Mitogen-activated Protein Kinase Inhibitors Induce Apoptosis and Enhance the Diallyl Disulfide-induced Apoptotic Effect in Human CNE2 Cells

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In this study, we investigated whether the inhibition of endogenous phosphorylation of mitogen-activated protein kinase (MAPK) and diallyl disulfide (DADS)-induced phosphorylation of MAPKs with MAPK specific inhibitors, SB203580 and U0126 (for phospho-p38 and phospho-p42/p44, respectively), can induce or enhance apoptosis in human CNE2 nasopharyngeal carcinoma cells. Our data demonstrate that MAPK inhibitors decrease the viability of CNE2 cells, stimulate typical apoptotic morphologic changes, and enhance DADS-induced apoptosis. The present findings indicate that phosphorylation of MAPKs plays an important cytoprotective role in CNE2 cell apoptosis and the DADS-induced apoptotic process.

Key words —— apoptosis, combined effect, CNE2 cell, mitogen-activated protein kinase inhibitor, phosphorylation

INTRODUCTION

Diallyl disulfide (DADS) from garlic (*Allium sativum*) has been shown to have an antiproliferative effect on human tumor cells including those of colon, lung, skin, breast and liver origins.[1–5] Cellular redox state, tyrosine kinases, and phosphatases are thought to be involved in the activation of stress responses. These represent the activation of various components of the mitogen-activated protein kinase (MAPK) family in different cell models after treatment with paclitaxel, TNF or TGF-1.[6–9] Among them, p38-MAPK and p42/44-MAPK play important roles in these apoptotic processes.[10–15]

P38-MAPK is more commonly activated in response to cytokines, stress and cellular damage; P44/42-MAPK is activated by growth stimuli. Both were generally considered to be pro-survival mediators.[16–18] P38-MAPK was initially proposed to mediate apoptosis in neuronal cells.[19,20] The use of kinase inhibitors and the over-expression of dominant negative mutant forms of MAPKs have also demonstrated the role of p38-MAPK in non-neuronal apoptosis induced by various stimuli, including estrogen, chelerythrine, UV-B radiation, thapsigargin, and singlet oxygen.[21–25] Under certain circumstances, the activation of p38-MAPK has been observed to be dependent on caspase activity, and therefore, kinase activation can be a consequence of apoptosis.[19] However, p38-MAPK has other roles dependent on cell type and other external stimuli, including promoting proliferation, differentiation, and survival.[1,26–29]

The p42/44-MAPK pathway may serve as mechanism opposing apoptosis.[30] Previous reports have shown that p42/44-MAPK activation contributes to cell differentiation, proliferation, and survival.[21,30,31] Apoptosis was induced when U0126, a p42/44-MAPK specific inhibitor, was added to HL60 cells.[32] U0126 significantly prevents kinase activation and greatly reduces apoptosis induced by Norcantharidin (NCTD), a potent anti-cancer drug, in human hepatoma HepG2 cells.[33] Interestingly, a combination of U0126 with paclitaxel (Taxol) results in a synergistic enhancement of apoptosis —

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four times that of the additive value.[31)]

DADS, an important component of garlic (*Allium sativum*), has been recently shown to inhibit the growth of human tumor cells from colon, lung, skin, breast and liver origins.[1, 34–37)] Although DADS' role and mechanisms as an anti-cancer agent remain unclear, there is increasing evidence for DADS-mediated modulation of signal transduction pathways.[35, 36, 38)] The anti-proliferative effect of DADS has been attributed to suppression in the rate of cell division and induction of apoptosis.

Apoptosis plays an essential role as a protective mechanism against neoplastic development by eliminating genetically damaged cells or excess cells that have been improperly produced. Many anticancer drugs act by stimulating apoptosis in cancer cells.[39–42)] The maintenance of homeostasis in normal mammalian tissues by apoptosis reflects a critical balance between cell proliferation and cell loss.

Inappropriate regulation of apoptosis is associated with a variety of diseases.[43–45)] In particular, the failure of dividing cells to initiate apoptosis in response to DNA damage has been implicated in the development and progression of cancer. However, since apoptosis represents an active, gene-directed mechanism, it may eventually be possible to control this process for therapeutic purposes.

