Dimethyl Fumarate Controls the NRF2/DJ-1 Axis in Cancer Cells: Therapeutic Applications

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Abstract

The transcription factor NRF2 (NFE2L2), regulates important antioxidant and cytoprotective genes. It enhances cancer cell proliferation and promotes chemoresistance in several cancers. Dimethyl fumarate (DMF) is known to promote NRF2 activity in noncancer models. We combined in vitro and in vivo methods to examine the effect of DMF on cancer cell death and the activation of the NRF2 antioxidant pathway. We demonstrated that at lower concentrations (<25 μM/L), DMF has a cytoprotective role through activation of the NRF2 antioxidant pathway. At higher concentrations, however (>25 μM/L), DMF caused oxidative stress and subsequently cytotoxicity in several cancer cell lines. High DMF concentration decreases nuclear translocation of NRF2 and production of its downstream targets. The pro-oxidative and cytotoxic effects of high concentration of DMF were abrogated by overexpression of NRF2 in OVCAR3 cells, suggesting that DMF cytotoxicity is dependent of NRF2 depletion. High concentrations of DMF decreased the expression of DJ-1, a NRF2 protein stabilizer. Using DJ-1 siRNA and expression vector, we observed that the expression level of DJ-1 controls NRF2 activation, antioxidant defenses, and cell death in OVCAR3 cells. Finally, antitumoral effect of daily DMF (20 mg/kg) was also observed in vivo in two mice models of colon cancer. Taken together, these findings implicate the effect of DJ-1 on NRF2 in cancer development and identify DMF as a dose-dependent modulator of both NRF2 and DJ-1, which may be useful in exploiting the therapeutic potential of these endogenous antioxidants.

Introduction

Dimethyl fumarate (DMF) is a derivative of fumaric acid registered for the treatment of relapsing forms of multiple sclerosis and psoriasis (http://www.fda.gov; refs. 1–3). Several studies have shown its cytoprotective and antioxidant effects in noncancer models (1–3), which appeared related to the induction of the nuclear factor erythroid 2 (NF-E2)-related factor 2 (NRF2) pathway (2–6). NRF2 is a ubiquitously expressed basic leucine zipper transcription factor (7–9). It is a “Master redox switch” as it is known to induce cytoprotective genes that render protection of cells from oxidative stress (OS; refs. 10, 11). At physiologic levels, NRF2 is held in the cytoplasm as an inactive complex bound to Kelch-like ECH-associated protein 1 (KEAP1), a repressor molecule that facilitates NRF2 ubiquitination. By inducing covalent modification of thiols in some of the cysteine residues of KEAP1, DMF leads to conformational changes in KEAP1 that ultimately result in disruption of the KEAP1–NRF2 interaction, thereby, warranting the translocation of NRF2 into the nucleus. Within the nucleus, NRF2 binds to the antioxidant response element (ARE) in the promoter region of phase II genes and stimulates their transcription (10, 11). On the other hand, the cytotoxic effect of DMF against several tumor cell lines has been suggested in vitro (12–16) and the induction of oxidative stress has also been reported (17, 18). These observations however, are somehow conflicting with the role of NRF2 in cancer cells. NRF2 accumulation was detected in many cancers, where it has been proven to act as an oncogene (7, 9). It could limit to an acceptable level the accumulation of reactive oxygen species produced in excess by the mitochondrial respiratory chain in hypermetabolic and proliferative cancer cells. Overexpression of the NRF2 protein has also recently emerged as a potential mechanism of resistance to platinum and other cytotoxicities (7–9).

DMF may have other molecular targets, such as DJ-1. DJ-1 is a multifunctional protein that is encoded by the PARK7 gene. It has antioxidant properties and it regulates NRF2-dependent antioxidant signaling by preventing its association with KEAP1 thereby promoting its stability and activation (19, 20).

In this report, we demonstrated that DMF has a dose-dependent effect in cancer cells, cytoprotective at lower concentrations by inducing NRF2; it inhibits at higher concentrations the NRF2 stabilizer DJ-1, which in turn inhibits NRF2 activation, induces OS, and subsequently promotes cancer cell death. These findings...
implicate the effect of DJ-1 on NRF2 in cancer development and identify DMF as not only an activator, but also an inhibitor of both NRF2 and DJ-1, which may be useful in exploiting the therapeutic potential of these endogenous antioxidants.

