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Original Article

In Vitro and In Vivo Studies of Non-Platinum-Based Halogenated Compounds as Potent Antitumor Agents for Natural Targeted Chemotherapy of Cancers

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ABSTRACT

Based on a molecular-mechanism-based anticancer drug discovery program enabled by an innovative femtomedicine approach, we have found a previously unknown class of non-platinum-based halogenated molecules (called FMD compounds) as potent antitumor agents for effective treatment of cancers. Here, we present in vitro and in vivo studies of the compounds for targeted chemotherapy of cervical, breast, ovarian, and lung cancers. Our results show that these FMD agents led to DNA damage, cell cycle arrest in the S phase, and apoptosis in cancer cells. We also observed that such a FMD compound caused an increase of reduced glutathione (GSH, an endogenous antioxidant) levels in human normal cells, while it largely depleted GSH in cancer cells. We correspondingly found that these FMD agents exhibited no or little toxicity toward normal cells/tissues, while causing significant cytotoxicity against cancer cells, as well as suppression and delay in tumor growth in mouse xenograft models of cervical, ovarian, breast and lung cancers. These compounds are therefore a previously undiscovered class of potent antitumor agents that can be translated into clinical trials for natural targeted chemotherapy of multiple cancers.

1. Introduction

The conquest of cancer continues to pose great challenges to medical science (Reese, 1995; Varmus, 2006; Alberts, 2011; Watson, 2013). There is a compelling need for innovative cancer research integrating biomedical sciences with new technology in order to ultimately conquer cancer. Femtosecond (fs) (1 fs = 10^{-15} s) time-resolved laser spectroscopy (fs-TRLS) is a direct technique to visualize molecular reactions in real time. Its application to chemical and biological systems gave birth to the fields of femtochemistry and femtobiology, with the pioneering contribution of Zewail (2000). Further, femtomedicine (FMD), which fuses ultrafast laser spectroscopic techniques with biomedical sciences, was recently coined to advance fundamental understanding and therapies of human diseases notably cancer (Wang et al., 2009; Lu, 2010a; Nguyen et al., 2011).

In particular, we proposed that dissociative-electron-transfer (DET) reactions may be exploited to improve cancer therapy (Lu, 2010a; Luo et al., 2012). Prior to our studies in FMD, it had strikingly been found that electron-induced dissociation of halogenated molecules were enhanced by up to 30,000 times with the presence of polar molecules such as NH_3 and H_2O, and a DET mechanism involving a prehydrated electron (e^-_{pre}) trapped in polar media was proposed to explain the results (Lu and Madey, 1999; Lu, 2010b). Employing fs-TRLS, we demonstrated that e^-_{pre} in liquid water has a lifetime of about 500 fs and is a weakly-bound excited state of the hydrated/solvated electron (Wang et al., 2008). We further discovered that the e^-_{pre} plays a key role in causing the biological effects of ionizing radiation: its ultrafast DET reaction leads to chemical bond breaks at the guanine base (Wang et al., 2009) and strand breaks in DNA (Nguyen et al., 2011). Our findings challenged the conventional notion that damage to the genome by ionizing radiation is mainly oxidative, induced by oxidizing $OH^•$, and might lead to improved strategies for radiotherapy of cancer (Lu, 2010a).

We have also discovered the DET mechanism of cisplatin (Lu, 2007; Lu et al., 2007). Platinum compounds as a class of antitumor agents were discovered unexpectedly by the biophysicist Rosenberg et al. (1965, 1969). Despite its severe toxicity (Reese, 1995), cisplatin is a widely-used drug in the treatment of a variety of cancer, including ovarian, testicular, cervical, bladder, lung, head and neck, lymphomas, and brain cancers, both as chemotherapy alone and in combination with radiotherapy. Although platinum compounds are well-known DNA-attacking agents, their precise molecular mechanism of action remained elusive until recently. Through fs-TRLS studies, we found that cisplatin is

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very effective for the DET reaction with a weakly-bound electron, such as an ultrashort-lived e^-_{pre} produced by radiolysis of water (Lu, 2007):

$$\begin{aligned} e_{pre}^- + Pt(NH_3)_2Cl_2 &\rightarrow [Pt(NH_3)_2Cl_2]^{*-} \rightarrow Cl^- + Pt(NH_3)_2Cl^{\bullet} \\ e_{pre}^- + Pt(NH_3)_2Cl &\rightarrow [Pt(NH_3)_2Cl_2]^{*-} \rightarrow Cl^- + Pt(NH_3)_2^{\bullet}. \end{aligned} \quad (1)$$

The resultant *cis*-$Pt(NH_3)_2Cl^{\bullet}$ and *cis*-$Pt(NH_3)_2^{\bullet}$ radicals are highly effectively in inducing DNA strand breaks. For cisplatin as a chemotherapeutic drug, its DET reaction with the G base in DNA was also observed (Lu et al., 2007). This DET mechanism has directly unraveled the radiosensitizing effect of cisplatin (Lu, 2007) and the long-existing mystery why the cisplatin-like drugs result in the preferential binding of the *cis*-$Pt(NH_3)_2$ to two neighboring G bases in DNA (Lu et al., 2007). Other researchers have subsequently confirmed this DET mechanism in experiments and theoretical calculations (Kopyra et al., 2009; Kuduk-Jaworska et al., 2011).

Similarly, we also found an ultrafast DET mechanism for a halopyrimidine family (XdUs, X = I, Br and Cl) as potential sensitizers for radiotherapy (Wang et al., 2006; Wang and Lu, 2007, 2010). Using fs-TRLS, we observed directly the ultrafast DET reactions of XdUs with e^-_{pre}, leading to the formation of the reactive radical ($dU^{\bullet}$), but they are far less efficient than that of cisplatin, due to the lack of the NH_3 groups as the effective electron-transfer promoter (Lu and Madey, 1999; Lu, 2010b), and thus have not sufficient radiosensitizing efficacies.

Our studies in FMD have led to the discovery of a reductive damaging mechanism in DNA (Wang et al, 2009; Lu, 2010a; Nguyen et al., 2011). More recently, we further demonstrated the reductive damaging mechanism in human lung and skin normal cells, which causes serious reductive DNA damage and genetic mutations and might be related to the pathology of diseases, especially cancer (Lu et al., 2013). We observed contrast differences in effects of an antioxidant (EGCG) on human normal and abnormal (cancer) cells: It caused DNA damage, mutations and cell death more effectively in normal cells than cancer cells. Our results provided a compelling explanation for the confirmed observation in clinical trials that increases of some cancers such as lung and skin cancers are associated with antioxidants (The ATBC, 1994; Albanes et al., 1996; Omenn et al., 1996). DeNicola et al. (2011) also recently showed evidence in mice that several oncogenes actively induce transcription of Nrf2 — the transcription factor that mainly regulates physiological antioxidant pathways — that is required for tumor initiation. This Nrf2 antioxidant pathway may therefore lead to a more reduced intracellular environment that enables tumor initiation, though its link to a promotion effect of antioxidant supplementation on human tumorigenesis has been questioned (e.g., Perera and Bardeesy, 2011). Moreover, researchers have also reviewed that the resistance or incurability of (later-stage) metastatic cancers are related to higher-level endogenous antioxidants (e.g., glutathione) in a variety of types of tumor cells/tissues (Calvert et al., 1998; Estrela et al., 2006; Traverso et al., 2013; Watson, 2013). Our finding of a reductive damaging mechanism may lead to effective prevention and therapies.