In this report we find that phospho-p38 and phospho-p42/44 specific inhibitors, SB203580 and U0126 respectively, significantly block both the endogenously activated MAPKs and DADS-induced activation of MAPKs. The inhibition of phosphorylated MAPKs causes a significant increase in CNE2 cell apoptosis and an enhancement of DADS-induced apoptosis. The phosphorylated MAPKs (phospho-p38 and phospho-p42/p44) appear to play a cytoprotective role, and MAPK-specific inhibitors may be used to enhance the apoptotic effect in CNE2 cells when combined with DADS treatment.

MATERIALS AND METHODS

Materials —— SB203580 [4-(4-fluoropheny)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) IH-imidazole] — a phospho-p38 specific inhibitor — was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine serum albumin (BSA) and Hoechst 33258 were also purchased from Sigma Chemical Co. U0126 [1,4-diamino-2,3-dicyano-1,4-bis(phenylthio)butadiene] — a phospho-p42/44 specific inhibitor — was a kind gift from Dr. J.M. Trzaskos (DuPont Merck Research Laboratory; Wilmington, DE, U.S.A.). DADS was purchased from Fluka Co. (Buchs, Switzerland). RPMI1640 medium was purchased from Gibco (Grand Island, NY, U.S.A.). P38-MAPK and p42/44-MAPK antibodies (total- and phospho-specific), Goat HRP–conjugated anti-rabbit secondary antibody were purchased from Santa Cruz Biotech (Santa Cruz,, CA, U.S.A.). The Phototope–HRP Western Detection Kit was purchased from New England Biolabs Inc. (Ipswich, MA, U.S.A.).

Cell Culture —— CNE2, a human nasopharyngeal carcinoma cell line was cultured in RPMI1640 with10% heat-inactivated fetal bovine serum (FBS), benzylpenicillin (100 kU/l), and streptomycin (100 mg/l) at 37°C in humidified air with 5% CO_2.

Cell Viability Assay —— To assess the cytotoxic effects of MAPK inhibitors in CNE2 cells, we used a tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.[46)] In this assay, MTT is used as a colorimetric substrate for measuring cell viability. When cells are injured there is an alteration in cellular redox activity such that cells are unable to reduce the dye. Cells were plated in 24-well plates and grown to 80% confluence. The cultures were then rinsed in phenol-free RPMI1640 media and incubated with specific test substances in phenol- and serum-free RPMI1640 for 10 min with inhibitors or 24 hr with DADS. At the end of this time, MTT was added to a final concentration of 0.5 g/l. After 1 hr incubation, cultures were removed from the incubator and the formazan crystals were solubilized using 10% (v/v) Triton X–100 and HCl 0.1 mol/l in isopropanol equal to the volume of the original culture. Colorimetric determination of the reduced MTT was made at 570 nm.

Cell Cycle Analysis —— CNE2 cells were grown to 60% confluence and culture media with final concentrations of 10 μmol/l SB203580 and 5 μmol/l U0126 was added and incubated for 10 min. For the combined assay, a final concentration of 100 μmol/l DADS was added and incubated for 24 hr after addition of the inhibitors. Afterwards, cells were harvested and fixed in 70% ethanol at 4°C for 24 hr. Immediately before analysis the fixed cells were treated with RNase (100 mg/l) at 37°C for 30 min, and then propidium iodide (10 mg/l) added. After incubating at 4°C for 30 min in darkness, samples (3×10^4 cells for each assay) were analyzed for

DNA content using an Epics Altra flow cytometer and software (Beckman-Coulter, Brea, CA, U.S.A.).

Apoptosis Analysis —— Two methods were used to detect apoptosis induced by MAPK inhibitors or the combination of inhibitors with DADS. First, after treatment, morphological changes of CNE2 cells were observed by fluorescence microscopy with Hoechst 33258 staining. Apoptosis was routinely determined by counting the numbers of cells with condensed or fragmented nuclei as described previously.[47] Six randomly chosen fields of view were examined with a minimum number of 500 cells scored for each condition. Secondly, a flow cytometric assay from Epics ALTRA (Beckman-Coulter), as described above, was used to analyze the fraction of apoptotic cells.