Materials and Methods

Reagents and antibodies

Protease inhibitor cocktail Complete was obtained from Roche Diagnostics, and paclitaxel was obtained from Fresenius Kabi. Buthanol was from Carbasynth. The Nucleobond AX-plasmid-purification kit was from Macherey-Nagel. NIH-OVCAR-3 Cell Avalanche Transfection Reagent was from EZ Biosystems. pGW1-Myc-DJ-1-WT and pDNN3A.1FLAGNRF2 were from Addgene (plasmids, #29347 and #36971). DJ-1 siRNA kit, antibodies against NRF2, DJ-1, β-actin, and GAPDH were all purchased from Santa Cruz Biotechnology. Anti-PARP, anti-caspase-3, anti-Myc, anti-Lamin A, and anti-Flag were purchased from Cell Signaling Technology. Goat, mouse, and rabbit secondary antibodies were all bought from Dianova. All other chemicals (except when and where stated) were from Sigma.

Cell culture

OVCAR3 (human serous ovarian carcinoma), TOV 21G (human clear ovarian carcinoma), CT26 (mouse colon carcinoma), HT29 (human colon carcinoma), SW40 (human colon carcinoma), Caco-2 (human colon carcinoma), A549 (human lung adenocarcinoma), MCF7 (human breast carcinoma), and Mia Paca-2 (human pancreatic carcinoma) were all obtained from ATCC. OVCAR3 cells were enriched with 5% CO2. They were passaged every 3 days and routinely tested to rule out mycoplasma infection. The seeding of the cells was done to evaluate the effect of DMF in vivo. Expression of NRF2 in mice tumors was evaluated by transmitted light microscopy, 50 nuclei were counted among 50 nuclei and in each treatment group. In each selected area, the number of NRF2-positive nuclei was counted from NRF2-positive staining sections in tumors (n = 6 in each group).

Histologic and immunohistochemical analysis

For histopathologic evaluation, colon samples were fixed in 4% formaldehyde and paraffin embedded. Sections (5 μm) were stained with hematoxylin and eosin before examining under a Nikon Eclipse 80i microscope with a Nikon PlanFluor 10× objective. Expression of NRF2 in mouse tumors was evaluated by IHC as described previously (25). Briefly, tumors were excised and fixed in 4% formaldehyde and paraffin embedded. Sections (5 μm) were cut and labeled with anti-NRF2 antibody (1:500, Santa Cruz Biotechnology, sc-722) and DAB staining. Images were collected using a Nikon Eclipse 80i microscope with a Nikon PlanFluor 40× objective. As NRF2 staining was patchy throughout the samples, tumors were scored as either positive or negative for NRF2. For the quantification of nuclear NRF2, 50 nuclei were selected from NRF2-positive staining sections in tumors (n = 6 by treatment group). In each selected area, the number of NRF2-positive nuclei was counted among 50 nuclei and in each treatment group and from this, localized nuclear NRF2 was counted and expressed as a percentage relative to DMSO-treated group.

Transient transfection

Transfection of cells with plasmids was performed by using the NIH-OVCAR-3 Cell Avalanche Transfection Reagent according to the manufacturer’s instructions. Briefly, OVCAR3 cells were

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seeded into either a 60-mm dish (1 × 10^5 cells) in a total volume of 2.5 mL of cell culture medium or in a 96-well plate (0.5 × 10^4 cells) in a total volume of 100 μL of cell culture. Cells were cultured overnight and were then transfected with the NIH: OVCAR-3 Cell Avalanche Transfection Reagent using a total of 0.1 μg (96-well plate) or 0.25 μg (6-well plate) of plasmid DNA. DJ-1 siRNA transfections were also done according to the manufacturer's instructions. After 24 hours of cultivation, the medium was replaced by a fresh one; the cells were cultured for an additional 12–18 hours before treatment and then harvested for Western blot analysis.

**Evaluation of cell viability and cell death**

Cancer cells lines were seeded at 0.5 × 10^5 cells per well to a final volume of 100 μL in a 96-well plate and incubated overnight. Cells were then treated with DMSO (solvent control), different concentrations of DMF, or left untreated as indicated in figures and/or corresponding figure legends. The number of adherent cells was determined by a crystal violet assay as described previously (26). Results are expressed as a percentage of either cell number ± SEM versus DMSO-treated cells or cell number ± SEM versus cells in culture medium alone. Cell death was assessed by the expression of cleaved caspase-3 and PARP.

