1 (NAG-1) expression (2), cyclin D1 downregulation (3), activation of p38 mitogen-activated protein kinase signaling pathway (4) and inhibition of tyrosine kinase (5-7).

Chitosan, a cationic natural polysaccharide present in the exoskeleton of crustaceans, has been widely used as a drug delivery system. The advantages of using chitosan in nanoparticles (NPs) are its biocompatibility, biodegradability, non-toxicity, non-immunogenicity and abundance of functional groups. Encapsulation of DAM improved the mode of action of the NPs and decreased their toxicity, indicating that there may be multiple potential applications of phytochemical delivery by chitosan (8).

One of the key proteins that regulates tumor suppressors in cancer cells is the chromosome maintenance protein 1 (CRM1, also known as exportin 1), which serves a pivotal function in tumorigenesis (9) and may be a target for anticancer drugs. CRM1 is a nuclear export receptor involved in the export of large macromolecules including RNA and protein from the nuclear membrane to the cytoplasm. Excessive nuclear export may be one of the factors contributing to resistance to

chemotherapy and cancer development (10). It has been identified that CRM1 is highly expressed in cancer, and a number of nuclear tumor suppressor proteins, including p53, p21 and NAG-1 (11,12), are translocated to the cytoplasm and are degraded. Overexpression of CRM1 has been associated with poor prognosis in patients with several types of cancer (13). Thus, CRM1 may be considered a promising therapeutic target for anticancer drug development.

The aims of the present study were to evaluate the effect of DAM and its nanoformulation on CRM1 expression and to elucidate the underlying molecular mechanisms of DAM-mediated anticancer activity.

Materials and methods

Materials. DAM was isolated from the roots of *Morinda citrifolia* and was purified as described previously (14). Chitosan with 90% deacetylation (M_v, 150,000) was purchased from Seafresh Chitosan (Lab) Co. Ltd. (Chumphon, Thailand). Cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). HCT-116 and U2OS cells were cultured in McCoy's 5A medium (Welgene Inc., Gyeongsan, Korea) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan UT, USA), 100 μg/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37°C under a humidified atmosphere containing 5% CO_2. Antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), except for the anti-NAG-1 antibody, which was obtained as previously described (15).

Preparation of DAM-NPs. DAM-NPs were prepared using deoxycholic and poly(ethylene glycol) methyl ether-grafted chitosan as a drug carrier. DAM was incorporated into the NPs using the dialysis method described previously (8). Briefly, the polymer was dissolved in water and mixed with DAM solution in dimethyl sulfoxide (DMSO). The mixture was ultrasonicated and dialyzed in 0.9% NaCl at 4°C. DAM-NPs were freeze-dried for further use.

Cell proliferation assay. The effect of DAM and DAM-NPs on the proliferation of HCT-116 and U2OS cells was investigated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA). The cells were seeded at a concentration of 3,000 cells/well in 96-well tissue culture plates. The cells were then treated with 1 or 10 μM DAM or DMSO as a control, and DAM-NPs (equivalent concentration of 50 μM DAM) or DAM-free NPs as controls. At 0, 1 and 3 days after treatment (for DAM-NPs and NPs) or 0, 1 and 4 days after treatment (for DAM and DMSO), 20 μl CellTiter 96 Aqueous One Solution was added to each well. The plate was then incubated at 37°C for 1 h. The absorbance at 490 nm was determined using an iMARK™ microplate absorbance reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Apoptosis assay. Apoptotic cells were detected using the Annexin V-Fluorescein Isothiocyanate Apoptosis Detection kit (BioVision, Inc., Milpitas, CA, USA). Briefly, cells were plated in 6-well culture dishes and treated with vehicle,

DAM, NPs and DAM-NPs followed by incubation at 37°C for 24 h. Samples were prepared according to the manufacturer's protocol. Apoptosis was detected using a Cell Lab™ Quanta SC flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) with excitation and emission settings of 488 and 530 nm, respectively. The images were captured with a Quanta SC flow cytometer (Beckman Coulter, Inc.) and processed with Flowing Software 2.5.1 (University of Turku, Turku, Finland).