Preparation of Lysates —— For MAPK detection, human CNE2 cells cultured in 6-well culture plates were grown to 80–90% confluence, then nutrient-starved for 24 hr in serum free RPMI1640. Indicated concentrations of inhibitors and DADS were added for the specified times. After three washes with ice-cold phosphate buffer solution (PBS), cells were lysed with 60 μl of ice-cold lysis buffer containing (in mmol/l) NaCl 50, Na_3VO_4 2, $Na_4P_2O_7$ 10, NaF 100, phenylmethylsulfonyl fluoride 1, DL-dithiothreitol (DTT) 2, and 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 50 at pH 7.5, along with 0.01% Triton X–100, leupeptin 20 μmol/l, and aprotinin 1×10^5 U/l. The lysates were obtained by centrifugation at 13000 *g* at 4°C for 10 min and the concentration of total cell protein was determined by spectrophotometry.[48]

Western Blot Analyses —— Sodium dodecyl sulfate (SDS) sample buffer containing Tris-HCl 0.33 mol/l, SDS 8% (w/v), glycerol 40% (v/v), and bromophenol blue 0.4% were added to cell lysates. After boiling the extracted protein for 5 min, 20 μg was resolved by SDS–polyacrylamide gel electrophoresis (PAGE). The protein was transferred to a nitrocellulose membrane which was then blocked at 25°C for 1 hr with 5% BSA in TBST (Tris–HCl 20 μmol/l, pH 7.5, NaCl 137 mmol/l containing 0.1% Tween–20). The blots were incubated with primary antibodies against phospho-p38, phospho-p42/p44, total-p38 MAPK, or total-p42/p44 MAPK at 1 : 1000 dilutions at 4°C overnight, followed by incubation at room temperature for 1 hr with secondary antibody (horseradish peroxidase conjugated) at 1 : 2000 dilutions. Immunoreactive signals were visualized by the Phototope Western Detection System (New England Biolabs). As a protein loading control, two gels for each group were loaded in parallel with the same protein samples and blotted for activated/phosphorylated MAPKs or total MAPKs. Protein bands were quantitatively determined by QuantiScan v2.1 (Biosoft, Ferguson, MO, U.S.A.).

Statistics —— Results are expressed as means ± SEM with N being the sample size. Statistical analysis was performed on SPSS statistical package v7.1 (SPSS Inc, Chicago, IL, U.S.A.). Values of $p < 0.05$ were considered statistically significant, on a 2-tailed *t*-test.

RESULTS

MAPK Inhibitors Decrease Cell Viability and Induce Apoptosis in Human CNE2 Cells

To test the effect of MAPK inhibitors on the viability of CNE2 cells, the MTT conversion assay was used (Table 1). After treated with 10 μmol/l SB203580 for 10 min, the viability of CNE2 cell was decreased by 7.3%. Similarly, treated with 5 μmol/l U0126 for 10 min decreased, the viability of CNE2 cell by 2.1% (0.739 ± 0.0015, $p < 0.05$). These results suggested that MAPK inhibitors were cytotoxicity to CNE2 cells.

Apoptosis of CNE2 cells induced by MAPKs was examined by fluorescence microscopy after Hoechst 33258 staining. After exposure to SB203580 (10 μmol/l) or U0126 (5 μmol/l) for 10 min, the CNE2 cells showed typical morphologic changes ofapoptosis. The number of apoptotic cells increased 3.6-fold (9.7%, $p < 0.01$) for SB203580 and 1.7-fold (4.5%, $p < 0.05$) for U0126, when compared with untreatedcontrol (2.7%) (Fig. 1 and Table 2).

