HHS Public Access

Author manuscript

Radiat Res. Author manuscript; available in PMC 2021 February 01.

Published in final edited form as:

Radiat Res. 2020 February ; 193(2): 107–118. doi:10.1667/RR15433.1.

Enhancement of Cytotoxicity of Enediyne Compounds by Hyperthermia: Effects of Various Metal Complexes on Tumor Cells

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Abstract

Enediyne natural products are a class of compounds that were recognized for their potential as chemotherapeutic agents many years ago, but found to be highly cytotoxic due to their propensity for low thermal activation. Bergman cyclization of the enediyne moiety produces a diradical intermediate, and may subsequently induce DNA damage and account for the extreme cytotoxicity. While difficulties in controlling the thermal cyclization reaction have limited the clinical use of cyclic enediynes, we have previously shown that enediyne activity, and thus toxicity at physiological temperatures can be modulated by metallation of acyclic enediynes. Furthermore, the cytotoxicity of "metalloenediynes" can be potentiated by hyperthermia. In this study, we characterized a suite of novel metallated enediyne motifs that usually induced little or no cytotoxicity when two different human cancer cell lines were treated with the compounds at 37°C, but showed a significant enhancement of cytotoxicity after cells were exposed to moderate hyperthermia during drug treatment. Cultured U-1 melanoma or MDA-231 breast cancer cells were treated with various concentrations of Cu, Fe and Zn complexes of the enediyne (Z)-N,N′-bis[1-pyridyl-2-yl-meth-(*E*)-ylidene]octa-4-ene-2,6-diyne-1,8-diamine (PyED) and clonogenic survival was assessed to determine the effects of the drugs at 37°C and 42.5°C. Toxicity at 37°C varied for each compound, but hyperthermia potentiated the cytotoxicity of each compound in both cell lines. Cytotoxicity was concentration-, time- and temperature-dependent. Heating cells during drug treatment resulted in enhanced apoptosis, but the role of cell cycle perturbation in the response of the cells to the drugs was less clear. Lastly, we showed that hyperthermia enhanced the number of DNA double-strand breaks (DSBs) induced by the compounds, and inhibited their repair after drug treatment. Thus, thermal enhancement of cytotoxicity may be due, at least in part, to the propensity of the enediyne moiety to induce DSBs, and/or a reduction in DSB repair

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efficiency. We propose that “tuning” of metalloenediyne toxicity through better-controlled reactivity could have potential clinical utility, since we envision that such compounds could be administered systemically as relatively non-toxic agents, but cytotoxicity could be enhanced in, and confined to a tumor volume when subjected to localized heating.

Editor’s note

The online version of this article (DOI: https://doi.org/10.1667/RR15433.1.S1) contains supplementary information that is available to all authorized users.

INTRODUCTION

Several compounds possessing the enediyne moiety (*Z*)-1,5-diyne-3-ene (“enediyne”) have been evaluated in the past for their potential as anti-cancer agents. The compounds undergo Bergman cyclization through the production of a 1,4-benzoid diradical intermediate (*1, 2*) that can abstract a H-atom from the ribose ring of DNA molecules. However, regulation of radical formation has proven to be difficult.

The Zaleski laboratory previously described the development of a small chelation-induced diradical-generating compound that is influenced by divalent metals which act either via Fenton chemistry or H-atom abstraction (*1, 3*). From this paradigm, the tetradentate ligand (*Z*)-*N,N*-bis[1-pyridin-2-yl-meth(*E*)-ylidene]oct-4-ene-2,6-diyne-1,8-diamine (PyED) was synthesized. This compound was shown to undergo Bergman cyclization, forming a 1,4-benzenoid diradical within 2.5 h at room temperature upon chelation of Mg(II) in methanol (3). Moreover, we found that exposure of HeLa cells to PyED in cell culture media (which contains divalent metal ions) or exposure of cells to our first-generation enediyne compounds at supraphysiological temperature for 1 h significantly reduced clonogenic cell survival, presumably through enhanced DNA damage (4,5).

Structurally-dependent diradical formation in biological systems represents a novel chemotherapeutic utility for metallated enediynes (metalloenediynes), especially when combined with hyperthermia treatment, which can accelerate compound activation. While hyperthermia is no longer envisioned to be an effective stand-alone therapy for most types of tumors, heat is known to greatly enhance the efficacy of some chemotherapeutic agents. In most cases, the hyperthermic treatment does not alter the drug itself; rather, cellular properties such as drug uptake or the kinetics of DNA repair may be influenced by heat (5–7). While we have shown that the killing of tumor cells *in vitro* was greatly potentiated when cells were treated with several first-generation metalloenediynes, many of the compounds synthesized previously are characterized by long reaction times (up to 48 h), inefficient DNA degradation, or do not show significant potentiation of cell killing *in vitro* after exposure to the compounds at elevated temperatures. Thus, the development of molecules with shorter reaction times or greater potency are required for these compounds to reach their full potential in the clinic.