Our mechanistic studies in FMD, described above, have now led to the discovery of a previously unknown class of non-platinum-based compounds, which essentially act as cisplatin analogues. Some general features of these compounds are that they comprise an aromatic ring (rather than a platinum coordinating ion), coupled to two NH_2 groups as the electron transfer promoter and one or more halogen atoms (Cl, Br or I), such as 4,5-dichloro/dibromo/diiodo-1,2-diaminobenzene (benzenediamine/phenylenediamine) and 4(3)-chloro/bromo/iodo-1,2-diaminobenzene (benzenediamine/phenylenediamine) (shortened as FMD-nX-DABs or $B(NH_2)_2X_n$ with X = Cl, Br or I, and n = 1, 2). Their structures are shown in Fig. S1 in Supplementary information. We have observed that such FMD compounds are highly effective in DET reaction with weakly-bound electrons (unpublished data; Wang et al.), which may be either intrinsically rich in tumor cells/tissues or exogenously produced by ionizing radiation of biological systems. The resultant radical $[B(NH_2)_2X_{n-1}]^{\bullet}$ can effectively lead to DNA damage and cell death. An advantage of these FMD agents over XdUs and cisplatin is that FMDs are effective in a DET reaction but are far less toxic due to the absence of the heavy metal (Pt). It has been shown that the cisplatin-induced nephrotoxicity is mainly due to the binding of Pt to proteins in kidneys of cisplatin-treated mice (Townsend and Hanigan, 2002; Zhang et al., 2006). It is the severe toxicity of Pt that led to the call to terminate the attempt to develop new Pt-based anticancer drugs (Reese, 1995). Thus, such non-Pt-based FMD compounds (FMD-nX-DABs) are highly desirable anticancer agents. This study presents in vitro and in vivo results of FMDs as translatable antitumor agents for natural targeted chemotherapy of cervical, breast, ovarian, and lung cancers with minimal toxicity.

2. Methods

Details for cell lines and culture conditions, cell viability assays, DNA DSB assays, apoptosis assays, and cell cycle analysis have been published previously (Luo et al., 2012; Lu et al., 2013). Only brief descriptions and different details are given here. ME-180 and MDA-MB-231 cells were cultured with the ATCC-formulated McCoy's 5A medium supplemented with 10% FBS, and L-15 Medium (Leibovitz) with 10% FBS, respectively. All the cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, except for MDA-MB-231 cells maintained at no CO_2 (according to ATCC's instruction). The cell growth and survival rates with various treatments were measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) assay (Invitrogen). DNA double-strand breaks (DSBs) were measured by detection of the phosphorylated H2AX foci using the HCS DNA Damage Kit (Invitrogen). The images of cells were acquired with a Nikon Eclipse TS100 fluorescence microscope; quantitative analyses of activated γ-H2AX (DNA DBS yield) in the cells were performed using an Image J software. The CellEvent™ Caspase-3/7 Green Detection Kit (Invitrogen) was used for the detection of activated caspases and apoptotic cells using fluorescence microscope, following the vendor's protocol, as described previously, while DNA fragmentation and cell cycle analysis were measured by flow cytometry using a standard APO-BrdU™ TUNEL assay kit (Invitrogen) (Luo et al., 2012). For the latter, the data were analyzed using a FlowJo software for quantitative measurements of apoptotic cells. The DNA histograms were also used for cell cycle analysis. The fits to the DNA histograms of the samples were performed by the Watson model to achieve a lowest RMS (root mean squared) score.

To show the reactivity of our FMD agents with antioxidants in human normal and cancer cells, we measured the intracellular reduced glutathione (GSH) levels of both human normal cells (GM05757) and human cervical cancer cells (ME-180) in control (with no FMDs) and treated by various concentrations of an exemplary FMD compound (FMD-2Br-DAB) using a GSH detection kit (Abcam), following the manufacturer's protocol. Briefly, 2×10^5 GM05757/ME-180 cells were plated into T25 flasks in 8 ml of MEM/McCoy's 5A with 10% FBS and were cultured overnight at 37 °C before the addition of the test FMD compound (0, 50, and 100 μM). Cells were incubated with the compound for 24 h, and were harvested and washed with PBS. For all samples, the cells were counted and lysed by 5% metaphosphoric acid at the concentration 8×10^5 cells/100 μl. The cell lysates were centrifuged at 12,000 rpm for 5 min and supernatants were collected and diluted using the assay buffer by 10 times for our experiments. The GSH levels were detected with a 96-well fluorometric microplate reader for an excitation wavelength at 485 nm and emission wavelength at 538 nm, which were close to the ex/em = 490 nm/520 nm suggested by the manufacturer.

To derive the mouse xenograft models of ME-180 cervical cancer, A549 lung cancer, MDA-MB-231 breast cancer and NIH:OVCAR-3 ovarian cancer for in vivo growth delay studies involving a FMD compound, 6–8 week old female SCID mice were injected s.c. in the flank with 1.5×10^6 ME-180 cells or 5×10^6 A549/MDA-MB-231/NIH:OVCAR-3 cells. The treatments were started when xenografts reached a volume of 100–150 mm^3. For each treatment, mice were allocated in complete

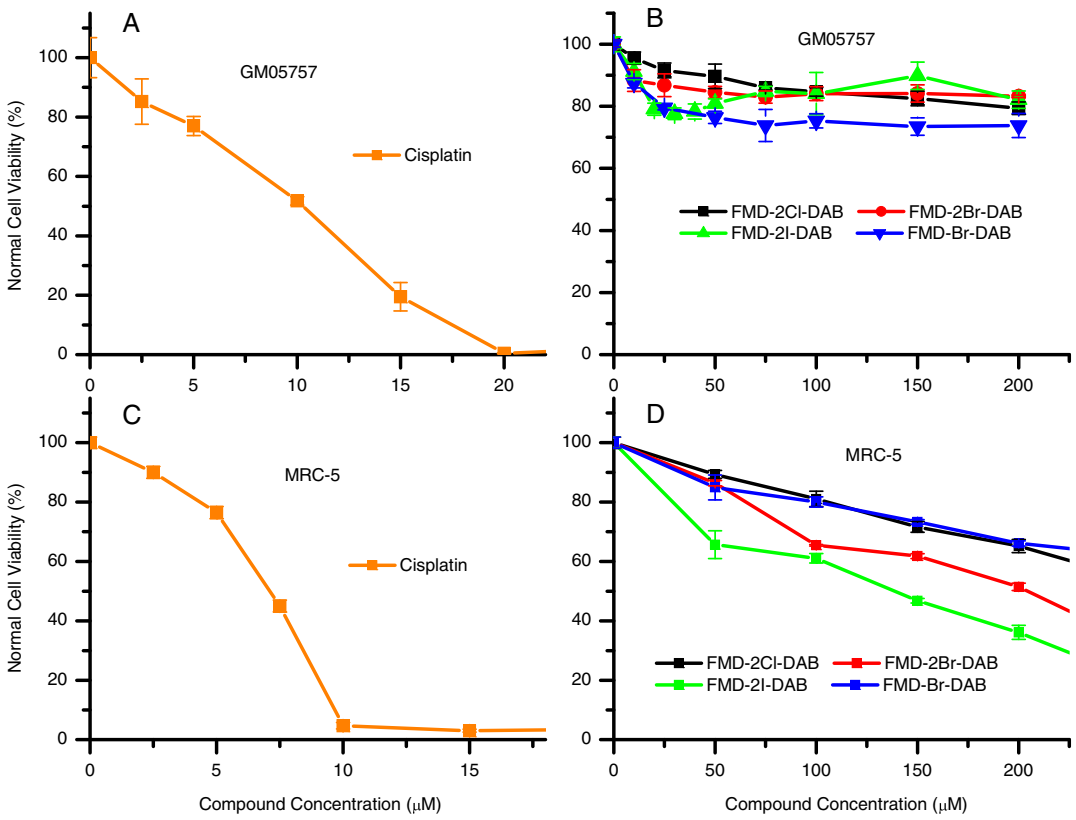


Fig. 1. In vitro toxicity assays of FMD compounds (FMD-nX-DABs) versus cisplatin. Cell viabilities of human normal cells (GM05757 and MRC-5) after 72-hr treatment of cisplatin or FMD compounds at various concentrations, where the clinically used chemotherapeutic drug and radiosensitizer cisplatin was used as a reference. The cell viability was measured by MTT. A: GM05757 normal cells treated by cisplatin, showing an IC50 value of ~10 μM. B: GM05757 normal cells treated by FMD compounds, showing an IC50 value over 200 μM. C: MRC-5 normal cells treated by cisplatin, showing an IC50 value of ~6 μM. D: MRC-5 normal cells treated by FMD compounds, showing an IC50 value over 200 μM.

randomness into two groups with 5 mice per group: (1) control (no FMD); (2) FMD-2Br-DAB at 7 mg/kg or FMD-2I-DAB at 5 mg/kg was administrated for totally 5/10 IP injections at each other day. Mice were weighed, and flank tumors were measured every 2–5 days with calipers, and tumor volumes were calculated. Growth delay was calculated as the time difference (in days) between treatment and control groups to grow to 500 mm^3.