Reverse transcription-semi-quantitative polymerase chain reaction (RT-PCR). HCT-116 and U2OS cells were grown to between 80 and 90% confluence in a 6-cm plate followed by treatment with 50 μM DAM or DAM-NPs (equivalent concentration of 50 μM DAM), and DMSO or NPs as vehicle controls. After 24 h, total RNA was extracted using TRIzol® LS reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then reverse-transcribed into cDNA using a Verso cDNA synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. PCR was performed for 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min with specific primers for human (h)CRM1 and hGAPDH (hCRM1 forward, 5'-AATGTGAGAGCCTGCAAAGC-3'; hCRM1 reverse, 5'-CGGCTCACCCAACCAGATAT-3'; hGAPDH forward, 5'-GACCACAGTCCATGCCATCACT-3'; hGAPDH reverse, 5'-TCCACCCTGTTGCTGTAG-3'). Each PCR product was electrophoresed on a 1.4% agarose gel with NEOgreen (NeoScience, Co., Ltd., Suwon, Korea) staining. Each value was normalized to the expression of hGAPDH.

Western blot analysis. HCT-116 and U2OS cells were grown to between 80 and 90% confluence in a 6-cm plate and treated with 50 μM DAM or DAM-NPs (equivalent concentration of 50 μM DAM), and DMSO or NPs as vehicle controls. Total cell lysates were isolated using radioimmunoprecipitation assay cell lysis buffer (1X) with EDTA supplemented with protease inhibitor (0.5 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitor (1 mM Na_3VO_4). Nuclear and cytoplasmic extracts were purified by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.) using bovine serum albumin (Pierce; Thermo Fisher Scientific, Inc.) as the standard. 