In an effort to further evaluate the geometric structure/Bergman cyclization relationship under physiological conditions, we recently reported the preparation of novel PyED-

metalloenediyne complexes containing Cu(II), Fe(II), and Zn(II). Bergman cyclization from thermal activation of each of these constructs resulted in diradical generation within 3 h at 37°C (8). A significant metal dependence on the rate of the reaction is observed with these complexes [Cu(II) > Fe(II) > Zn(II)], and reaction rate was directly correlated to efficiency of DNA degradation. Considerable DNA degradation was observed after exposure of naked DNA to PyED, at slightly supraphysiological temperatures, in the presence of all metals within 1 h after treatment.

We now report the results of our initial characterization of this new suite of compounds. We demonstrate that treatment with these metallated enediyne motifs results in a significant enhancement of cytotoxicity in two different tumor cell lines when cells are exposed to drug at elevated temperatures, but that little or no cytotoxicity results when cells are treated at 37°C. Furthermore, we describe the potential mechanisms by which heat enhances metalloenediyne cytotoxicity and reveal insight into the mode of death after drug treatment at supraphysiological temperature. We envision potential clinical utility of metalloenediynes, since they could be administered systemically without side effects, but their cytotoxicity may be enhanced in, and confined to a tumor exposed to localized heating.

MATERIALS AND METHODS

Synthesis of Ligands and Metal Complexes

All syntheses of compounds and subsequent complexations were performed under nitrogen atmosphere employing standard Schlenk and dry box techniques using chemicals of the highest purity available and purchased from commercial sources. The ligand (Z)-N,N′-bis[1-pyridin-2-yl-meth-(*E*)-ylidene]oct-4-ene-2,6-diyne-1,8-diamine (PyED) and metal complexes were prepared using a previously published synthesis (8). Briefly, a cooled methanol solution of PyED (1 eq) was added to a stirring solution of $MSO_4 \cdot \times H_2O$ or $MCl_2 \cdot \times H_2O$ (M=Cu(II), Fe(II), Zn(II); 1 eq) in methanol and stirred for 4 h at 0°C. The solvent was removed *in vacuo* at 0°C, and diethyl ether was then added to the crude product. The suspension was stirred for 2 h and filtered to yield the solid products $FeCl_2$-PyED, $FeSO_4$-PyED, $CuCl_2$-PyED, $CuSO_4$-PyED, $ZnCl_2$-PyED and $ZnSO_4$-PyED. Structures of these compounds are shown in Fig. 1.

Cell Culture and Drug Preparation

U-1 melanoma or MDA-231 breast cancer cells were cultured in McCoy’s 5A (Mediatech Inc./Corning® Inc., Manassas, VA) or DMEM media (Mediatech/Corning), respectively, supplemented with 10% iron-supplemented calf serum (Hyclone™ Laboratories/GE Healthcare Life Sciences, Logan, Utah). Unless otherwise indicated, cells were trypsinized from stock flasks and then cells were plated into T-25 flasks three days prior to day of each experiment. Cells in logarithmic growth were allowed to reach ~90% confluency by the day of each experiment. Compounds were dissolved in either sterile water (sulfate derivatives) or DMSO (chloride derivatives).

Clonogenic Cell Survival Assay

Clonogenic cell survival was measured as described elsewhere (5). Briefly, U-1 or MDA-231 cells were treated with either vehicle (1.5% by volume) or various concentrations of one of the above compounds for various times at either 37°C or while being heated at temperatures up to 43.5°C in a precision-controlled water bath. After the treatment, the drug or vehicle was washed off the cells with fresh media, and the cells were trypsinized, counted and finally plated into new T-25 flasks. Surviving cells were allowed to form colonies in a 5% CO_2 incubator for 10 days before being fixed, stained and counted. Surviving fractions were calculated and normalized to the plating efficiency of cells treated with vehicle only at the respective treatment temperature, or to the plating efficiency of cells treated at 37°C with their respective drug treatment. In some experiments, cells were treated with drug or vehicle alone for 0, 15, 30 or 60 min at a specific temperature. Surviving fractions were calculated and normalized to the plating efficiency of cells that were only washed with the treatment media and then washed with fresh media before trypsinization.

Assessment of Apoptosis

For studies in which apoptotic fraction was determined, $2.5–3\times10^5$ cells were plated into T-25 flasks 2–3 days prior to the day of the experiment (cells were confirmed to be growing logarithmically at the time all experiments were initiated). On the day of each experiment, compounds were prepared in either sterile water or DMSO. After addition of drug (25 μ*M* $CuCl_2$-PyED, 75 μ*M* $ZnCl_2$-PyED or 100 μ*M* $FeSO_4$-PyED) or vehicle (1.5% by volume) to cell culture flasks, the cells were then incubated at 37°C or heated in a 42.5°C water bath for 1 h. We chose to use a drug concentration in these experiments that yielded maximal enhancement of killing when cells were treated with drug at 42.5°C, but which caused minimal or no cytotoxicity if treated at 37°C. After treatment, the vehicle- or drug-containing media was removed, the monolayer washed, and then fresh drug-free media was added back to flasks. Flasks were placed in a 37°C (5% CO_2) incubator for various times, and the cells were then trypsinized and counted. We then used the Annexin V-EGFP Apoptosis Kit (BioVision Inc., Milpitas, CA) to quantify the number of apoptotic cells at various times after treatment. Five-hundred thousand cells were processed as per manufacturer's recommendation. Briefly, cells were re-suspended in binding buffer, Annexin V-EGFP and propidium iodide (PI). They were incubated in the dark for at least 5 min at room temperature and then analyzed for green and red fluorescence using a BD Accuri™ flow cytometer (BD Biosciences, San Jose, CA). Results were analyzed using the FlowJo program (Ashland, OR).