**Intracellular ROS measurement**

Cells were seeded at 1 × 10^4 cells per well to a final volume of 100 μL in a 96-well plate and incubated overnight. Cells were then treated with DMSO (solvent control), different concentrations of DMF, or left untreated for different time periods as indicated in the figures. ROS was then assessed spectrophotometrically by oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) as described previously (26). Fluorescence intensity was recorded every 1 hour for 6 hours at excitation and emission wavelengths of 485 and 530 nm, respectively. The number of cells was evaluated by the crystal violet assay and the level of ROS in each sample was calculated as follows: ROS levels (arbitrary units min^-1 10^4 cells^-1) = [fluorescence intensity (arbitrary units) at T360 min - fluorescence intensity (arbitrary units) at T0 min] per 60 minutes per number of cells as measured by the crystal violet assay. The final ROS figure (arbitrary units min^-1 10^4 cells^-1) was then expressed as a percentage relative to control.

**Intracellular GSH measurement**

Intracellular glutathione (GSH) levels were assessed as described previously (27). Briefly, cells seeded in 96-well plates were washed in PBS and incubated with 100 μL/well of 50 μM monochlorobimane in PBS. To measure total oxidized GSH levels, free sulfhydryl groups were masked with 1.25% 2-vinyl-pyrindine, dissolved in 125 mmol/L Na2HPO4 supplemented with EDTA 6.3 mmol/L (pH 7.5) for 1 hour and then processed as described previously (28). GSH levels were then assayed using a fusion spectrofluorimeter. Fluorescence intensity was recorded every 1 hour for 6 hours at excitation and emission wavelengths of 405 and 460 nm, respectively. The number of cells was evaluated by the crystal violet assay and the level of GSH in each sample was calculated as follows: GSH levels (arbitrary units min^-1 10^4 cells^-1) = [fluorescence intensity (arbitrary units) at T360 min - fluorescence intensity (arbitrary units) at T0 min] per 60 minutes per number of cells as measured by the crystal violet assay. The final total GSH figure (arbitrary units min^-1 10^4 cells^-1) was then expressed as a percentage relative to control.

**Western blot analysis and immunoprecipitation**

Extraction of total, nuclear, and cytosolic cellular proteins was performed as described previously (29, 30). The protein content of the supernatant was determined according to the Bradford method using the Bio-Rad protein assay reagent (Bio-Rad). SDS-PAGE and immunoblotting was also performed as described previously (29, 30). Briefly, proteins separated on a 7.5%, 10%, 12.5%, or 15% SDS-PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane. Membrane was blocked with 5% dry milk in PBS containing 0.1% Tween-20 (PBST) for 1 hour at room temperature and then incubated with the specific primary antibody, which was diluted in PBST containing 1% dry milk powder. The membrane was washed with PBST (3 × for 10 minutes), before being incubated with a peroxidase-coupled secondary antibody diluted in PBST for 1 hour at room temperature. The membrane was washed again in PBST (3 × for 10 minutes). Signals were developed, visualized, and quantified using the Fujifilm LAS – 3000 imaging system. Immunoprecipitation (IP) was performed to detect the interaction between NFR2 and DJ-1. The immunoprecipitation procedures are provided in the Supplementary Data.

**Statistical analysis**

GraphPad Prism (GraphPad Inc.) software was used to analyze the data. All values are averages of at least three independent experiments made in triplicates, except when specified. Error bars shown in the figures represent SEM and all results were expressed as arithmetic mean ± SEM. Differences between the experimental groups were analyzed using one-way ANOVA or Student t test (two-tailed, unpaired), and statistically significant differences were shown as follows: * , P < 0.01; **, P < 0.05.

**Results**

High concentrations of DMF display cytotoxicity in several cancer cell lines and induce parallel increase of oxidative stress and GSH depletion

Previous studies have shown that at higher concentrations (50–200 μmol/L), DMF is capable of inhibiting melanoma and colon cancer cell growths (12, 13). In the current study, we therefore asked whether DMF treatment might have an influence on the viability of other cancer cell types. Hence, eight different human cancer cell lines, namely OVCAR3, TOV 21G, MiaPaca2, HT29, Caco-2, SW-480, MCF7, A549, and primary lung adenocarcinoma (P) along with one mouse CT26 cells were all treated with 100 μmol/L of DMF for 24, 48, and 72 hours. Cell viability was then evaluated by the crystal violet assay. As shown in Fig. 1A, DMF treatment resulted in loss of cell viability in a time-dependent manner in all the cell lines. The degree of cytotoxicity on the selected cell lines varied, and it was found to be most potent on Mia Paca-2 pancreatic carcinoma cells. In addition, the cleavage of caspase-3 (Fig. 1B, top) along with its substrate PARP (Fig. 1B, bottom), well-known events in the apoptotic process, were induced by high-dose DMF (100 μmol/L). Interestingly, lower concentrations of DMF (0.25–5 μmol/L) did not have any significant effect on cell viability (Supplementary Fig. S1A–S1D), caspase-3, or PARP cleavage (Supplementary Fig. S2A–S2D) in any of the selected cell lines.