Table 1. Effect of MAPK Inhibitors on the Growth of CEN2 Cells

Substances (μmol/l)	MTT absorbance (570 nm)	Normalized (%) with Control	Normalized (%) with DADS
Control (0)	$0.755 \pm 5.77 \times 10^{-3}$		
DADS (100)	$0.650 \pm 1.00 \times 10^{-3a)}$	13.9	
SB (10)	$0.700 \pm 1.53 \times 10^{-3b)}$	7.3	
SB+DADS	$0.626 \pm 5.77 \times 10^{-3c)}$		3.7
U (5)	$0.739 \pm 5.77 \times 10^{-3a)}$	2.1	
U+DADS	$0.641 \pm 3.79 \times 10^{-3d)}$		1.4

Human CEN2 cells were treated with inhibitor for 10 min, followed by DADS for 24 hr or not. The cell viability was determined. Cell viability is normalized (%) to control (treated with solvent) or DADS. Data are shown as means ± SEM, $n = 3$ experiments. *a*) $p < 0.01$, *b*) $p < 0.05$ *vs.* control; *c*) $p < 0.01$, *d*) $p < 0.05$ *vs.* DADS (100 μmol/l).

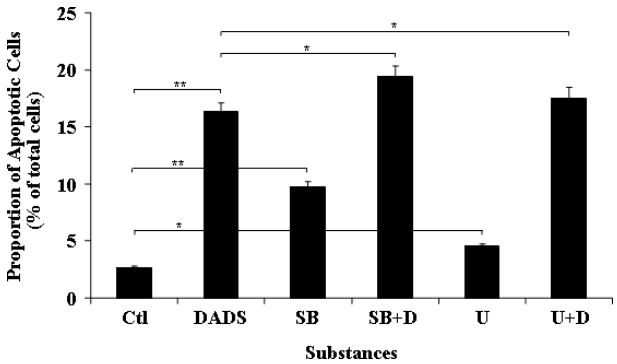

Fig. 1. Effect of MAPK Inhibitors on Cell Apoptosis

CNE2 cells were treated with MAPK inhibitors, SB203580 (10 μmol/l) and U0126 (5 μmol/l) for 10 min, followed by DADS (100 μmol/l) or control for 24 hr. After staining with Hoechst 33258 (1 μM in phosphate-buffered saline) six randomly-chosen fields of view were observed with a minimum number of 500 cells scored under each condition. Apoptotic cells (condensed with fragmented nuclei) were calculated as a ratio to the total number of cells counted. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* DADS or MAPK inhibitor alone. Ctl: Control (treated with solvent); D: DADS (100 μmol/l); SB: SB203580; U: U0126; SB+D: Combination of SB203580 and DADS (100 μmol/l); U+D: Combination of U0126 and DADS (100 μmol/l).

Table 2. Effect of MAPK Inhibitors on CEN2 Cells Apoptosis

Substances (μmol/l)	Percentage of apoptotic cells (% of total CEN2 cells)
Control (0)	2.71 ± 0.25
DADS (100)	16.30 ± 0.91[a)]
SB (10)	9.72 ± 0.75[b)]
SB+DADS	19.43 ± 1.04[c)]
U (5)	4.52 ± 0.33[a)]
U+DADS	17.64 ± 0.96[d)]

Human CEN2 cells were treated with inhibitor for 10 min, followed by DADS for 24 hr or not. The number of apoptotic cells was determined by direct apoptotic cell counting after cell staining. The percentage of apoptotic cells was calculated with total CNE2 cells. C Data are shown as means ± SEM, $n = 3$ experiments. *a)* $p < 0.01$, *b)* $p < 0.05$ *vs.* control; *c)* $p < 0.01$, *d)* $p < 0.05$ *vs.* DADS (100 μmol/l).

Similar apoptotic results were observed by DNA flow cytometric analyses. SB203580 treatment decreased 4.8-fold (8.7%), and U0126 decreased 3.2-fold (5.8%) in CNE2 cell apoptosis when compared with control treated with solvent (1.8%) (Table 3). Western blot analysis also confirmed that MAPKs blocked activation of endogenous p38-MAPK and p42/44-MAPK in CNE2 cells (Fig. 2 A-I and B-I, Lane3). Since inhibiting p38- and p42/44-MAPKs increased apoptosis, we concluded that these pathways represented a cytoprotective mechanism.