Flow Cytometry

U-1 (2×10^5) and MDA-231 (1.75×10^5) cells were plated into T-25 flasks three days prior to the experiment. On the day of the experiment, compounds were prepared in either sterile water or DMSO. Cells were then exposed to various concentrations of the compounds or vehicle (1.5% by volume) during incubation at 37°C or during heating in a 42.5°C water bath for 1 h. After treatment, the drug-containing media was washed from the flasks and replaced with fresh media. Cells were returned to a 37°C incubator for various lengths of time, and then trypsinized, centrifuged, fixed with 100% ethanol and kept at 4°C until

staining and analysis. Just prior to analysis, the cells were washed free of fixative and stained using a DNA staining buffer containing sodium citrate (1 mg/ml), Triton™ X-100 (0.3%), ribonuclease A (0.02 mg/ml) and PI (50 μg/ml). For cell cycle analysis, either a BD Accuri or BD LSR II flow cytometer (BD Biosciences) was used to measure the DNA content in at least 10,000 cells per sample. The ModFit™ program (Verity Software House, Topsham, ME) was used to fit curves to the data and determine the percentage of cells in each cell cycle phase.

Detection of Gamma-H2AX Foci

Two days prior to each experiment, 30,000–40,000 U-1 cells were plated into each well of Lab-Tek® or Lab-Tek II four-well chamber slides. On the day of the experiment, cells were treated with 100 μ*M* $FeSO_4$-PyED or vehicle (1.5% by volume) for 1 h at either 37°C or 42.5°C. After treatment, media containing the drug was removed, wells washed and fresh media was added to each well. The slides containing the cells were then placed in a 37°C CO_2 incubator for up to 240 min. At various times after treatment, the media was removed and cells were rinsed with phosphate buffered saline (PBS) prior to fixation with 4% paraformaldehyde at room temperature for 15 min in the dark. After fixation, cells were washed with PBS and treated with permeablizing solution (20 m*M* HEPES, 50 m*M* NaCl, 3 m*M* $MgCl_2$, 300 m*M* sucrose, 0.5% Triton X-100) for 7 min at room temperature. Cells were then washed with PBS before blocking the cells in PBS with 5% goat serum and 0.5% Tween® 20 for at least 60 min at room temperature. Cells were labeled with an anti-γ-H2AX mouse antibody (diluted 1:500; EMD Millipore, Burlington, MA) overnight at 4°C in the dark. The next day, the primary antibody was washed off with PBS and the cells labeled with a fluorescently-conjugated secondary antibody (Alexa Fluor® 488 goat anti-mouse, diluted 1:1,000; Thermo Fisher Scientific™ Inc., Waltham, MA) for 1 h at room temperature in the dark. The secondary antibody was washed off with PBS and ProLong Gold Anti-Fade Reagent (Carlsbad, CA) was used to mount the coverslip to the slide before drying slides in the dark overnight at room temperature. Images of the stained cells were obtained 1–10 days later using a Leica TCS SP8 confocal microscope (Buffalo Grove, IL). Foci [a surrogate measure for double-strand breaks (DSBs)] were counted or nuclear fluorescence per cell was measured using the FindFoci plug-in in FIJI (https://bit.ly/2q8REgx) (*9–11*). For each treatment, the total number of γ-H2AX foci was divided by the number of nuclei present to determine the average number of foci/nucleus. Due to differences in background staining between experiments, each experiment was normalized to the average number of foci per nucleus across all of the “vehicle at 37°C” time points for that experiment before the data from the experiments could be combined. The mean number of foci per nucleus in samples treated with vehicle only (at 37°C) was 6.9, 2.5 and 6.4 for the three experiments. To determine fluorescence intensity per nucleus, FindFoci was used to calculate the total intensity of pixels in the peak of each focus. For each treatment, fluorescence intensities from all foci for that treatment were added and then divided by the number of nuclei to determine the mean relative fluorescence value per nucleus. This number was also normalized to the average of the “vehicle at 37°C” time points for that experiment.