As shown in Fig. 1C, treatment of cancer cells with 100 μmol/L DMF induced a time-dependent increase in ROS, in parallel to total GSH depletion (Fig. 1D), the body's master antioxidant. This
DMF effect is also dose-dependent as treatment of OVCAR3 cells with different concentrations of DMF resulted in both a dose-dependent increase in ROS, in parallel to total GSH depletion (Supplementary Fig. S3A and S3B). Moreover, DMF caused a significant increase in oxidized GSH (GSSG) and a significant decrease in reduced GSH; both of which were concentration dependent (Supplementary Fig. S3C). Our results therefore suggest that DMF-induced ROS formation can lead to thiol oxidation and depletion in cancer cells. Next, we analyzed whether we could prevent DMF-induced cell death by pretreatment of cells with the GSH precursor N-acetyl cysteine (NAC). Indeed, NAC significantly prevented the DMF-induced oxidative stress (Supplementary Fig. S3D) and cytotoxicity at 24 hours (12.6%, \( P < 0.05 \)) and even more so at 48 hours (27.3%, \( P < 0.01 \); Supplementary Fig. S3F). These data strongly suggest that cancer cell death induced by high concentration of DMF is related to oxidative stress.

Low concentrations of DMF induce NRF2 activation and are cytoprotective

As we observed no significant change in cell viability with lower concentrations of DMF (0.25–5 \( \mu \text{mol/L} \)), but a rather significant increase in GSH levels in OVCAR3 cells (Supplementary Fig. S3B and S3C), we hypothesized that low concentrations of DMF may be cytoprotective through activation of the NRF2 antioxidant pathway. To test this hypothesis, we first treated OVCAR3 cells with DMF alone or in combination with the NRF2 small-molecule inhibitor brusatol (Bn; ref. 31). OVCAR3 cells were chosen for all subsequent experiments because the cytotoxicity of DMF in this cell line appeared as the mean of all tumor cell lines and OVCAR3 cells were also more sensitive to paclitaxel, a drug we were also interested in testing. Furthermore, OVCAR3 cells express high levels of NRF2. As shown in Fig. 2A, 5 \( \mu \text{mol/L} \) DMF induced nuclear accumulation of NRF2 protein which was abrogated when cells were treated with brusatol in addition to DMF. To further showcase the cytoprotective capability of low-dose DMF, OVCAR3 cells were treated with DMSO (ctrl), paclitaxel, or brusatol, alone or in combination with 5 \( \mu \text{mol/L} \) DMF. As a positive control, we treated the cells with epigallocatechin-3-gallate (EGCG), which is known to induce ROS formation and cause cell death. Compared with DMSO control-treated cells, paclitaxel and brusatol significantly reduced cell viability. Peculiarly, 5 \( \mu \text{mol/L} \) DMF significantly reduced the cytotoxicity of paclitaxel (Fig. 2B). In parallel, DMF also significantly reduced paclitaxel-induced ROS, confirming the cytoprotective capability of low-dose DMF. In contrast, DMF was unable to prevent oxidative stress and cytotoxicity induced by brusatol (Fig. 2B and C). Thus, low concentrations of DMF display cytoprotective and antioxidant effects that appear related to NRF2 activation.

DMF cytotoxicity is dependent on NRF2 depletion

Given the cytotoxic effect of high concentrations of DMF, we wondered whether NRF2 expression and activity is dependent on DMF concentration. Indeed, we observed that total NRF2
expression was increased by low concentration of DMF but at a decreasing level with subsequent increase in DMF concentration in OVCAR3 cells (Fig. 3A). Moreover, it was evident in OVCAR3 (Fig. 3B) and TOV 21G (Supplementary Fig. S4A) cells that only the nuclear, active, fraction of NRF2 was decreased by high concentration of DMF, while the cytoplasmic, inactive, fraction was not. A similar pattern of dose-dependent DMF effect was observed for both HO-1 and γGCS proteins, two antioxidant enzymes which are downstream targets of NRF2, observed for both HO-1 and γGCS (Fig. 3B). Moreover, it was evident in OVCAR3 cells (Fig. 3A). The concentration of DMF, while the cytoplasmic, inactive, fraction of NRF2 was decreased by high concentration of DMF, while the cytoplasmic, inactive, fraction was not. A similar pattern of dose-dependent DMF effect was observed for both HO-1 and γGCS (Fig. 3B). Moreover, it was evident in OVCAR3 cells (Fig. 3A).