10% SDS-PAGE was used to separate 60 or 30 μg of proteins from the cell lysates. These proteins were then transferred onto a nitrocellulose membrane. The blot was blocked with 5% skimmed milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T), applied overnight. The membranes were incubated at room temperature with primary antibodies against NAG-1, CRM1 (sc-74454), β-actin (sc-47778), α-tubulin (sc-398103) and lamin A/C (sc-376248), diluted in 5% skimmed milk in TBS-T solution (1:1,000) for 1 h. Following washing with TBS-T four times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Thermo Fisher Scientific, Inc.; cat. no. 31460) or HRP-conjugated goat anti-mouse (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. 62-6520) for 1 h and washed with TBS-T six times. The proteins were detected by chemiluminescence using Enhanced Chemiluminescence Western
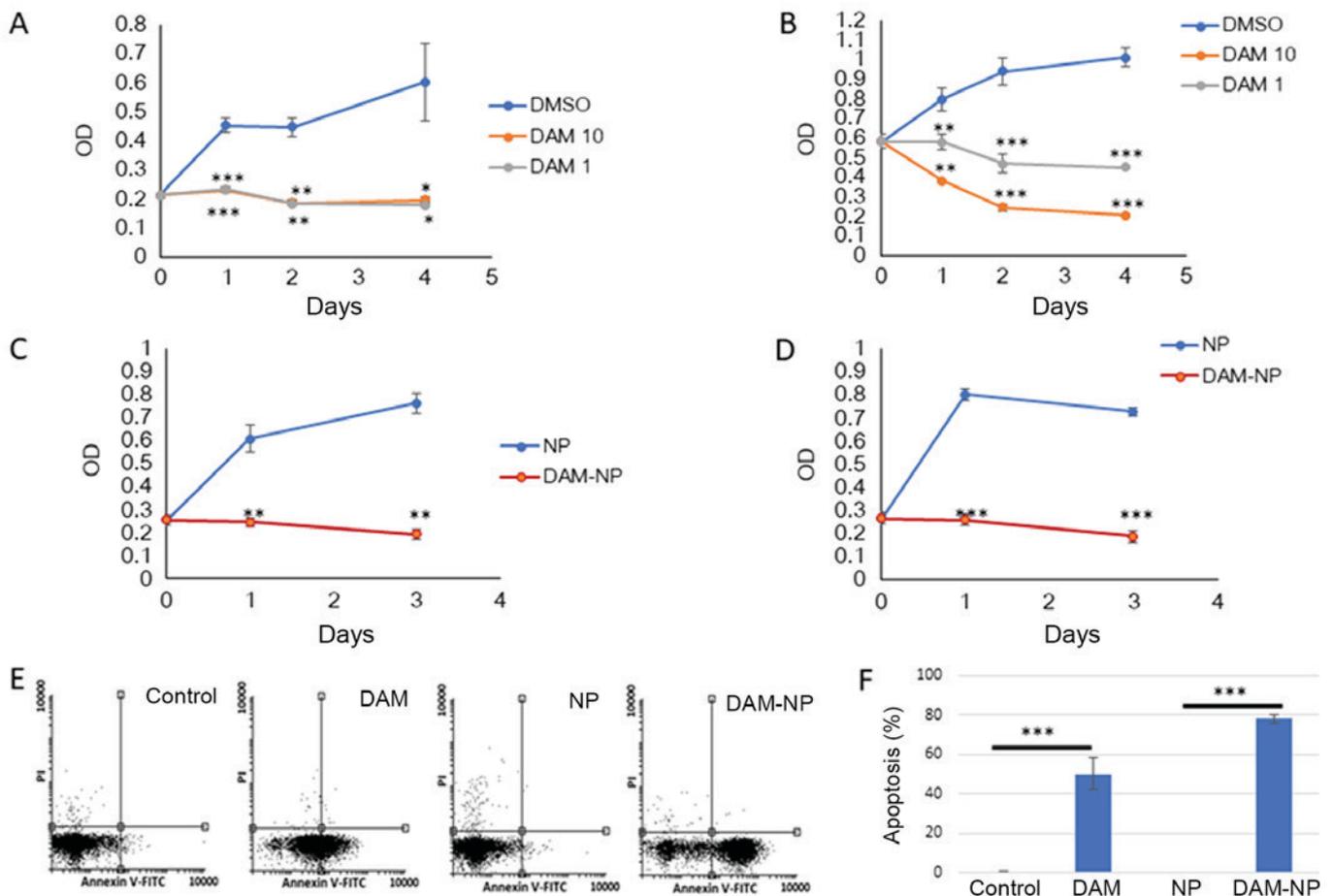
Figure 1. Effect of DAM and DAM-NPs on HCT-116 and U2OS cell proliferation. (A) HCT-116 and (B) U2OS cells were treated with DAM or DMSO for 4 days, and (C) HCT-116 and (D) U2OS cells were also treated with NPs or DAM-NPs (50 μM final DAM concentration) for 3 days. Results are expressed as the mean ± standard deviation of four independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. DMSO or NP-treated cells. U2OS cells were plated at 1x10^6 cells/well in 6-well plates, incubated with vehicle or 50 μM DAM or DAM-NPs for 24 h and analyzed for apoptosis. (E) Representative flow cytometric profiles. (F) Early apoptosis rate (Annexin V-positive and PI-negative). Results are expressed as the mean ± standard deviation of three independent experiments. ***P<0.001 vs. DMSO-treated cells. DAM, damnacanthal; DAM-NPs, DAM nanoparticles; DMSO, dimethyl sulfoxide; PI, propidium iodide; FITC, fluorescein isothiocyanate; NPs, nanoparticles; OD, optical density.

Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) and visualized using MicroChemi (software 4.2; DNR Bio-Imaging Systems, Ltd., Neve Yamin, Israel).

Statistics. Statistical analysis was performed with SSPS software (version 25; SPSS, Chicago, IL, USA). Statistical significance was determined by analysis of variance and Scheffe's test. P<0.05 was considered to indicate a statistically significant difference. Results are expressed as the mean ± standard deviation.

Results

DAM-NPs inhibit cell proliferation. It has been identified previously that treatment with DAM inhibits cancer cell proliferation (2). In the present study, the anti-proliferative effect of DAM and DAM-NPs on human colorectal and osteosarcoma cancer cells, HCT-116 and U2OS, respectively, was identified. The two cell lines, which each express wild-type p53, were used in this assay because it is known that DAM induces p53 expression and its induction leads to cell proliferation inhibition in cancer cells (16). The cells

were treated with 1 or 10 μM DAM for 1, 2 and 4 days. The cells were also treated with DAM-NPs at the equivalent DAM concentration (50 μM) for 1 and 3 days. The results indicated that DAM and DAM-NPs significantly decreased the proliferation of HCT-116 (P<0.001 and P<0.01, respectively) and U2OS (P<0.01 and P<0.001, respectively) cells after 1 day of treatment, compared with control cell proliferation (Fig. 1A-D). Furthermore, cell proliferation continuously decreased after 4 days of treatment. It was also identified that cell proliferation arrest by DAM and DAM-NPs resulted from induction of early apoptosis, as assessed using an Annexin V assay (Fig. 1E and F). These results indicated that DAM-NPs exhibit a similar activity to that of DAM in that they inhibit the proliferation of cancerous cells (2).

DAM and DAM-NPs inhibit CRM1 expression. To investigate the effect of DAM and DAM-NPs on CRM1 expression, HCT-116 and U2OS cells were treated with 50 μM DAM and DAM-NPs at the equivalent DAM concentration (50 μM). Western blots from total cell lysates with 60 μg protein were performed to determine CRM1 expression. As presented in Fig. 2, CRM1 was downregulated in HCT-116 and U2OS
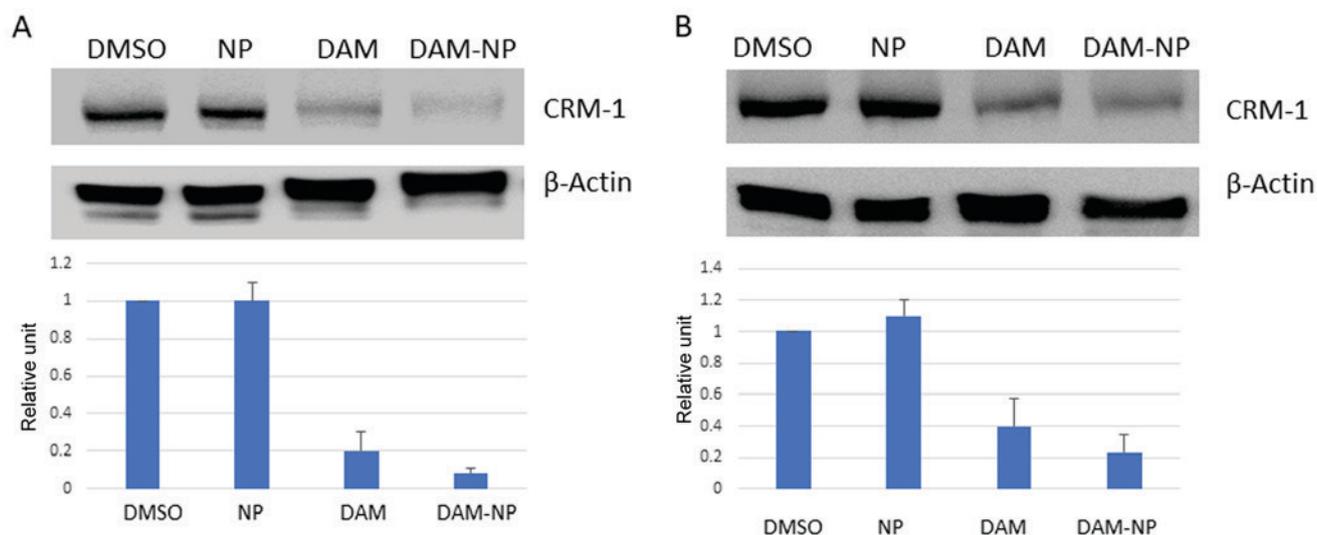
Figure 2. Effect of DAM and DAM-NPs on CRM1 expression. (A) HCT-116 and (B) U2OS cells were treated with control (0.1% DMSO or NPs), DAM (50 μM) or DAM-NPs (50 μM final DAM concentration) for 24 h. Cell lysates were subjected to western blot analysis using anti-CRM1 and anti-β-actin antibodies. Results are representative of three independent experiments. Densitometry results are expressed as fold induction relative to vehicle-treated cells normalized to β-actin. DAM, damnacanthal; DAM-NPs, DAM nanoparticles; DMSO, dimethyl sulfoxide; CRM1, chromosome maintenance protein 1; NPs, nanoparticles.
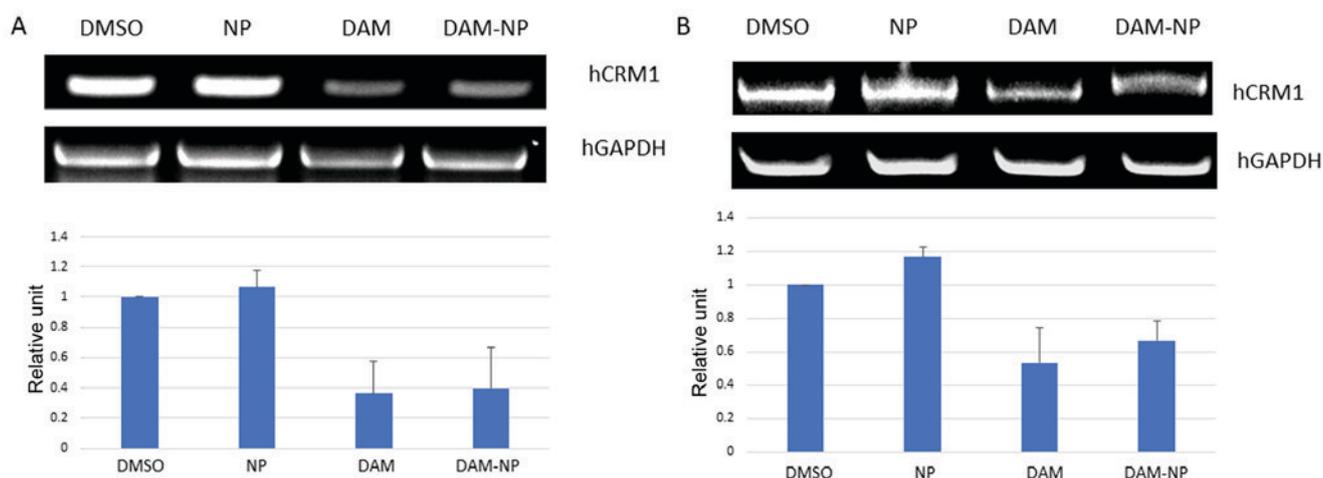