RESULTS

Cytotoxic Effects of Novel Enediyne Complexes Containing Cu(II), Fe(II) and Zn(II) after Treatment at Physiological or Supraphysiological Temperatures

Initial dose-response studies were performed to assess the biological effects of a novel suite of divalent metal complexes of the enediyne PyED on human U-1 melanoma cells after treatment at physiological or hyperthermic temperatures. These initial studies were performed to identify candidate compounds for future mechanistic and potential *in vivo* studies. U-1 cells were treated with various concentrations of $CuCl_2$-PyED, $CuSO_4$-PyED, $FeCl_2$-PyED, $FeSO_4$-PyED, $ZnCl_2$-PyED or $ZnSO_4$-PyED at 37°C or 42.5°C, and clonogenic survival was determined (Fig. 2A–C). The compounds varied considerably with respect to their toxicity at 37°C. $ZnSO_4$-PyED and $CuCl_2$-PyED were more cytotoxic at 37°C than any of the other compounds; the surviving fraction was less than 0.4 after treatment with 20 or 25 μ*M* $CuCl_2$-PyED, while treatment with three- to-fourfold-greater concentrations of $CuSO_4$-PyED, $FeCl_2$-PyED, $FeSO_4$-PyED, or $ZnCl_2$-PyED still failed to reduce cell survival to that level. $FeSO_4$-PyED was the least toxic of all of the compounds when cells were treated at 37°C; surviving fraction was approximately 1.0 even after treatment with 85 μ*M*, and cell survival remained high (surviving fraction ~0.7 or greater) after treatment up to 100 μ*M* (Fig. 2C). After treatment at 37°C for 1 h, we found that $CuCl_2$-PyED was more cytotoxic than $CuSO_4$-PyED at concentrations ≥25 μ*M*, $FeCl_2$-PyED cytotoxicity was slightly greater than that of $FeSO_4$-PyED, but only at higher concentrations (100 μ*M*), and $ZnSO_4$-PyED was much more cytotoxic than $ZnCl_2$-PyED at concentrations of >15 μ*M*.

Hyperthermia potentiated the cytotoxicity of all six metalloenediyne compounds, even at concentrations that were either only mildly or not cytotoxic at 37°C (Fig. 2A–C). We noted that $CuCl_2$-PyED or $ZnCl_2$-PyED treatments at 42.5°C resulted in a greater enhancement of cell killing than their respective sulfate counterparts. Conversely, when the iron complexes were compared, $FeSO_4$-PyED showed a greater enhancement of cytotoxicity when cells were treated at 42.5°C than its chloride counterpart. That is, a greater differential in cytotoxicity was observed when survival of heated and non-heated cells was measured after treatment with $ZnCl_2$-PyED, $CuCl_2$-PyED & $FeSO_4$PyED compared to $ZnSO_4$-PyED, $CuSO_4$-PyED and $FeCl_2$-PyED, respectively. It is worth noting that $ZnCl_2$-PyED, $CuCl_2$-PyED and $FeSO_4$-PyED cytotoxicity was enhanced by heat even when cells were treated at relatively low concentrations. The greatest differential in cell killing when drug treatments were administered at 37°C vs. 42.5°C was achieved using $ZnCl_2$-PyED; more than a 100-fold increase in killing was noted when cells were treated with 75 μ*M* $ZnCl_2$-PyED at 42.5°C compared to 37°C. More than a 10-fold increase in killing was noted when cells were treated with 25 μ*M* $CuCl_2$-PyED and 100 μ*M* $FeSO_4$-PyED at 42.5°C compared to 37°C. Because of the relative lack of cell killing at 37°C and/or the differential in cell killing (between treatment at 37°C and 42.5°C) generally at lower concentrations than the other compounds, $ZnCl_2$-PyED, $CuCl_2$-PyED and $FeSO_4$-PyED were chosen for further cytotoxicity testing and mechanistic studies.

Time-Temperature Dependence of Metalloenediyne Cytotoxicity

The time- and temperature dependence of potentiation of $CuCl_2$-PyED, $ZnCl_2$-PyED and $FeSO_4$-PyED cytotoxicity was investigated by determining the surviving fraction of U-1 cells after treatment with the compounds for up to 60 min at physiological temperature or at hyperthermic temperatures ranging from 38.5 to 43.5°C (Fig. 3A–C). Generally, no significant cytotoxicity was noted after subjecting U-1 cells to heat alone for 15, 30 or 60 min until cells were challenged with a treatment temperature of 42.5°C or greater, and only after a 30- or 60-min heat treatment. As shown in Fig. 3A, heating cells during treatment with 25 μ*M* $CuCl_2$-PyED for only 15 min at 42.5°C significantly potentiated cytotoxicity when compared to treatment at 37°C (Fig. 3A). Fifteen-minute exposures to 75 μ*M* $ZnCl_2$-PyED and 100 μ*M* $FeSO_4$-PyED were not toxic to cells at temperatures lower than 43°C (Fig. 3B and C). Of the three compounds, the cytotoxicity of $CuCl_2$-PyED was the most time- and temperature-dependent. For $CuCl_2$-PyED, enhanced cytotoxicity was observed when cells were treated with the compound for 30 or 60 min at temperatures as low as 38.5°C; these treatments produced similar toxicity to that observed when cells were heated for 15 min at 42.5°C (Fig. 3A). A significant time- and temperature dependence was noted for $ZnCl_2$-PyED after treating for 15–60 min at temperatures 42.5°C compared to treatment at 37°C (Fig. 3B), and for cells treated with $FeSO_4$-PyED for 30–60 min at 41.5°C or 15–60 min at 42.5°C or higher (Fig. 3C). In general, potentiation of cytotoxicity was observed to increase with duration of treatment with the drug at 41.5°C and higher. As evidenced in the full time-temperature curves generated for $CuCl_2$-PyED and $FeSO_4$-PyED (and the more limited 60-min data for treatment with $ZnCl_2$-PyED at 42.5°C), the greatest enhancement of cytotoxicity was observed after 30- and 60-min drug treatments at temperatures greater than 41.5°C (Fig. 3A–C).