For acute toxicity assay, liver and kidney toxicity biomarkers were measured through proteins, enzymes, metabolites, and electrolytes. Blood samples were collected by a terminal cardiac puncture of the mice at the end of the treatment with 0 and 7 mg/kg FMD-2Br-DAB or 5 mg/kg FMD-2I-DAB daily for 10 days. Analyses of blood serum samples were provided by the Animal Health Laboratory of the University of Guelph. Moreover, mice were observed for any physical toxicity. These physical indicators included: body weight changes, dull sunken eyes, rapid/shallow breathing, hunched back, and lethargy.

Wax-embedded tumor tissue samples from SCID mice in the control group and treatment groups (FMD-2Br-DAB at 7 mg/kg × 5; FMD-2I-DAB at 5 mg/kg × 5) were obtained at 24 h post-treatment and sectioned at 4 μm onto polarized slides for colorimetric TUNEL assay. To visualize and quantify apoptotic cells in tumor tissue, TUNEL assay was performed using the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Apoptotic cells were visualized by staining them in brown color. Nuclei were counterstained using Harris-modified hematoxylin, and slides were mounted. Percentage of apoptotic cells is expressed as a percentage of total cells in the measured regime.

All the animal experiments were conducted in compliance with the Animals for Research Act of Ontario, the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care and the University of Waterloo's Guidelines for the Care and Use of Animals in Research and Teaching (AUPP No. 13-30).

Data are presented as mean value ± SEM, and statistically analyzed with two-tailed and paired t-tests. A P value < 0.05 was considered statistically significant ($P^* < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

3. Results

3.1. In Vitro Toxicity Tests of FMD-nX-DABs vs Cisplatin

The in vitro toxicity of FMD-nX-DABs versus cisplatin was investigated in human skin normal cells (GM05757) and lung normal cells (MRC-5), which are sensitive to the toxicity of exogenous chemicals (Lu et al., 2013). The cells were incubated with each of the agents for 72 h, and then the cell viability was measured by MTT assay, one of the most commonly used cell viability assays. As shown in Fig. 1A and C, the normal cells were effectively killed by cisplatin in a dose-dependent manner with measured IC50 values of about 10 and 6 μM (at which the cell survival rate is 50% with respect to untreated cells) for GM05757 and MRC-5 normal cells, respectively. This confirms that cisplatin is indeed a highly toxic drug. In contrast, the data plotted in Fig. 1B and D show that the treatment of FMD compounds led to little toxicity toward human normal cells with measured IC50 values of ≥200 μM (except IC50 = 150 μM for FMD-2I-DAB on MRC-5 cells), which were more than 20 times the IC50s of cisplatin.

3.2. In Vitro Anti-Cancer Effect Tests of FMD-nX-DABs vs Cisplatin

The in vitro anti-tumor effects of FMD agents vs cisplatin were investigated in human cervical cancer (HeLa or ME-180), breast cancer

(MDA-MB-231), lung cancer (A549) cell lines and the cisplatin-resistant human ovarian cancer (NIH:OVCAR-3, HTB-161) cell line. As shown in Fig. 2A–D, these FMD agents effectively killed various cancer cells in a dose-dependent manner at 0–200 (250) μM. As also shown in Table S1, the FMD compounds had IC50 values far below 200 μM against HeLa, ME-180, MDA-MB-231, A549 and NIH:OVCAR-3 cancer cells for the 72-hr treatment. In particular, cervical cancer (ME-180) and lung cancer (A549) cells were most sensitive to these FMD compounds. Among the FMD compounds, FMD-2Br-DAB and FMD-2I-DAB exhibited the strongest antitumor effect, with IC50 values of 15–75 μM, except IC50 = 175 μM for FMD-2Br-DAB on NIH:OVCAR-3 cells. The cell viability results of these cancer cell lines treated by cisplatin are shown in Figs. S2A and B, and the corresponding IC50s are also listed in Table S1. It is particularly interesting to observe that FMDs as small molecules exhibited a natural selective killing of cancer cells (Figs. 1A and B, 2A–D). In contrast, cisplatin killed both normal cells and cancer cells more effectively than FMDs, but it had unfortunately poor selectivity and therefore severe toxicity (Fig. S2A and B). Note that for the ovarian cancer NIH:OVCAR-3 cell line that is a well-established cisplatin-resistant cell line, cisplatin exhibited a high killing efficiency at low doses (≤5 μM) for 72-hr incubation, but a certain fraction of cancer cells survived event at very high drug doses, showing the drug resistance. This is more obviously shown in Fig. S2B for the 48 hr-incubation, which shows that about 12% NIH:OVCAR-3 cancer cells survived from the high-dose cisplatin treatment. Similarly, a certain fraction of A549 cancer cells also exhibited resistance to cisplatin even at high doses.

3.3. In Vitro DNA DSB Measurements in Cancer Cells

To understand the antitumor activity, we further measured DNA DSBs in cancer cells treated by FMD-2Br-DAB as an exemplary FMD compound, using the HCS DNA Damage (γH2AX) Kit (Invitrogen) (Luo et al., 2012; Lu et al., 2013). Fig. 3 shows the fluorescence images of ME-180 cancer cells and the yield of DNA DSBs detected by γH2AX foci with/without the treatment of 0–200 μM FMD for 12 h. It is seen that the treatment of the FMD induced a significant amount of DSBs in genomic DNA in cervical cancer cells ($P < 0.005$); an increase by a factor of 3–5 in DNA DSB yield was observed with the presence of the FMD compound at ≥50 μM. Note that at higher concentrations ≥100 μM, no further increases in the counted number of γH2AX foci were obtained, but this was due to the severe damage to the DNA by the FMD, leading to DNA fragmentation. It is well-known that DNA DSBs are difficult to repair and thus potent inducers of apoptosis (cell death).

3.4. In Vitro Apoptosis Measurements by Assays of Activated Caspases and DNA Fragmentation

We also measured apoptosis-related caspase activation and DNA fragmentation in human normal cells (MRC-5) and human cervical cancer cells (ME-180) treated by cisplatin or FMD-2Br/I-DAB for 48 h, using a CellEvent™ Caspase-3/7 Green Detection Kit and a standard APO-BrdU™ TUNEL Assay Kit (both from Invitrogen), respectively (Luo et al., 2012; Lu et al., 2013). The results are shown in Fig. 4A–P. First, Fig. 4A–D, M and N show that cisplatin at 5–15 μM induced a significant activation of caspase-3 and -7 in MRC-5 normal cells, which was evident from a significant enhancement in green fluorescence, compared with untreated cells ($P < 0.001$). In contrast, the FMD compounds induced negligible caspase activation (apoptosis) in normal cells at concentrations up to 200 μM for FMD-2Br-DAB and 150 μM for FMD-2I-DAB. Second, in striking contrast, the results in Fig. 4E–H, and O for cancer cells show that the treatment of a FMD compound resulted in a significant activation of caspase-3 and -7 in cancer (ME-180) cells ($P < 0.001$). Moreover, the FMD compound also caused clear changes on nuclear morphology, showing condensed nuclei in the treated cancer cells, which is typical of late-stage apoptosis. Third, a landmark of cellular