Figure 3. (A) HCT-116 and (B) U2OS cells were treated with control (0.1% DMSO or NPs), DAM (50 μM) or DAM-NPs (50 μM of final DAM concentration) for 24 h. hCRM1 mRNA levels were quantified using the reverse transcription-semi-quantitative polymerase chain reaction. Results are representative of three independent experiments. Densitometry results are expressed as fold induction relative to vehicle-treated cells adjusted to hGAPDH. DAM, damnacanthal; DAM-NPs, DAM nanoparticles; DMSO, dimethyl sulfoxide; NPs, nanoparticles; hCRM1, human chromosome maintenance protein 1; hGAPDH, human GAPDH.

cells treated with DAM and DAM-NPs compared with that in control cells. This result indicated that DAM and DMA-NPs decrease CRM1 expression at the protein level. To further verify the CRM1 downregulation at the transcriptional level, mRNA expression from HCT-116 and U2OS cells following treatment with DAM and DAM-NPs for 24 h was analyzed. As presented in Fig. 3A and B, the level of CRM1 mRNA decreased in the two cell lines following treatment. These results indicated that DAM and DAM-NPs also downregulate CRM1 expression at the transcriptional level.

CRM1 downregulation results in the accumulation of NAG-1. It has been identified that NAG-1 is a tumor suppressor protein translocated from the nucleus to the cytoplasm by CRM1 (12). To confirm that the decrease in CRM1 expression led to the nuclear accumulation of NAG-1, western blot

analyses for NAG-1 in nuclear as well as in cytoplasmic extracts were performed. As presented in Fig. 4, an increase in the NAG-1 level in the nuclear fraction was observed in DAM- and DAM-NP-treated cells. This may have resulted from the inhibition of CRM1 and may be mediated by DAM or DAM-NPs.