A Comparison of Metalloenediyne Cytotoxicity in Two Different Tumor Cell Lines

We next determined the effects of various concentrations of $CuCl_2$-PyED, $FeSO_4$-PyED and $ZnCl_2$-PyED on a second cell line, the MDA-231 breast cancer cell line, and compared cytotoxicity, or enhancement of cytotoxicity, with that of U-1 cells after treatment with the compounds at 37° or 42.5°C (Fig. 4A–C). Similar to what was observed for U-1 cells, the compounds varied considerably with respect to their toxicity when MDA-231 cells were treated at 37°C, and hyperthermia potentiated the cytotoxicity of each of the compounds when MDA-231 cells were treated with the drugs at 42.5°C. MDA-231 cells were very resistant to exposure to high concentrations of $FeSO_4$-PyED and $ZnCl_2$-PyED at 37°C. U-1 cells were found to be more sensitive than MDA-231 cells to $FeSO_4$-PyED and $ZnCl_2$-PyED at 37°C, and greater thermal enhancement of cytotoxicity was also noted in U-1 cells compared to MDA-231 cells for these compounds. However, MDA-231 cells were slightly more sensitive to treatment with lower concentrations of $CuCl_2$-PyED at both temperatures, compared to U-1 cells.

Perturbation of Cell Cycle Progression by $CuCl_2$-PyED, $FeSO_4$-PyED and $ZnCl_2$-PyED

We analyzed the effects of $CuCl_2$-PyED, $FeSO_4$-PyED and $ZnCl_2$-PyED on cell cycle progression of U-1 melanoma cells and MDA-231 breast cancer cells. Heat alone resulted in an increased number of U-1 cells in the G_2 phase of the cycle at 8–12 h after treatment,

suggesting the presence of a G_2 block during that period after treatment. Heating U-1 cells during treatment with $CuCl_2$-PyED or $FeSO_4$-PyED appeared to largely eliminate the G_2 block normally induced by heat alone at 8–12 h after treatment (Fig. 5). $ZnCl_2$-PyED, when administered during heating, induced a significant G_2 block 24 h after treatment. $ZnCl_2$-PyED treatment at 37°C caused cells to block in S phase 8 h after drug treatment, but the other compounds did not induce significant cell cycle perturbations when administered to cells at 37°C. Generally, there were no significant differences noted in cell cycle progression for cells treated with $CuCl_2$-PyED or $FeSO_4$-PyED at 37°C, or treated with either of the drugs during heating (compared to untreated controls) in U-1 cells (Fig. 5).

Similar to U-1 cells, heat alone induced a G_2 delay in MDA-231 cells, although a greater percentage of cells were arrested in that phase, and the block did not occur until longer times had elapsed after heating. This was most notably accompanied by a concomitant decrease in G_1 phase cells. In MDA-231 cells, all of the compounds generally appeared to mitigate the G_2 arrest induced by heat when cells were treated with drugs at 42.5°C (Fig. 6). Thus, in general, both the U-1 and MDA-231 cell lines responded similarly in regard to cell cycle arrest when the drugs were administered at an elevated temperature. However, due to the generally greater sensitivity of U-1 cells to the effects of at least two of the drugs when exposed to elevated temperatures, U-1 cells were chosen for subsequent mechanistic studies, using the compounds at a concentration which resulted in minimal cytotoxicity after treatment at 37°C, while also showing significant potentiation of cytotoxicity at a designated temperature and duration of heating (60 min at 42.5°C).