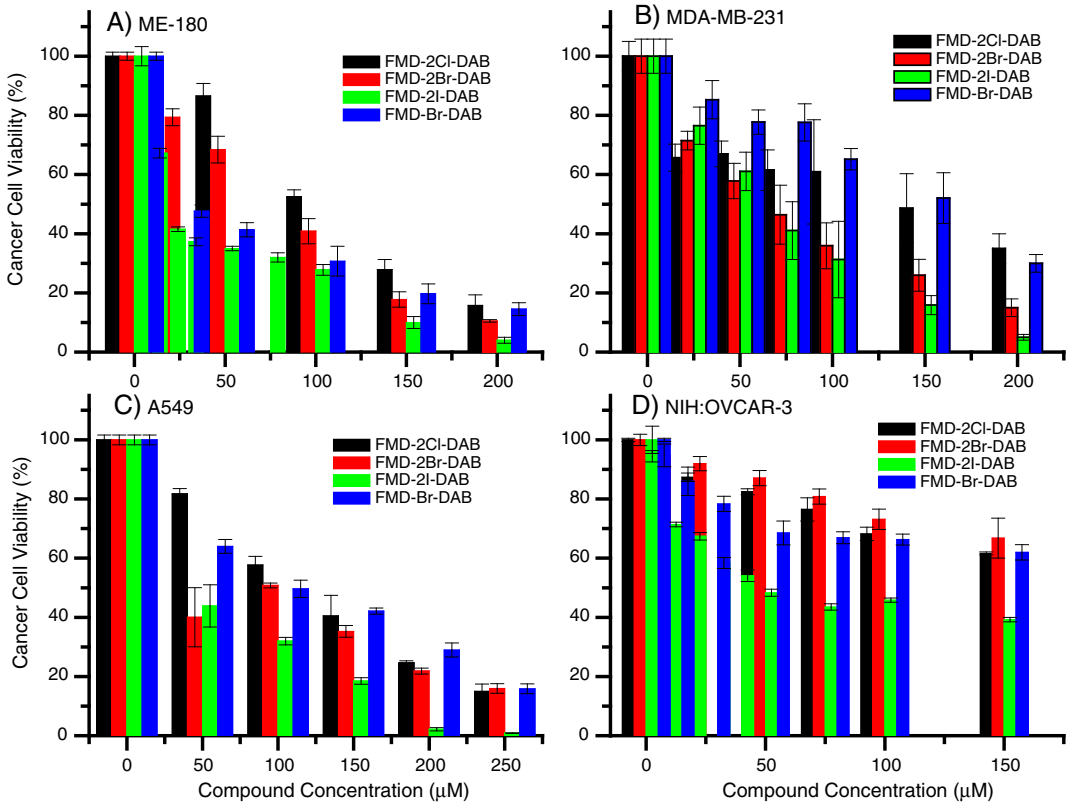


Fig. 2. Cell viability results of various human cancer cells treated by FMD compounds with various concentrations for 72 h. The cell viability rates were measured by MTT assay. A. Cervical cancer (ME-180). B. Breast cancer (MDA-MB-231). C. Lung cancer (A549). D. Cisplatin-resistant human ovarian cancer (NIH:OVCAR-3).

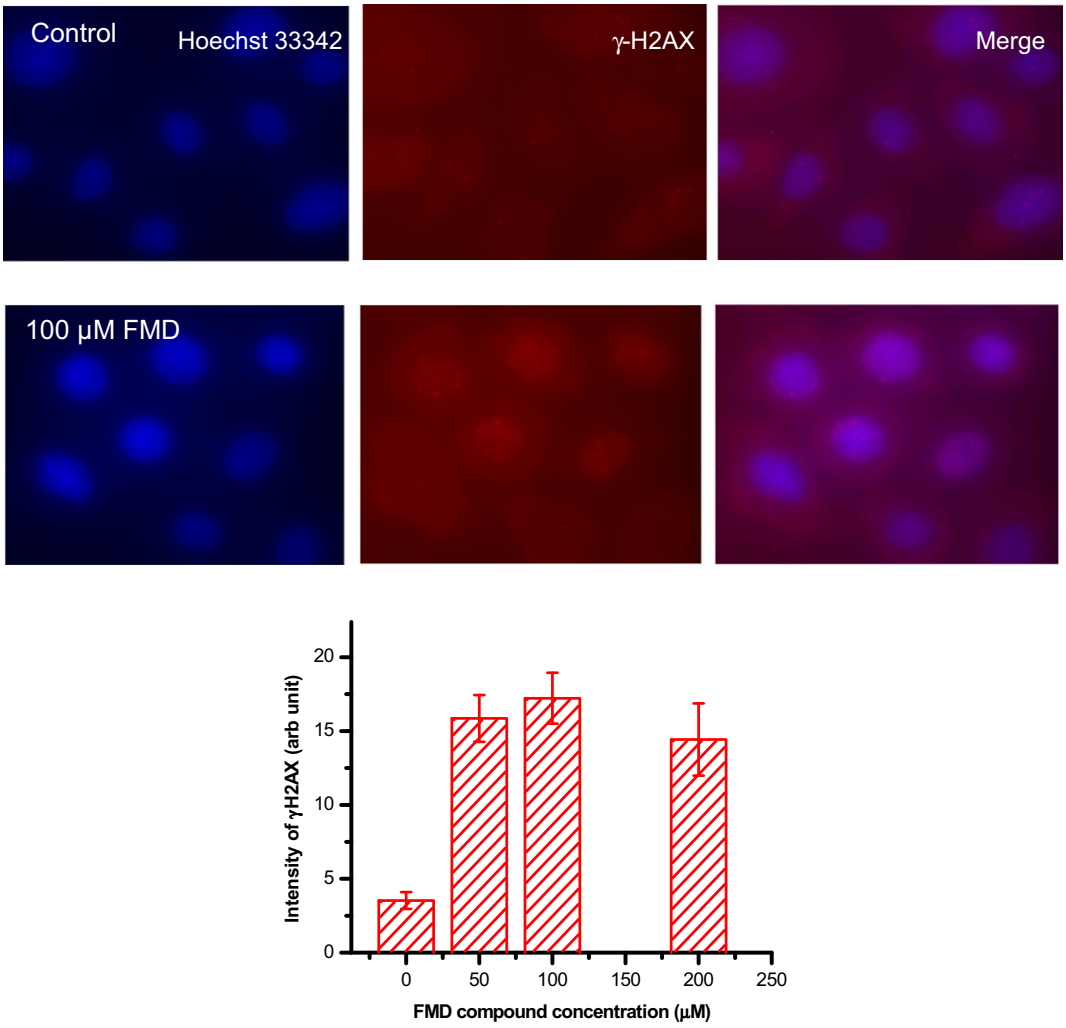


Fig. 3. Measurements of DNA double-strand breaks (DSBs) in human cervical cancer cells (ME-180) treated by FMD-2Br-DAB. The cancer cells were treated with various concentrations of the FMD compound for 12 h, and then imaged by the HCS (γ-H2AX) DNA Damage Kit (Invitrogen) using fluorescence microscopy. The yields of DNA DSBs versus compound concentration were obtained by quantitative analyses of activated γ-H2AX using an Image J software.

self-destruction by apoptosis is also the activation of nucleases that eventually degrade the nuclear DNA into fragments. Detection of these fragments is a straightforward and reliable method to quantify apoptotic cells. As shown in Fig. 4I–L and P for ME-180 cancer cells, the treatment of the FMD resulted in an enhancement of apoptosis in a dose-dependent manner. The fraction of BrdU-positive (apoptotic) cells was increased from 0.5 ± 0.4% for the cancer cells in control (with 0 μM FMD) to 40.7 ± 2.6% for the cancer cells treated with 150 μM FMD for 48 h. These data clearly show that the FMD compound resulted in large enhancements in apoptosis of cancer cells but no normal cells. We therefore conclude that the FMD compound led to DNA damage and apoptosis preferentially in cancer cells.