Figure 2.
Cytoprotective effects of low concentrations of DMF. A, OVCAR3 cells were treated with 5 μmol/L DMF alone, 5 μmol/L DMF in combination with 0.25 μmol/L brusatol (Bru), 0.25 μmol/L brusatol alone or cells left untreated for 24 hours. Nuclear and cytosolic protein lysates were prepared and analyzed on a 10% SDS-polyacrylamide gel followed by Western blotting using an anti-NRF2-specific antibody. Lamin A was used as a nuclear marker, while β-actin was used as a loading control. One representative of at least two Western blots is shown here. B and C, OVCAR3 cells were seeded in 96-well plates overnight and treated the following day with 0.05% DMSO (ctrl), 0.25 μmol/L DMF, 5 μmol/L DMF, 0.25 μmol/L brusatol, 0.25 μmol/L paclitaxel (Pac), 5 μmol/L DMF, 0.25 μmol/L brusatol, 0.25 μmol/L paclitaxel (Pac) in combination with 5 μmol/L DMF or with 0.25 μmol/L brusatol in combination with 5 μmol/L DMF for the indicated times shown in the figure. Epigallocatechin-3-gallate (EGCG) was used as a positive control for ROS production. Cell viability (B) was determined using the crystal violet assay method. In all the experiments, data are expressed as a percentage change relative to control. In each case, the mean of three independent experiments is shown. *P < 0.05; **P < 0.01; ns, not statistically significant.

DMF downregulates the NRF2 protein stabilizer DJ-1 in OVCAR3 cells
Chemical structure and previous proteomic data strongly suggest that NRF2 is not a direct target of DMF (32). We therefore hypothesized that DJ-1, which is a "SH"-rich protein known to interact with NRF2 and affect its protein stability and activity (32, 33), could be a target of succination by fumarate. So we first investigated the effect of DMF on DJ-1 in OVCAR3 ovarian cancer cells. As shown in Fig. 3D, a decrease in DJ-1 expression, which correlates with increase in DMF concentration, was observed. Time dependency of this effect was also evaluated and a decrease in DJ-1 expression was observed from 8 hours posttreatment (Supplementary Fig. S4B). A decreased expression of DJ-1 was also observed in a primary lung cancer cell line treated with increasing concentration of DMF (Supplementary Fig. S4E).
Expression level of DJ-1 modulates cellular effects of high concentrations of DMF in OVCAR3 cells

To understand the role of DJ-1 in DMF cytotoxicity, expression of DJ-1 was modulated in OVCAR3 cells by DJ-1 siRNA and wild-type DJ-1 expression vector (wt DJ-1). As shown in Fig. 3E, compared with non-siRNA and control siRNA groups, transfection of DJ-1 siRNA in OVCAR3 cells caused not only a decrease in the endogenous protein levels of DJ-1, but also in total and nuclear fractions of NRF2. Lamin A and GAPDH were used as nuclear and cytosolic markers, respectively. Also, compared with the empty vector, overexpression of wt DJ-1 showed an increase in the protein levels of both DJ-1 and NRF2 (Fig. 3F). Similarly, in DMF-treated cells, DJ-1 downregulation induced depletion of nuclear and cytosolic NRF2 fractions, while expression of wt DJ-1 prevented it (Fig. 3E and F). Furthermore, we observed an interaction between NRF2 and DJ-1 (Supplementary Fig. S4F) through immunoprecipitation experiments. These results confirmed the previous observation that DJ-1 favors accumulation of NRF2 and its nuclear translocation. Moreover, DJ-1 level controls NRF2 depletion induced by DMF. We then examined the effect of DJ-1 levels in DMF cytotoxicity.

In cells not treated with DMF, downregulation of DJ-1 did not have any significant effect on cell viability (data not shown) but induced a small amount of apoptosis as evaluated by caspase-3 (Supplementary Fig. S3) and PARP cleavage (Fig. 4A, top left). However, compared with control siRNA–treated cells, downregulation of endogenous DJ-1 made the cells more susceptible to DMF-induced ROS production (Fig. 4B, P < 0.01) and decreased GSH (Fig. 4B, P < 0.01), decreased cell viability (Fig. 4B, P < 0.01) and induced apoptosis (Fig. 4A, top right). Overexpression of wt DJ-1 did not modify the amount of caspase-3 (Supplementary Fig. S4A) and PARP (Fig. 4A, bottom left) cleavages in untreated OVCAR3 cells but countered the observed high-dose DMF effect.