Mode of Cell Death after Metalloenediyne Treatment

Since our previous studies with first-generation metalloenediyne compounds indicated that the primary mode of death induced by this class of compounds was apoptosis, flow cytometric analysis of annexin V and PI staining was used to confirm the mode of death and identify the number of apoptotic U-1 cells at various times after treatment with 25 μ*M* $CuCl_2$-PyED, 75 μ*M* $ZnCl_2$-PyED or 100 μ*M* $FeSO_4$-PyED at 37°C or 42.5°C for 1 h (Fig. 7). In the annexin V/PI staining assay, apoptotic cells are positive for annexin V staining (due to flipping of phosphatidyl serine from the inner to outer leaflet of the plasma membrane), but do not take up PI, while necrotic cells stain positive for both annexin V and PI. Hyperthermia alone did not induce apoptosis in a significant fraction of U-1 cells throughout the 24-h period of observation. Similarly, treatment of cells with any of the drugs at 37°C did not result in an increase in the apoptotic fraction except at 24 h after treatment with 25 μ*M* $CuCl_2$-PyED. Heating cells in the presence of any of the compounds resulted in an increase of the apoptotic fraction 18–24 h after treatment. In the case of $CuCl_2$-PyED, heating during drug treatment appeared to induce slightly more than an additive effect, but heating during treatment with 75 μ*M* $ZnCl_2$-PyED or 100 μ*M* $FeSO_4$-PyED significantly increased the apoptotic fraction 18–24 h after treatment, and by 24 h after $ZnCl_2$-PyED or 100 μ*M* $FeSO_4$-PyED treatment, the number of apoptotic cells was ~12–21% greater than the percentage of apoptotic cells from the “heat only” and “drug only” groups combined (Fig. 7).

DSB Induction and Repair after Treatment with Metalloenediynes

Our previously published study indicated that hyperthermia potentiated metalloenediyne-induced DNA damage and inhibited its repair in U-1 cells (5). However, in that study, the alkaline comet assay was used to measure DNA damage (single-strand breaks, DSBs, alkali labile sites), and therefore we were not able to assess individual species of DNA damage. To determine whether potentiation of cytotoxicity of metalloenediynes by hyperthermia could be attributed to a greater induction of initial DSBs or alteration of the kinetics of DSB repair after treatment, we used the γ-H2AX assay to investigate DSB induction and repair dynamics in U-1 cells that were treated with 100 μ*M* $FeSO_4$-PyED for 1 h at either 37° or 42.5°C (Fig. 8). Gamma-H2AX foci (a biomarker of DSBs) were scored or total nuclear γ-H2AX fluorescence measured in nuclei of cells that were fixed and stained with an antibody against γ-H2AX after allowing cells to recover from heat and/or drug treatment for various times.

Compared to cells treated with vehicle only (control) for 1 h at 37°C, cells treated with $FeSO_4$-PyED for 1 h at 37°C contained twice as many γ-H2AX foci per nucleus immediately after treatment (Fig. 8A). Cells that received only a heat treatment or that were treated with $FeSO_4$-PyED during the heat treatment both showed approximately a four-fold increase in γ-H2AX foci (compared to the control) when foci were measured immediately after the respective treatments; this also represented a two-fold increase compared to cells treated with drug alone at 37°C. The mean number of foci per cell rapidly decreased within 1 h after treatment of cells with $FeSO_4$-PyED at 37°C to a level that was not significantly different from the unheated vehicle. Interestingly, cells that were only heated at 42.5°C for 1 h showed an initial decrease in foci to near-normal levels within 30 min after treatment, but then within 60 min after treatment, the number of foci increased to levels originally observed immediately after treatment, before again approaching levels similar to the untreated control by 2 h after treatment.

The initial increase in γ-H2AX foci in nuclei of cells treated with $FeSO_4$-PyED at 42.5°C (as measured immediately after the treatment) appears to simply be attributed to DSBs that form in response to the heat treatment alone. Cells treated with 100 μ*M* of $FeSO_4$-PyED at 42.5°C for 1 h showed significantly more foci per nucleus than cells treated with $FeSO_4$-PyED at 37°C or only the vehicle at both temperatures throughout the period of post-treatment incubation at 37°C (Fig. 8A). The number of γ-H2AX foci per nucleus in cells treated with drug at 42.5°C significantly increased and reached a plateau starting at 30 min after treatment; the plateau was maintained through 120 min after treatment. After 240 min post-treatment, the number of foci per nucleus had dramatically decreased (indicative that DSB repair had occurred in heated, drug-treated cells), but was still slightly higher than that of cells treated with $FeSO_4$-PyED or vehicle at 37 or 42.5°C. Similar trends were observed when cells were analyzed for total nuclear γ-H2AX fluorescence. U-1 cells treated with 100 μ*M* of $FeSO_4$-PyED at 42.5°C for 1 h showed significantly more fluorescence per nucleus than cells treated with $FeSO_4$-PyED at 37°C or vehicle at either 37° or 42.5°C (Fig. 8B).

DISCUSSION

During our initial characterization of first-generation metalloenediyne compounds, we demonstrated that the cytotoxic effects of PyED (which undergoes complexation with divalent metals, notably the Mg(II) ion present in cell culture media) were significantly potentiated when the compound was administered to tumor cells at hyperthermic temperatures (4). This enhancement of cytotoxicity was observed when cells were treated with concentrations of the compound that were minimally cytotoxic after treatment at 37°C.