3.5. Cell Cycle Analysis of Cancer Cells Using Flow Cytometry

Using the same APO-BrdU™ TUNEL Assay Kit (Invitrogen), we performed cell cycle analysis of human cervical cancer cells (ME-180) treated by FMD-2Br-DAB at 0 and 100 μM for 48 h. The results are shown in Fig. 5. The cell cycle histograms contain statistics giving percentages for G1, S, and G2 phases of the cycle. It was observed that the treatment of the 50 μM FMD compound led to a slight decrease in the percentage of G1 phase from ~33% to ~30%, a decrease in the percentage of G2 phase from ~24% to ~13% and an increase in the percentage of S phase from ~36% to ~54%. These results show that the FMD agent induced DNA damage and blocked the cells in the DNA synthesis (S phase) ($P < 0.005$), in good agreement with the observed DNA damage caused by the FMD (Fig. 3). These results indicate that the FMD-induced DNA damage activated cell-cycle checkpoints, which induced a S-phase arrest. This is consistent with the generally accepted notion that DNA damage leads to cell cycle arrest; when repair of damage is difficult or incomplete, the cells undergo apoptosis. Thus, the induced DNA damage is closely associated with activation of cell-cycle checkpoints and apoptosis.

3.6. Measurements of Reduced Glutathione Levels in Normal and Cancer Cells Treated by a FMD

We measured the intracellular reduced glutathione (GSH) levels of both human normal cells (GM05757) and human cervical cancer cells (ME-180) in control (with no FMDs) and treated by an exemplary FMD compound (FMD-2Br-DAB) at various concentrations (0, 50, and 100 μM) for 24 h using a GSH detection kit (Abcam), following the manufacturer's protocol. The results are shown in Fig. 6. It is interesting to observe that the FMD caused an increase by about 40% of the GSH level in normal cells at 100 μM FMD. In contrast, the FMD significantly decreased the GSH level in cancer cells, compared with the untreated

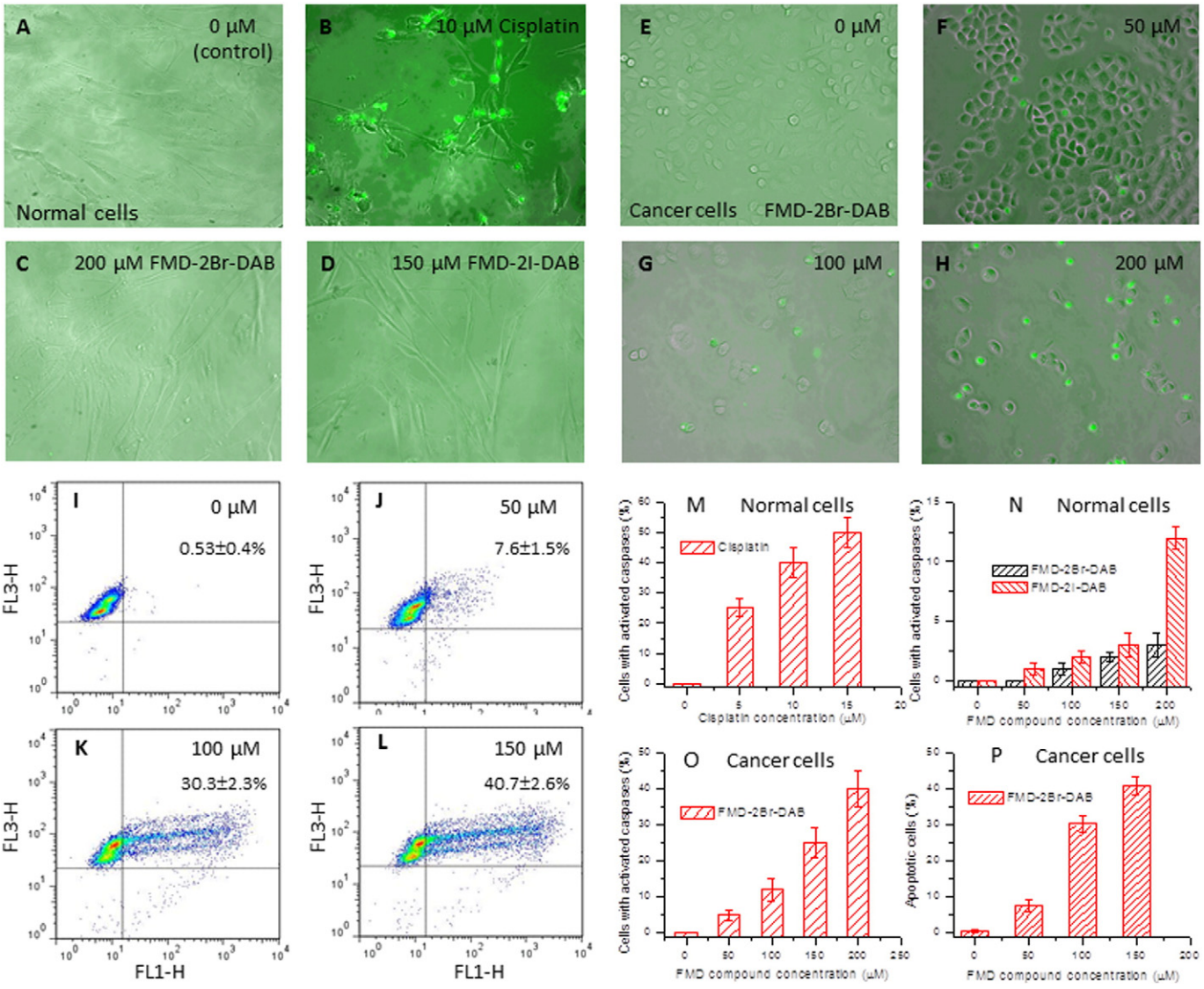


Fig. 4. Measurements of apoptosis in human normal cells (MRC-5) and human cervical cancer cells (ME-180) treated by cisplatin or FMD-2Br/I-DAB. The cells were treated at various agent concentrations for 48 h, and then analyzed by the CellEvent™ Caspase-3/7 Green Detection Kit using fluorescence microscopy and the APO-BrdU TUNEL Assay Kit using flow cytometry. A–D: Apoptosis in MRC normal cells with activated caspases represented by green fluorescence. E–H: Apoptosis in ME-180 cancer cells with activated caspases represented by green fluorescence. I–L: TUNEL assay of DNA fragmentation in ME-180 cancer cells; the cells in the right side of the vertical line are BrdU-positive cells (apoptotic cells) with percentages indicated. M–N: Percentages of MRC normal cells with activated caspases 3/7 versus cisplatin/FMD compound concentration. O–P: Percentages of activated caspases 3/7 and apoptosis (DNA fragmentation) in ME-180 cancer cells with versus FMD compound concentration.

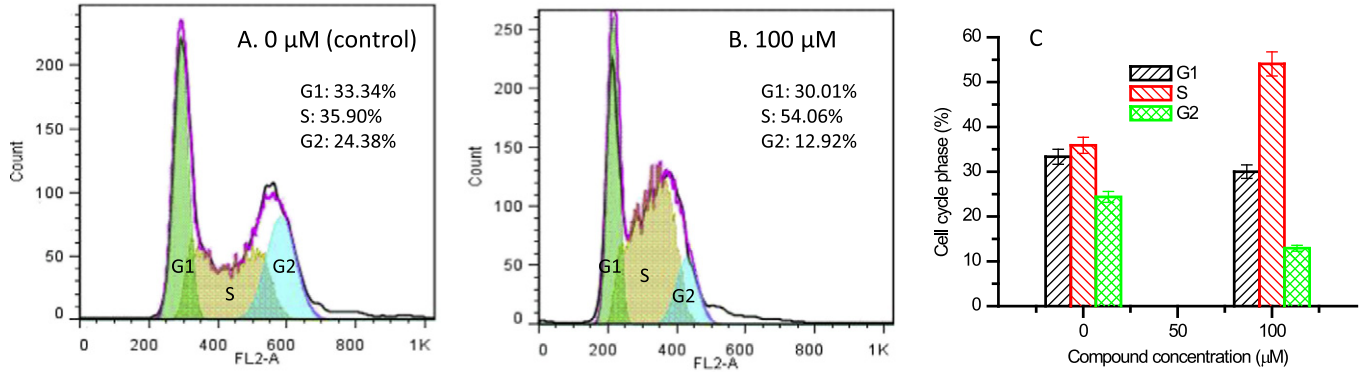


Fig. 5. Cell cycle analysis by flow cytometry of human cervical cancer cells (ME-180) treated by FMD-2Br-DAB for 48 h. The cell cycle histograms contain statistics giving percentages for G1, S, and G2 phases of the cycle, analyzed using a FlowJo software. The fits to the DNA histograms of the samples were performed by the Watson model. A: ME-180 cancer cells in control. B: ME-180 cancer cells treated by 100 μM FMD. C: Percentages of cell-cycle phases versus FMD concentration.