Figure 3.
Modulation of NRF2 expression and activity by DMF in OVCAR3 cells. Cells were either treated with 0.05% DMSO or with increasing concentrations of DMF for 24 hours. Cell lysates were prepared and analyzed on a 10% SDS-polyacrylamide gel followed by Western blotting using an anti-NRF2, HO-1, or γ-GCSc-specific antibody. Relative densitometry for NRF2 is shown (A), while the protein levels of HO-1 and γ-GCSc are shown in (B, bottom). Lamin A was used as a nuclear marker; GAPDH was used as a loading control. One representative of at least two independent Western blots is shown here. C, OVCAR3 cells were either left untransfected or transfected, with a control empty vector or the NRF2 plasmid as described in the “Materials and Methods”. After transfection, cells were further incubated with or without DMSO and DMF for 24 hours. Cell viability was then determined using the crystal violet assay method (C). Cells were also assayed for ROS production and GSH depletion (C). Cell lysates from A were again analyzed on a 10% SDS-polyacrylamide gel followed by Western blotting using an anti-DJ-1 antibody. Relative densitometry for DJ-1 is shown in D. In all the experiments, data are expressed as a percentage change relative to control. In each case, the mean of three independent experiments is shown. *P < 0.05; **P < 0.01. Modulation of cellular effects of high concentrations of DMF by expression level of DJ-1 in OVCAR3 cells. E and F, OVCAR3 cells were left un transfected (no siRNA) or transfected with nontargeting scramble siRNA (ctrl siRNA), DJ-1 siRNA, wild-type DJ-1 (wt DJ-1), or the control vector pGWI (EV) with the NIH OVCAR-3 Cell Avalanche Transfection Reagent. Twenty-four hours after transfection, the cells were left untreated; treated with 0.05% DMSO (ctrl), or with 100 μmol/L DMF for an additional 24 hours. Whole cell, nuclear, and cytosolic lysates were prepared and analyzed on a 10% SDS-polyacrylamide gel followed by Western blotting using the appropriate antibody. E and F, immunoblots showing the expression of nuclear and cytosolic fractions of NRF2, total NRF2, DJ-1 and KEAP1 in OVCAR3 cells that were left untreated after transfection (left). Right, immunoblots showing the expression of nuclear and cytosolic fractions of NRF2 in OVCAR3 cells that were treated with 100 μmol/L DMF for 24 hours following transfection. Lamin A was used as a nuclear marker, whereas GAPDH was used as a loading control and in each case one representative of at least two independent Western blots is shown.
on ROS (Fig. 4B) induction and GSH (Fig. 4B) levels, cell viability (Fig. 4B), and apoptosis (Fig. 4A, bottom right).

**DMF has antitumor effects in mice models of colon cancer**

To confirm the observations made on cancer cells in vitro, the anticancer property of DMF was assessed in two mice models. The preventive effect of DMF was assessed in a first model in which colon cancer was chemically induced in mice by the AOM/DSS combination (Supplementary Fig. S6A; Table 1). After week 20, the incidence of colon cancer in DMF-treated mice appeared lower than in DMSO-treated control mice, compared with DMF-treated mice (Fig. 5A, top; Table 1). These tumors were further histologically diagnosed as tubular adenoma and were more pronounced in DMSO-treated control group and combination of paclitaxel and DMF did not induce further decrease. The nuclear, activated, form of NRF2 protein expressions, as described in the Materials and Methods section. As shown in Fig. 5C, decreased expression of total NRF2 and DJ-1 were observed in the DMF-treated group compared with the control group. Paclitaxel treatment alone was associated with a slight decrease expression of total NRF2 and DJ-1 compared with control group and combination of paclitaxel and DMF did not induce further decrease. The nuclear, activated, form of NRF2 was further analyzed by IHC in tumors excised from animals in the CT26 mouse model. The percentage of stained nuclei (Fig. 5D; Supplementary Fig. S6A) was significantly lower in mice treated with DMF alone compared with control mice (−14%, respectively; P<0.05) and in mice treated with DMF + paclitaxel.

### Table 1. The preventive effect of DMF against colon cancer chemically induced in mice by AOM/DSS

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Cancer induction</th>
<th>Treatment</th>
<th>No. of mice examined</th>
<th>Body weight (g)</th>
<th>Av. length of bowel (mm)</th>
<th>No. of mice with visible prolapses</th>
<th>Av. no. of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM/DSS</td>
<td>DMSO</td>
<td>9</td>
<td>19.92 ± 1.96</td>
<td>69.72 ± 4.70</td>
<td>9 ± 16.86</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>AOM/DSS</td>
<td>DMF, 20 mg/kg</td>
<td>9</td>
<td>21.46 ± 0.89*</td>
<td>80.30 ± 2.10**</td>
<td>1</td>
<td>3 ± 7.50**</td>
</tr>
<tr>
<td>3</td>
<td>Non</td>
<td>Drinking water</td>
<td>4</td>
<td>23.67 ± 3.35**</td>
<td>82.99 ± 7.16**</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**NOTE:** * and ** indicates statistically significant difference from group 1 (*, P < 0.05 and ***, P < 0.001).*