The Zaleski laboratory recently synthesized a suite of six novel metal complexes of PyED ($CuCl_2$-PyED, $CuSO_4$-PyED, $FeCl_2$-PyED, $FeSO_4$-PyED, $ZnCl_2$-PyED or $ZnSO_4$-PyED) in an effort to develop compounds with greater enhancement of triggering diradical reactivity, with the intention being that toxicity and efficacy could be more efficiently tuned (8). Much like the predecessor compounds, Bergman cyclization from thermal activation of each of the complexes resulted in diradical generation. The order of diradical cyclization reactivity from these divalent complexes, as well as the efficiency of degradation of DNA *in vitro* was metal-dependent (Cu > Fe > Zn). However, cytotoxicity at 37°C was not always correlated with reactivity and efficiency of DNA degradation *in vitro*, since the ranking of surviving fraction at the same concentration (25 μ*M*) from lowest to highest, for all of the sulfate compounds, is Zn(0.28) > Cu(0.82) > Fe(0.96), although the ranking for all of the chloride compounds is Cu(0.39) > Fe(0.67). Zn(0.84). Moreover, while the survival curves for cells treated at 37°C with either derivative of the Fe complexes were similar to each other, and cells showed similar potentiation after treatment at 42.5°C, the Cl^- and SO_4^- derivatives of the Cu and Zn derivatives varied greatly with respect to both cytotoxicity induced at 37°C (Fig. 2) and extent of potentiation of cytotoxicity when cells were treated with the compounds at 42.5°C.

The extensive cell killing after treatment at 42.5°C (e.g., enhancement of cell killing) with low concentrations of $CuCl_2$-PyED appeared promising for potential future *in vivo* work when this result was taken at face value (Fig. 2A). Unfortunately, cell survival when treated with the same concentrations at 37°C that resulted in significant enhancement of killing at 42.5°C (e.g., greater than an order of magnitude difference in surviving fraction) was less than ideal, since the goal in an animal model would be to show sparing of normal tissue effects (presumably reflecting a sparing of cytotoxicity) at physiological temperature. Similar results were obtained, albeit at higher concentrations, after treatment with the $ZnCl_2$ and $FeCl_2$ derivatives of PyED, and lower concentrations of the $CuSO_4$ and $ZnSO_4$ derivatives (Fig. 2). However, $FeSO_4$-PyED was non-toxic after treatment with high concentrations at 37°C, and cell killing was greater than one order of magnitude when cells were treated with drug during hyperthermia treatment. Because $FeSO_4$-PyED provided the “best of both worlds” (e.g., little or no toxicity after treatment at 37°C, but at least an order of magnitude difference in cell survival upon treatment at elevated temperature), we eventually confined mechanistic studies to this compound.

Three compounds were tested for cytotoxic effects in two cell lines. All three compounds showed enhanced efficacy in killing cells when cells were treated with the drugs at elevated temperature (Fig. 4), but there was variability in the magnitude of cytotoxic effects between

the cell lines. It is not clear why MDA-231 cells were more resistant than U-1 cells to $ZnCl_2$-PyED or $FeSO_4$-PyED after treatment with the compounds at either 37° or 42.5°C, but were more sensitive to $CuCl_2$-PyED than U-1 cells during treatment with the drugs at both temperatures. It is attractive to speculate that the MDA-231 breast cancer cells may take up less of the $ZnCl_2$-PyED or $FeSO_4$-PyED compounds or may have a higher rate of drug efflux at both temperatures compared to U-1 cells, and the U-1 cells may likewise take up less of the $CuCl_2$-PyED complex or extrude it more rapidly compared to the MDA-231 cells.

The cell cycle progression studies (Figs. 5 and 6) performed in the current study with our next-generation metalloenediynes, when taken together with our previous studies with the first-generation compounds (4), indicate that metalloenediynes, when administered to cells under hyperthermic conditions, significantly reduce the G_2 block that is normally observed after heating alone (12). The current studies also confirm the mode of death induced by this class of compounds when administered at elevated temperatures. In our original characterization of first-generation metalloenediynes in mode-of-death studies, we found that the compounds (when used at concentrations that produced similar cytotoxicity at 37°C as the second-generation compounds tested in the current study) induced apoptotic death in over one third of the population of cells analyzed 24 h after a 1 h treatment at 37°C (4). In the current study, only $CuCl_2$-PyED induced apoptosis in a significant fraction of cells (17%) by 24 h after treatment at 37°C; after treatment with $FeSO_4$-PyED or $ZnCl_2$-PyED, less than 5% of cells were found to be apoptotic at any point within the 24-h period of observation. Consistent with our previous study, an appreciable enhancement of apoptotic death was detected during the 24-h period of observation when cells had been treated with any of the three compounds at 42.5°C (Fig. 7).