MAPK Inhibitors Enhance DADS-induced Apoptosis in CNE2 Cells

As discussed previously, DADS also acts in the MAPK pathway. Whether would these inhibitors have a synergistic effect with DADS in inducing apoptosis? We carried out a combined treatment MTT assay. Statistically significant decrease of CNE2 cell viability was observed: 3.7% for SB203580 plus DADS when compared with DADS-treatment alone ($p < 0.01$) and 1.4% for U0126 plus DADS when compared with DADS-treatment alone ($p < 0.05$) (Table 1). Next, apoptosis was specifically examined under the above treatment conditions. Combined treatment increased the proportion of apoptotic CNE2 cells by an absolute 3.1% with SB203580 plus DADS when compared with DADS-treatment alone ($p < 0.05$) and 9.7% when compared with inhibitor-treatment alone ($p < 0.01$). CNE2 cell apoptosis was increased by 1.3% with U0126 plus DADS when compared with DADS-treatment alone ($p < 0.05$) or 13.1% when compared with inhibitor-treatment alone ($p < 0.01$) (Fig. 1 and Table 2).

This increase of apoptosis in CNE2 cells was also found by DNA flow cytometric analy-

Table 3. Effect of MAPK Inhibitors on the Cell Cycle Distribution in CEN2 Cells

Substances (μmol/l)	%			
	G1	S	G2/M	Apoptosis
Control (0)	45.0 ± 2.1	45.0 ± 1.8	9.9 ± 0.7	1.8 ± 0.1
DADS (100)	37.1 ± 1.2[a)]	53.0 ± 2.5[a)]	9.9 ± 0.5	15.1 ± 0.8[a)]
SB (10)	41.4 ± 1.8[b)]	49.3 ± 2.0[b)]	9.3 ± 0.5	8.7 ± 0.5[a)]
SB+DADS	18.6 ± 0.2[c)]	73.5 ± 3.5[c)]	7.9 ± 0.3[c)]	23.4 ± 1.1[c)]
U (5)	46.3 ± 2.2[a)]	43.6 ± 2.0[a)]	10.1 ± 0.6[a)]	5.8 ± 0.3[a)]
U+DADS	32.9 ± 1.0[d)]	58.3 ± 2.1[d)]	8.8 ± 0.4[d)]	17.4 ± 0.9[d)]

Human CEN2 cells were treated with inhibitor for 10 min, followed by DADS for 24 hr or not. Cell cycle distribution was analyzed by flow cytometry. Data are shown as means ± SEM, $n = 3$ experiments. *a)* $p < 0.01$, *b)* $p < 0.05$ *vs.* control; *c)* $p < 0.01$, *d)* $p < 0.05$ *vs.* DADS (100 μmol/l).

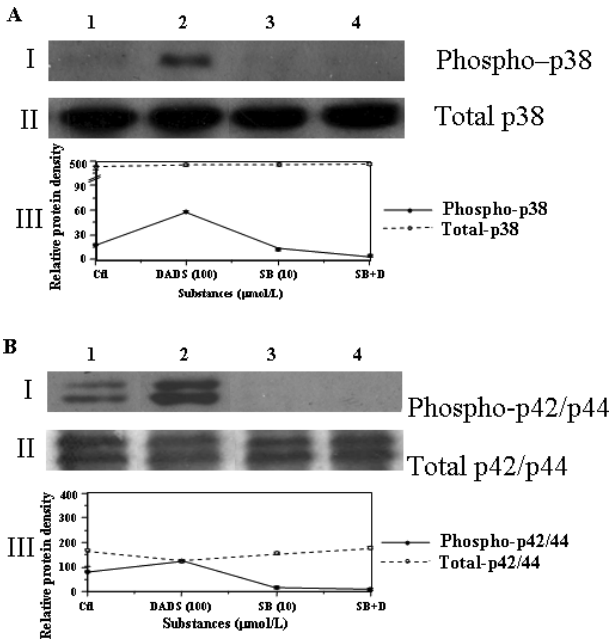

Fig. 2. MAPKs Inhibitors Block the Endogenous and DADS-induced Activation of MAPKs in CNE2 Cells

Cells were treated with MAPK inhibitors, SB203580 (10 μmol/l) and U0126 (5 μmol/l) for 10 min, followed by DADS (100 μmol/l) or control for 24 hr, and Western blot analysis was performed. Extracts (20 μg protein) from CNE2 cells were resolved by SDS-PAGE and probed with anti-phospho–p38 antibody (A-I), or with anti-phospho–p42/p44 antibody (B-I). In parallel experiments, the amount of total-p38-MAPK or p42/p44-MAPK was determined in the same cell extracts with anti-total-p38-MAPK antibody (A-II) or anti-total-p42/p44-MAPK antibody (B-II). Lane 1: control (treated with solvent), Lane 2: 50 μmol/l DADS, Lane 3: 100 μmol/l DADS, Lane 4: 150 μmol/l DADS. Representative graphs of Western blot densities are shown in A-III and B-III. Results were confirmed in triplicate.