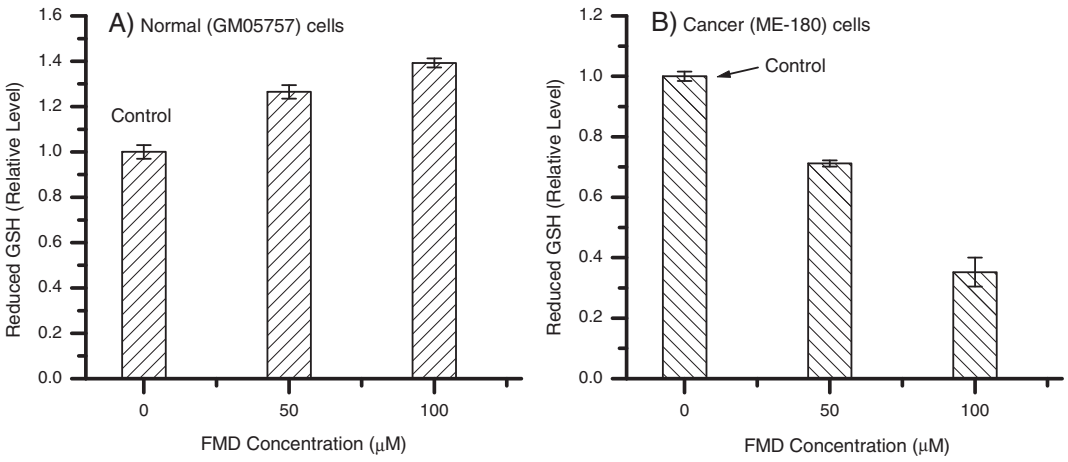
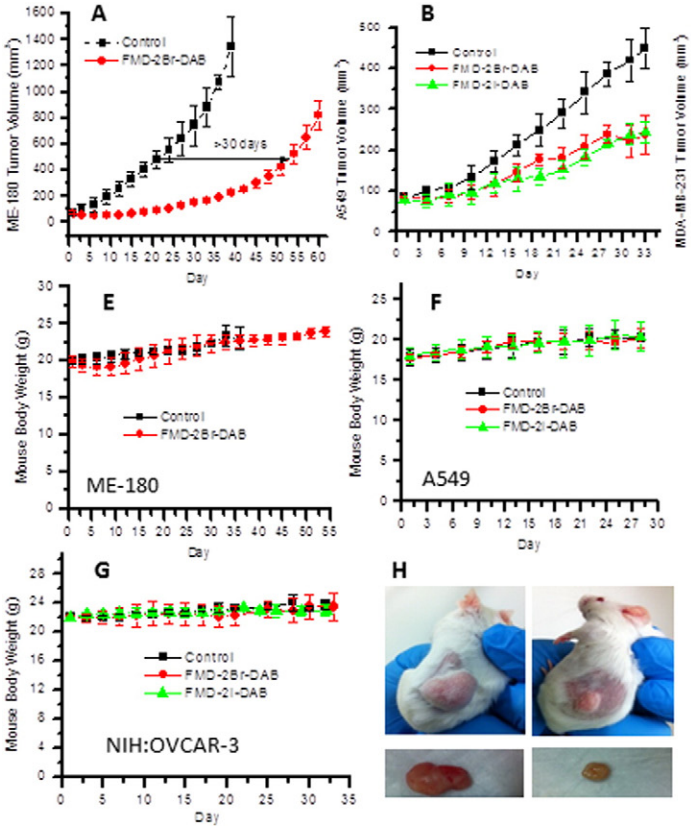


Fig. 6. Changes of the reduced glutathione (GSH) in human normal cells and human cervical cancer (ME-180) cells treated by FMD-2Br-DAB for 24 h. Both human normal cells (GM05757) and human cervical cancer cells (ME-180) were treated by FMD-2Br-DAB at various concentrations (0, 50, and 100 μM) for 24 h. The intracellular reduced glutathione (GSH) levels in the cells were then measured by a GSH detection kit (Abcam). The values were normalized to those of the cells in control.

cells ($P < 0.001$); the GSH level was decreased by about 65% (to ~35%) at 100 μM FMD. The marked selective depletion of the GSH is consistent with the expected DET reaction between a FMD and a GSH molecule preferentially in cancer cells. This is also consistent with the observed selective cytotoxic effects of the FMDs shown in Figs. 1–5 above and Figs. 7 and 8 below.



I. Toxicity Test Results

	0mg/kg	7mg/kg FMD-2Br-DAB	5mg/kg FMD-2I-DAB
Hepatotoxicity			
ALP	48 (+/-11)U/L	37 (+/-14)U/L	60 (+/-6)U/L
ALT	50 (+/-42)U/L	44 (+/-41)U/L	61 (+/-39)U/L
TBIL	1.3 (+/-0.9) umol/L	1 (+/-0.0)umol/L	1 (+/-0.0)umol/L
Nephrotoxicity			
	0mg/kg	7mg/kg FMD-2Br-DAB	5mg/kg FMD-2I-DAB
Creatinine	6.4 (+/-4.7)umol/L	3.0 (+/-1.4)umol/L	10.6 (+/-1.5)umol/L
Urea	6.6 (+/-1.3)mmol/L	7.1 (+/-0.5) mmol/L	10.2 (+/-2.5) mmol/L
Electrolytes			
	0mg/kg	7mg/kg FMD-2Br-DAB	5mg/kg FMD-2I-DAB
Chloride	114 (+/-2.1)mmol/L	116 (+/-1.0)mmol/L	114 (+/-0.6)mmol/L
Potassium	4.4 (+/-0.7)mmol/L	5.0 (+/-0.8)mmol/L	4.3 (+/-0.4)mmol/L
Sodium	150 (+/-3.1)mmol/L	150 (+/-1.7)mmol/L	154 (+/-1.7)mmol/L

Fig. 7. Mouse xenograft models of human cervical (ME-180) cancer, lung (A549) cancer, breast (MDA-MB-231) cancer and ovarian (NIH:OVCAR-3) cancer treated by a FMD compound. For chemotherapy, FMD-2Br-DAB at 7 mg/kg/day or FMD-2I-DAB at 5 mg/kg/day was administrated by IP injection into the mice, with totally 5/10 treatments at each other day. A: FMD-2Br-DAB with totally 5 treatments significantly suppressed cervical (ME-180) tumor growth, compared with the control group with no treatment ($P < 0.001$). B: FMD-2Br-DAB or FMD-2I-DAB with totally 5 treatments significantly suppressed lung (A549) tumor growth, compared with the control group with no treatment ($P < 0.001$). C: FMD-2Br-DAB with totally 10 treatments significantly suppressed breast (MDA-MB-231) tumor growth, compared with the control group with no treatment ($P < 0.001$). D: FMD-2Br-DAB or FMD-2I-DAB with totally 10 treatments significantly suppressed cisplatin-resistant ovarian (NIH:OVCAR-3) tumor growth, compared with the control group with no treatment ($P < 0.001$). E: Mouse body weight variations in the xenograft model of cervical (ME-180) cancer for the control group and the group treated by FMD-2Br-DAB ($P > 0.05$). F: Mouse body weight variations in the xenograft model of lung (A549) cancer for the control group and the groups treated by FMD-2Br-DAB or FMD-2I-DAB ($P > 0.05$). G: Mouse body weight variations in the xenograft model of ovarian (NIH:OVCAR-3) cancer for the control group and the groups treated by FMD-2Br-DAB or FMD-2I-DAB ($P > 0.05$). H: Representative pictures of xenograft ME-180 tumors in mice for the control group and the treated group on day 40, both in situ and after surgical resection. I. Acute toxicity tests of FMD-2Br-DAB and FMD-2I-DAB in mice: the hepatotoxicity (ALT, ALP, TBIL), nephrotoxicity (blood urea, creatinine), and electrolytes (Na, K, etc.) were measured from the blood samples collected from the mice at the end of the 10 treatments (7 mg/kg FMD-2Br-DAB × 10; 5 mg/kg FMD-2I-DAB × 10), where ALP = lkaline phosphatase, ALT = alanine aminotransferase, and TBIL = total bilirubin.