Discussion

Cancer is the primary cause of mortality in Thailand, and the second most common cause of mortality worldwide (17,18). In the last few decades, potential therapeutics for cancer have been investigated in a number of ways; however, extensive efforts are required to decrease the incidence of cancer and associated mortality. It has been indicated that plant phytochemicals serve an important function in anti-carcinogenesis (1). DAM,
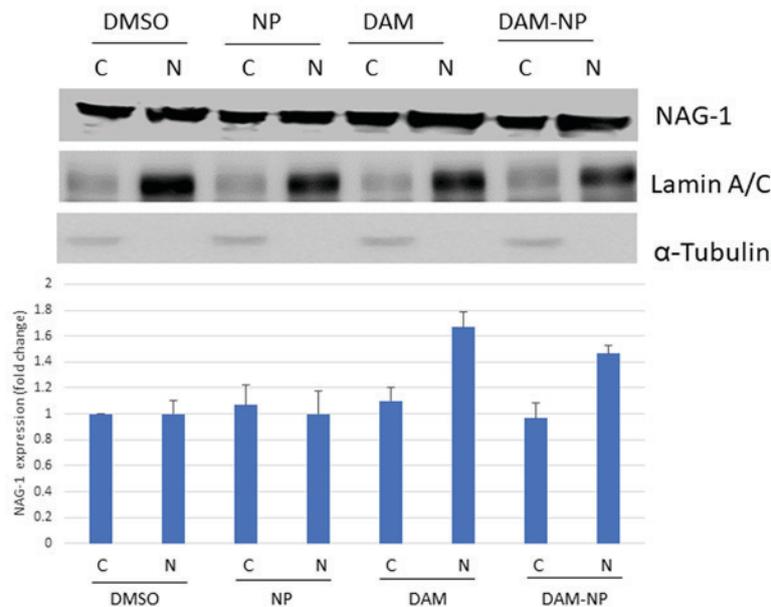
Figure 4. DAM and DAM-NPs increase NAG-1 expression in the nucleus of HCT-116 cells. HCT-116 cells were treated with control (0.1% DMSO or NPs), DAM (50 μM) or DAM-NPs (50 μM final DAM concentration) for 24 h. Nuclear and cytoplasmic extracts were subjected to western blot analysis using anti-NAG-1, anti-lamin A/C and anti-α-tubulin antibodies. Results are representative of three independent experiments. The densitometry represents NAG-1 fold induction, compared with DMSO-treated cytoplasmic NAG-1 expression. NAG-1, non-steroidal anti-inflammatory drugs-activated gene 1; DAM, damnacanthal; DAM-NPs, DAM nanoparticles; DMSO, dimethyl sulfoxide; NPs, nanoparticles; C, cytoplasmic; N, nuclear.

extracted from *M. citrifolia*, commonly called noni (19), has potential anticancer effects. In the present study, it was identified that DAM inhibits the proliferation of HCT-116 and U2OS cells. DAM and encapsulated DAM inhibited CRM1 expression in the two cell lines, which may be key to its anticancer activity.

CRM1 is an export protein that facilitates the transport of large molecules including tumor suppressor proteins from the nucleus to the cytoplasm (20). Previous studies have identified that CRM1 overexpression occurs in various types of cancer such as osteosarcoma, ovarian cancer, pancreatic cancer, glioma, cervical cancer and renal cell carcinoma (21-26), making CRM1 a focal target for anticancer drugs. In the present study, it was identified that DAM and DAM-NPs downregulate CRM1 expression in HCT-116 and U2OS cells. This result was corroborated by analyzing the expression of NAG-1 protein in the nucleus and cytoplasm. Following treatment with DAM and DAM-NPs, NAG-1 expression was increased in the nucleus compared with in the cytoplasm. This may be a consequence of CRM1 downregulation and the associated decrease in NAG-1 transportation from the nucleus to the cytoplasm. CRM1 is highly expressed in several types of cancer, and its inhibition is beneficial. Although it requires further investigation, DAM may affect CRM1 activity by directly binding to CRM1, similar to leptomycin B (a synthetic CRM1 inhibitor). These results are of importance because DAM is a natural compound, which is associated with fewer side effects compared with synthetic CRM1 inhibitors and may be used to develop potent anticancer drugs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NC, PR, KS and SJB designed and conceived the present study. NC, YY and TN performed the experiments. NC and SJB wrote the manuscript. WG, SC, JKS and SJB analyzed the data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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