sis. Treatment with SB203580 plus DADS increased apoptosis by an absolute 8.3% when compared with DADS-treatment alone ($p < 0.01$) and 14.7% when compared with inhibitor-treatment alone ($p < 0.01$). Treatment with U0126 plus DADS increased apoptosis by 2.3% when compared with DADS-treatment alone ($p < 0.05$) and 11.6% when compared with inhibitor-treatment alone ($p < 0.01$) (Table 3).

To test whether MAPK inhibitors block DADS-induced activation of MAPKs in CNE2 cells, we examined MAPK protein expression by Western blot analysis. When SB203580 or U0126 was added to CNE2 cells 10 min prior DADS-treatment, DADS-induced activation of p38-MAPK (Fig. 2A) and p42/p44-MAPK (Fig. 2B) was markedly decreased. Levels were so low that phospho-p38 and phospho-p42/p44 could not be detected using phospho-specific antibodies (Lane 4, Fig. 2A and 2B).

MAPK Inhibitors Enhance DADS-induced Cell Cycle Arrest in CNE2 Cells

The cell cycle effects of MAPK inhibitors followed by DADS treatment in CNE2 cells were analyzed by cytofluorometric analysis. We detected cell cycle distribution after a combined treatment with SB203580 (10 μmol/l) and DADS (100 μmol/l), or a combined treatment with U0126 (5 μmol/l) and DADS (100 μmol/l). As displayed in Table 3, the percentages of S-phase cells were significantly increased when combined with DADS, but not when given alone. These results indicate that MAPK inhibitors enhance DADS-induce cell cycle arrest in S-phase in CNE2 cells, although they appear to have no significant effect by themselves.

DISCUSSION

A number of pathways are thought to lead to DADS-induced apoptosis. First, it has been shown that DADS-induced apoptosis is mediated via activation of caspase-3,[37] an important regulator of apoptosis.[49,50] In DADS-treated HL-60 cells, caspase-3 activation is evidenced by an increase in protease activity and proteolytic cleavage activity of the proenzyme.[36] Secondly, excessive intracellular calcium is known to lead to apoptosis in several *in vitro* models.[34] In HCT–15 cells, DADS is found to cause a sustained and dose-dependent increase in intracellular calcium.[51,52] Our previous work has demonstrated that DADS transiently activates both p38-MAPK and p42/p44-MAPK while it induces apoptosis in a time- and concentration-dependent manner in human HepG2 hepatoma cells.[1] The present study focuses on the role of MAPKs and MAPK inhibitors in cell apoptosis and DADS-induced apoptosis. Activation of p38-MAPK is generally associated with induction of apoptosis, whereas phospho-p42/44 is thought to exert a cytoprotective effect.[21] Interestingly, we have found that reduction of these activated/phosphorylated MAPKs by specific inhibitors (SB203580 and U0126), markedly increases apoptosis in CNE2 cells and enhances DADS-induced apoptosis (Fig. 1, Tables 2 and 3). These data suggest that basal levels of endogenously activated MAPKs and DADS-induced activation of p38-MAPK and p42/44-MAPK (Fig. 2A and 2B) may play a role in maintaining the survival of CNE2 cells. The results presented in this study established a potential role for MAPK inhibitors in

DADS-induced apoptosis. First, inhibitor treatment not only blocks constitutive levels of phospho-p38 and phospho-p42/44, but also completely blocks DADS-induced MAPK activation (Fig. 2). Second, a combination treatment with both DADS and MAPK inhibitor (SB203580 or U0126) decreases the viability of CNE2 cells and leads to increased apoptotic activity compared with DADS or inhibitor alone (Table 1, Fig. 1, Tables 2 and 3). The combined effect suggests a co-chemocytotoxic value in human nasopharyngeal carcinoma.

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