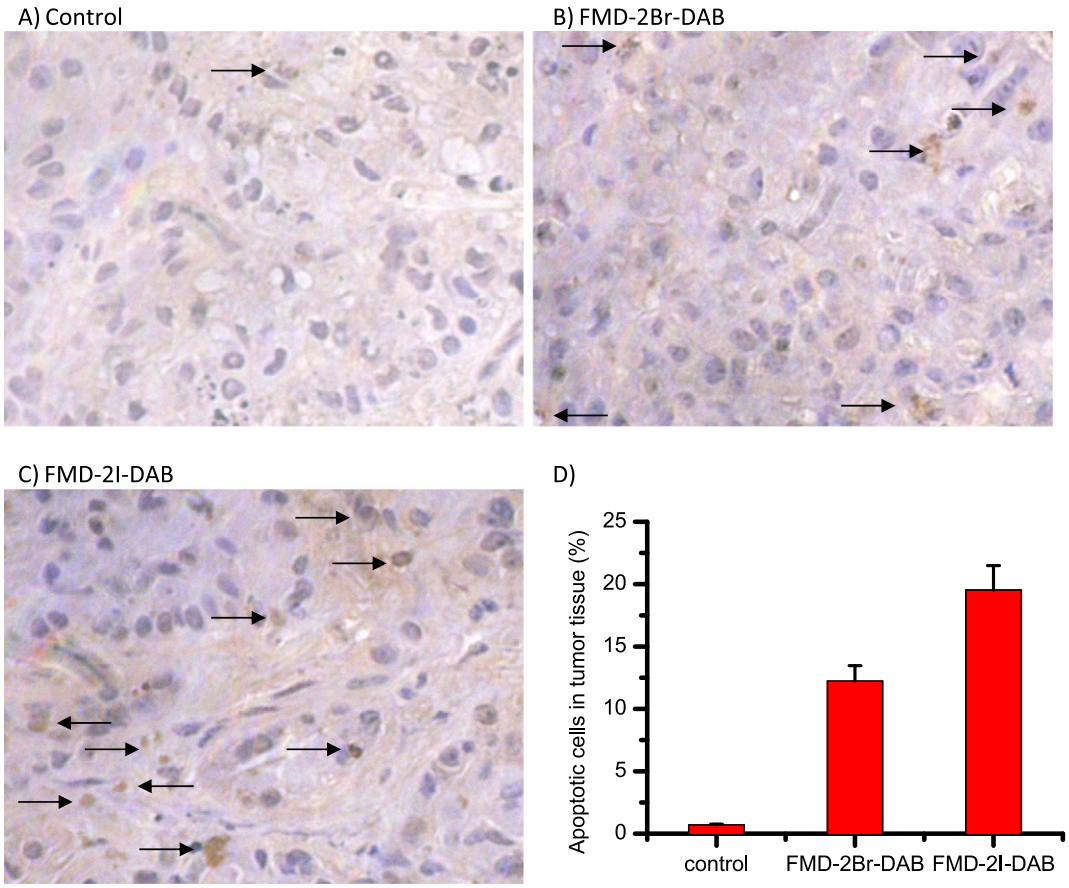


Fig. 8. Apoptosis measurements in lung cancer (A549) xenograft model. Tissue samples were collected from the tumors grown in mice in the control group and treatment groups (7 mg/kg FMD-2Br-DAB × 5; 5 mg/kg FMD-2I-DAB × 5) at 24 h post-treatment and sectioned at 4 μm onto polarized slides. A colorimetric TUNEL assay of the tissue slides was then performed using the DeadEnd™ Colorimetric TUNEL System to visualize and quantify apoptotic cells. The arrows in the images indicate apoptotic cells. A: Tumor tissue from mice in control with no FMD treatment. B: Tumor tissue from mice treated by FMD-2Br-DAB. C: Tumor tissue from mice treated by FMD-2I-DAB. D: Percentages of apoptotic cells in tumor tissue with various treatments.

3.7. In Vivo Anti-Cancer Effect Tests in Xenograft Mouse Models of Cancers

The in vivo anti-cancer effects of FMD-2Br-DAB and FMD-2I-DAB were investigated in the xenograft mouse models of human cervical cancer (ME-180), lung cancer (A549), breast cancer (MDA-MB-231) and ovarian cancer (NIH:OVCAR-3). It was reported that mice treated with a daily dose of 2.3 mg/kg cisplatin for 5 days, followed by 5 days of rest, for two cycles (similar to those used in patients), showed a significant decrease in mean body weight compared to vehicle control after the first and second treatment cycle (Ta et al., 2009). Half the mice treated with a single dose of 15 mg/kg cisplatin alone died within 5 days, while the cisplatin-treated mice sacrificed at 5 days had significantly elevated levels of blood urea nitrogen (BUN) (Townsend and Hanigan, 2002). The typical dose of cisplatin for chemotherapy alone or in combination with another agent or therapy is 2–7.5 mg/kg per treatment in xenograft mouse tumor models (Lee et al., 2007; Coxon et al., 2012). For our present experiments, FMD-2Br-DAB and FMD-2I-DAB were administrated at 7 mg/kg/day and 5 mg/kg/day, respectively: totally 5 treatments (days) for ME-180 and A549 models and 10 treatments for MDA-MB-231 and NIH:OVCAR-3 models (given in each other day). The results are shown in Fig. 7A–I. Fig. 7A–D show that the FMDs significantly suppressed cervical, lung, breast and ovarian tumor growths, respectively, compared with the control group with no FMD ($P < 0.001$). It was also observed in Fig. 7A that the FMD resulted in a significant cervical tumor regrowth delay of more than 30 days in vivo. Fig. 7H shows the pictures of xenograft cervical tumors in mice for the control group and the treated group on day 40, both in situ and after surgical resection. Fig. 7B–D also shows that the FMD agents effectively suppressed the lung, breast and ovarian tumor growths with the observations up to 33–51 days. Remarkably, FMD-2I-DAB effectively suppressed the cisplatin-resistant NIH:OVCAR-3 ovarian tumor growth. These results exhibited a large difference between the control group and the treated group ($P < 0.001$), indicative of a high drug efficacy.

3.8. In Vivo Acute Toxicity and Physical Toxicity Measurements in Xenograft Mouse Models of Cancers

For acute toxicity assay, blood samples were collected by a terminal cardiac puncture of the mice at the end of the treatment with 0 and 7 mg/kg FMD-2Br-DAB or 5 mg/kg FMD-2I-DAB daily for 10 days. The acute drug toxicity was assessed through measurements of the hepatotoxicity (ALT, ALP, total bilirubin), nephrotoxicity (blood urea, creatinine), and electrolytes (Na, K, Cl). The overall drug toxicity was studied through body weight measurements. Fig. 7E, F and G shows that there were no effects of the FMD compounds on the weight of mice with time, i.e., the FMD exhibited no physical toxicity. Fig. 7I shows that the FMDs induced no observable changes in biomarkers of hepatotoxicity and nephrotoxicity and no changes in electrolytes. These results indicate that the FMD compounds induced no overall and acute toxicity ($P > 0.05$).

3.9. In Vivo Apoptosis Measurements in Lung Cancer Xenograft Model

The in vivo cell death induced by FMD-2Br-DAB at 7 mg/kg × 5 and FMD-2I-DAB at 5 mg/kg × 5 was investigated in the xenograft mouse tumor model of human lung cancer (A549). Apoptotic cells in tumor

tissue were measured at 24 h post-treatment by the TUNEL assay. As shown in Fig. 8, there was a significant enhancement in apoptosis in the tumor tissue treated by the FMD compounds, compared with the untreated group ($P < 0.001$). Among the FMD agents, FMD-2I-DAB is the most effective inducer of apoptosis. These results indicate that FMDs induced apoptosis in the tumor and are therefore effective anti-cancer agents.