In a second model, mice bearing CT26 tumors were treated with DMSO i.p. once daily, “force feeding” DMF once daily, i.p. paclitaxel or a combination of DMF and paclitaxel (Fig. 5C). Mice treated with DMF alone developed smaller tumors than mice treated by DMSO alone from day 9 (P < 0.05; Fig. 5C). Paclitaxel was also associated with smaller tumors from day 9 (P < 0.05). Tumor sizes were significantly smaller from day 12 in mice treated with DMF + paclitaxel than in mice treated with DMSO (P < 0.01), paclitaxel (P < 0.01), or DMF (P < 0.01) alone. AOPP levels were also significantly increased in DMF- or DMF in combination with paclitaxel-treated mice compared with DMSO control mice (data not shown for CT26 model). To determine the influence of DMF on the NRF2/DJ-1 axis in the CT26 mouse model, Western blot analysis was performed to detect changes in NRF2 and DJ-1 protein expressions, as described in the Materials and Methods section. As shown in Fig. 5C, decreased expression of total NRF2 and DJ-1 were observed in the DMF-treated group compared with the control group. Paclitaxel treatment alone was associated with a slight decrease expression of total NRF2 and DJ-1 compared with control group and combination of paclitaxel and DMF did not induce further decrease. The nuclear, activated, form of NRF2 was further analyzed by IHC in tumors excised from animals in the CT26 mouse model. The percentage of stained nuclei (Fig. 5D; Supplementary Fig. S6A) was significantly lower in mice treated with DMF alone compared with control mice (−14%, respectively; P<0.05) and in mice treated with DMF + paclitaxel...
compared with mice treated by paclitaxel alone (12%, respectively; \( P < 0.05 \)).

Taken together, these \emph{in vivo} data show that daily administration of DMF decreases growth of established tumor and prevents tumor formation induced by chronic colitis in inflammation. Moreover, the observed increase of plasma AOPP, the decrease expression of total and nuclear NRF2 and DJ-1 in CT26 tumors from DMF-treated mice is consistent with the \emph{in vitro} findings regarding modulation of NRF2/DJ-1 axis by DMF.

**Discussion**

The data presented in this report supports the antitumor effect of DMF and discloses the molecular mechanism model for DMF-induced cancer cell death involving the relative depletion of NRF2 antioxidant system and increased ROS production. DMF is well known for its immunomodulatory effects and has recently been approved for the treatment of psoriasis and relapsing-remitting multiple sclerosis (1–3). It is now also emerging as an anticancer agent as it has been shown to inhibit melanoma (13) and more recently, colon cancer cell growth (12). Nevertheless, the underlying molecular mechanisms involved still remain elusive.

DMF is a methyl-ester of fumarate, an intermediate in the Kreb cycle used by cells to metabolize pyruvate to form ATP. As an endogenous electrophile, fumarate is involved in protein succination, wherein, it spontaneously reacts with cysteine (SH) residues in proteins by a Michael addition reaction to form S-(2-succinyl) cysteine (2SC); and studies have shown that succination of these proteins in cells may impact their functions (32). KEAP1 has been shown to be succinated on two critical cysteine residues by fumarate, which disrupts its interaction with NRF2, resulting in stabilization and accumulation of nuclear NRF2 (ref. 15; Fig. 6).

Thus, low concentrations of DMF were reported to exert neuroprotective effects in several noncancer cell models via activation of the NRF2 antioxidant pathway (6, 33). In agreement with these findings, we observed that DMF at lower concentrations (<10 \( \mu \text{mol/L} \)) induces nuclear translocation of NRF2 and expression of downstream target genes, HO-1 and \( \gamma \)-GCS, in ovarian cancer cells. Moreover, by inducing NRF2, low-dose DMF prevents oxidative stress and cell death induced by paclitaxel.