Overall, the observed in vivo results (Figs. 7 and 8) are consistent with the in vitro results (Figs. 1-6 and Table S1). The in vitro results demonstrate that the presence of a FMD compound effectively killed tumor cells but no normal cells by inducing significant DNA DSBs and apoptosis in cancer cells. This is consistent with the proposed DET mechanism for the FMD compounds with weakly-bound electrons that may intrinsically exist in cancer cells. Correspondingly, the in vivo results in mice confirm that the FMDs indeed generated sufficient anti-cancer effects while inducing minimal or no toxic side effects.

4. Discussion

Our studies of FMD in cancer have led to the discoveries of a reductive damaging mechanism in DNA (Wang et al., 2009; Nguyen et al., 2011) and living cells (Lu et al., 2013) and of the molecular mechanisms of existing anti-cancer agents (Lu, 2007; Lu et al., 2007; Wang et al., 2006; Wang and Lu, 2007, 2010). These have offered unique opportunities to develop new effective drugs for targeted chemotherapy of cancer. Our recent results on cell experiments showed that an exogenous antioxidant induced significant DNA damage and apoptosis (cell death) in human normal cells through a reductive mechanism (Lu et al., 2013). Antioxidants may have a promotion effect in causing cancer (The ATBC, 1994; Albanes et al., 1996; Omenn et al., 1996; DeNicola et al., 2011; Lu et al., 2013), and reduce the effect of an exogenous reducing agent in killing tumor cells (Lu et al., 2013). This is probably because weakly-bound electrons in antioxidants can cause serious reductive DNA damage, which if not repaired properly, can lead to apoptosis, genetic mutations and likely diseases notably cancer. Thus, it is reasonable to speculate that abnormal (cancer) cells may have a more reduced intracellular/intranuclear environment and may therefore have some resistance to an exogenous reducing agent (antioxidant). Our finding of a reductive DNA-damaging mechanism might be an important step for effective prevention and therapy of cancer.

We have now successfully found a previously unknown family of non-platinum-based anticancer compounds (called FMD compounds) as potent antitumor agents for natural targeted chemotherapy without the need of using an antibody to bind to cancer cells. Upon entering normal and abnormal (cancer) cells, the FMD compounds, which are highly oxidizing agents, have effects just opposite to those induced by antioxidants and are capable of generating the anti-cancer effects on cancer cells while having minimal toxicity toward normal cells. They are highly reactive with weakly-bound electrons rich in the more reduced intracellular/intranuclear environment of cancer cells. In contrast, the FMDs are much less toxic toward normal cells due to the lack of a reduced intracellular/intranuclear environment in normal cells. Therefore FMDs have no or low systemic or acute toxicity in the body. They are effective anti-cancer agents that can preferentially kill cancer cells and are therefore useful for natural targeted chemotherapy of cancer and potentially other disorders treatable by chemotherapy. The FMDs are superior to cisplatin, which has poor target selectivity and is highly toxic due to the containing of the heavy metal (Pt) readily binding to proteins in kidney (Townsend and Hanigan, 2002; Zhang et al., 2006), though cisplatin has also generally stronger toxicity against cancer cells.

To the best our knowledge, no previous studies showing considerable toxicity of our identified FMD compounds have been reported in the literature. A previous study by Yu et al. (2008) showed that all six non-halogenated and halogenated (chlorinated) phenylenediamines (PD) as dye precursors used to manufacture hair dyes are not toxic/mutagenic in Salmonella typhimurium TA 102 bacteria or skin keratinocyte cells, and do not cause DNA cleavage in ΦX 174 phage DNA. Their phototoxicity test in skin cells showed that without light irradiation, all the PD compounds are not toxic to the keratinocytes up to 1000 μM; only at an extremely high PD concentration (1000 μM) and with extensive exposure to light irradiation (at a large light dose of 3.3 J/cm^2 of UVA and 6.3 J/cm^2 of visible light), two chlorinated PDs and two non-chlorinated PDs show modest phototoxicity. However, the phototoxicity is a subject that has no direct relevance to the work reported in our present manuscript.

In the present studies, our in vitro results from cell viability measurements show that the FMD compounds significantly enhanced the killing of both cisplatin-sensitive cancer cells and cisplatin-resistant cancer cells. Furthermore, large enhancements in DNA damage and apoptosis in the treated cancer cells but no normal cells were also observed. Our cell cycle measurements also correspondingly show that the FMD compounds significantly inhibited DNA synthesis in the cell cycle, resulting in cell arrest in the S phase. We also found that the FMD compounds exhibited little toxicity toward human normal cells with measured IC50 values ≥200 μM, in contrast to the clinically widely-used but highly-toxic cisplatin with IC50s ≤10 μM for 72-hr incubation. Very interestingly, we also observed the marked difference in modifying the levels of the reduced glutathione (GSH, an endogenous antioxidant) in normal and cancer cells. A FMD up to 100 μM caused a significant increase of the GSH level in normal cells, while it largely depleted the GSH in cancer cells. Although further studies as to how the FMD compound enhances the GSH level in normal cells are needed, these results indicate a selective strong reaction between the FMD and the GSH within cancer cells and to kill the latter. Thus, these FMD agents are ideal potent anticancer agents.

Correspondingly, our in vivo results have clearly confirmed that FMD-2Br-DAB and FMD-2I-DAB as exemplary FMD compounds exhibited no or minimal toxicity in mice, no overall physical toxicity and no acute toxicity (no hepatotoxicity, no nephrotoxicity, and no changes in electrolytes). These results are in excellent agreement with the in vitro results observed in human normal cells. Furthermore, the in vivo results from the xenograft mouse tumor models of human cervical cancer, lung cancer, breast cancer and ovarian cancer exhibited significant tumor growth inhibition and regrowth delay due to the anti-cancer effect of the FMD compounds. These results demonstrate that the FMDs are indeed potent anti-cancer agents for natural targeted chemotherapy. Given that only a small dose (5 or 7 mg/kg) of the FMDs were used in the present experiment and that the FMDs showed little or no toxicity at much higher doses in vitro, it is reasonably expected that these results can be extrapolated to larger doses or more frequent treatments so that a maximal therapeutic efficacy can be achieved.

Finally, it is expected that our in vitro and in vivo results obtained in this study may be applied to other cancer types and models beyond those exemplified here. This innovative study in FMD represents an efficient and economical rational approach for discovery of novel anticancer agents, which is superior to the screening strategy of a large library of compounds. Discovered on a well-established DET mechanism of action, these FMD compounds being essentially non-toxic, small molecules and therefore readily for delivery to cancer sites should have large opportunities for success in clinical trials and ultimate use in cancer patients in the clinic. The present study also demonstrates the great potential of FMD as a new frontier in biomedical research to bring breakthroughs in fundamental understandings of major human diseases such as cancer and ultimately in therapies of diseases in clinical settings.

Author Contributions

Q.B.L., C.R.W. and Q.R.Z. performed spectroscopic measurements. N.O., Q.R.Z. and C.R.W. performed in vitro DNA DSBs, apoptosis, cell cycle, and cell viability experiments. Q.R.Z. and N.O. conducted the GSH measurements in the cells. J.W., N.O. and Q.R.Z. performed in vivo

mouse experiments. Q.B.L. initiated and established the DET mechanism, identified the FMD compounds and designed the study. All authors were involved in the data analyses and discussion. Q.B.L. wrote the manuscript.

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Author Information

Q.B.L. declares that there was a submitted patent application associated with the data reported in this manuscript. Correspondence and requests for materials should be addressed to Q.B.L. (qblu@uwaterloo.ca).

Appendix A. Supplementary Data

Supplemental information includes materials and extended description of methods, Figs. S1 and S2, and Table S1 can be found with this article online at http://dx.doi.org/10.1016/j.ebiom.2015.04.011.

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