However, our data indicate that at higher concentrations (>25 \( \mu \text{mol/L} \)), DMF displays cytotoxicity in several cancer cell lines, induced parallel increase of ROS, elevated levels of oxidized glutathione, and reduced glutathione depletion. Cotreatment with N-acetylcysteine demonstrates that DMF cytotoxicity is related to oxidative stress. Surprisingly, such high-dose DMF (25 \( \mu \text{mol/L} \)) was associated with a decrease in total NRF2 protein levels and its nuclear (active) fraction with increasing DMF concentrations, while the (inactive) cytosolic fraction remained unchanged. A similar pattern was observed for both HO-1 and
γGCSc, confirming the decrease of NRF2 transcriptional activity. The results therefore suggest that at lower concentrations, DMF activates the NRF2 signaling pathway compared with untreated cells, whereas at higher concentrations, this effect disappears and the NRF2 activity tends to be lower than in untreated cells. The transfection of OVCAR3 ovarian cancer cells with the NRF2 cDNA shows that the observed DMF-induced oxidative stress and cytotoxicity is indeed dependent of NRF2 depletion. Consistent with this finding, results from Western blot analysis and IHC experiments performed in tumor samples from DMF-treated mice showed that the daily administration of 20 mg/kg DMF decreased total NRF2 expression and its nuclear localization. It is likely that the nuclear localization of this pool of NRF2 requires the contribution of other factors such as the NRF2 protein stabilizer DJ-1.

Chemical structure and previous proteomic data (33, 34) strongly suggest that NRF2 is not a direct target of DMF, while the NRF2 protein stabilizer DJ-1 could be. Thus, modifications of its Cys 106 have been associated with a loss of DJ-1 functions and degradation by proteasome (35). Indeed, we observed that high concentration of DMF induced an early concentration-dependent decrease in DJ-1 protein expression in OVCAR3 ovarian cancer cells. DJ-1 has been shown to stabilize the NRF2 protein and increase its nuclear translocation and transcriptional activity by preventing its association with KEAP1 (20). As previously reported (20), we observed by immunoprecipitation experiments a direct interaction between NRF2 and DJ-1. In cancer cells treated with high concentration of DMF, a causal relationship was demonstrated by siRNA and expression vector experiments between decrease DJ-1 protein level in one hand, and nuclear NRF2 depletion, oxidative stress, GSH depletion, and finally cancer cell death, on the other.

These data suggest that not only does high concentration of DMF inhibit KEAP1, but also DJ-1, preventing NRF2 activation (Fig. 6). In this situation, the lethal oxidative stress and glutathione depletion are the result of the impairment of NRF2-mediated antioxidant defense, probably associated with the formation of covalent adducts between fumarate and glutathione, as reported previously (17). NRF2 has long been established as a key molecule for redox homeostasis in cancer cells which produce a large amount of ROS, due to high metabolic activity, mitochondrial dysfunction, or oncogenic activation. DMF could be specifically
active in cancers which develop high NRF2 activity in response to oxidative stress.

We report the antitumoral effects of DMF in two mouse models. In the syngeneic CT26 mouse model, DMF on its own, and to an even greater extent, in combination with paclitaxel, significantly reduces tumor growth. The daily dose of DMF administered to mice was the same as in previous studies of DMF-induced neuro- and cardioprotection (4, 6) but the length of treatment was much shorter in these studies (1–5 days). These observations could be related to DMF tissue accumulation, which is associated with longer treatment or alternatively, to a lesser DMF toxic effect on noncancer cells compared with the malignant ones. This differential effect of DMF may be related to the lower level of oxidative stress in noncancer cells that render the cells more permissive to NRF2 depletion. In addition, previous report showed that activation of the NRF2 antioxidant response pathway is independent of DJ-1 in primary neural cells and tissues, suggesting that the protective role of DJ-1 may be less important for NRF2 function in noncancer cells (36). Even more interesting is the fact that the daily administration of DMF to animals in this CT26 mouse model attenuated the protein levels of both NRF2 and its stabilizer DJ-1, which was yet again consistent with the in vitro findings.

In the chemically induced mouse model, DMF prevented weight loss, colon shortening, and rectal prolapse that were associated with colorectal cancer occurrence in DMF-treated animals compared with controls. This clinical effect could in fact be related to inhibition of chronic inflammation induced by DSS (through preventive effect) or to tumor growth inhibition as described in the CT26 model (antitumoral effect). The significant increase of advanced oxidized protein products in the sera of DMF-treated mice indicates that DMF causes oxidative stress in these animals and favors the antitumoral effect hypothesis.

In conclusion, our data support the development of DMF as a new anticancer agent. Its antitumoral effect was observed in two mice models at a dose well-tolerated by humans and applicable to clinical practice (37). The elucidation of the underlying molecular mechanisms involving NRF2–DJ1 interaction may help to identify most sensitive tumors.

Disclosure of Potential Conflicts of Interest

F. Goldwasser is a consultant/advisory board member for Fresenius Kabi. No potential conflicts of interest were disclosed by the other authors.

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Molecular Cancer Therapeutics

Dimethyl Fumarate Controls the NRF2/DJ-1 Axis in Cancer Cells: Therapeutic Applications

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