The observation that heating cells during treatment with $FeSO_4$-PyED resulted in an increase of γ-H2AX foci (a biomarker of DSBs) (Fig. 8) is not surprising; we observed that heat increased DNA damage and inhibited DNA repair when cells were treated with one of the first-generation compounds we previously tested (PyED, which in the presence of magnesium in cell culture media promotes formation of the metalloenediyne construct). However, in these previous studies, DNA damage was assessed using the comet assay, which limited us to detection of a combination of DNA single-strand breaks, DSBs and alkali-labile sites in the DNA (13). Because accurate counting of γ-H2AX foci can be challenging due to the irregular size and morphology of foci, which may be attributed in part to cell cycle phase, we opted to also measure pan-nuclear γ-H2AX (fluorescence per nuclei, as an indication of the pan-nuclear γ-H2AX phosphorylation that occurred), especially since we noted that fluorescence was not totally confined to foci. Although the relationship between pan-nuclear γ-H2AX response and foci formation remains unclear, there is a strong correlation between the number of γ-H2AX foci per cell and total γ-H2AX fluorescence after inducing DSBs either by ionizing radiation or clastogenic drugs (20, 21). Thus, other investigators have concluded that total γ-H2AX fluorescence can be used as a reliable and sensitive measurement of induction of DNA DSBs as well as DSB repair kinetics. We found the same trends when both γ-H2AX foci and total γ-H2AX nuclear fluorescence were measured for all treatment groups. A limited analysis of fluorescent foci from images accumulated after U-1 cells were treated with either 25 μ*M* $CuCl_2$-PyED or 75 μ*M* $ZnCl_2$-

PyED at 37°C or 42.5°C (Supplementary Fig. S1, data from one experiment with each compound) indicated a pattern of formation and lack of disappearance of γ-H2AX foci that was similar to what we observed when cells were treated with 100 μ*M* $FeSO_4$-PyED at the respective temperatures (Fig. 8); that is, treatment of cells with $CuCl_2$-PyED or $ZnCl_2$-PyED, under hyperthermic conditions, resulted in an enhanced number of γ-H2AX foci induced by the compounds when compared to treatment at 37°C, and the number of foci per cell increased or remained elevated even after cells were allowed to recover at 37°C for 2 h after the respective drug treatments.

Data from our γ-H2AX experiments suggest that potentiation of metalloenedyine-induced cell death by hyperthermia is mediated via enhanced production of DSBs or inhibition of DSB repair, the latter of which has been advanced to explain the radiosensitizing effect of hyperthermia (*14–19*). This notion is supported by several previous observations by Porter *et al.* (*8, 22*). For example, the reaction rates of the various metallated compounds differ, and the reaction rates show significant temperature-dependence and have been directly correlated to efficiency of DNA degradation. However, no degradation is observed when DNA is incubated with the cyclized control compound $FeSO_4$-PyBD (the non-reactive version of $FeSO_4$-PyED); this indicates that the enediyne moiety is responsible for the production of DSBs. While it is attractive to speculate that the enhanced cytotoxicity and induction of DSBs in heated, drug-treated cells (compared to cells treated with the compounds at 37°C) may be due, at least in part, to enhanced formation of diradical intermediates, further experiments will be required to definitively determine this.

Our findings of the reduction of the G_2 block (normally induced by heat) taken together with the increase in, and lack of disappearance of γ-H2AX foci and pan-nuclear γ-H2AX staining in heated, drug-treated cells suggest that the enhanced cell killing when cells are treated with $FeSO_4$-PyED at 42.5°C is due to an increase in the initial induction of DSBs or inhibition of repair. It is therefore attractive to speculate that progression of U-1 cells into mitosis with unrestituted DSBs may account for the potentiation of cytotoxicity observed for heated drug-treated cells.

Experiments in which time and temperature were varied during treatment with $CuSO_4$-PyED, $FeCl_2$-PyED or $FeSO_4$-PyED confirmed that metalloenediyne cytotoxicity was both time- and temperature-dependent (Fig. 3). These findings have potential clinical relevance and could spur renewed interest in hyperthermia, particularly the expanded use of thermal ablation as a cancer modality, when exploited for chemosensitization. During thermal ablation therapy, tumors are subjected to brief localized heat treatments at temperatures ≥50°C. While tumor cells around the central zone of coagulation will be readily killed, the technique allows for normal tissues to be largely unaffected, since they are expected to lie outside the temperature gradient of the ablation zone. However, tumor cells which lie at the periphery of the tumor, outside of the coagulation zone but within the lower side of the temperature gradient created during the ablation procedure, may not have reached a sufficient temperature, or may not have been heated to the required temperature long enough to have received a lethal heat dose. Thus, such cells that receive a sublethal heat dose could provide the basis for tumor regrowth. We envision that metalloenediynes could be useful for eliminating cells that would normally not be killed by a sublethal heat dose, since even

sublethal heat doses might be sufficient to activate the metalloenediyne compounds and potentiate cytotoxicity of the drugs.

In summary, we found that hyperthermia potentiated the cytotoxicity of new metalloenediyne complexes at concentrations that are either only mildly or not cytotoxic at 37°C, and that the potentiation of cytotoxicity by heat appears to be mediated by increased induction of DSBs and inhibition of DSB repair. Since our *in vitro* experiments have been confined to tumor cells, it will be important to assess the toxicity of metalloenediyne compounds on normal cells when the cells are treated under normothermic or hyperthermic conditions; determination and then comparison of thermal enhancement ratios for tumor and normal cells will provide insight regarding potential dose limitations for future clinical applications in which the tumor and, potentially, surrounding normal tissues, are heated during drug treatment. Likewise, further testing of these heatactivatable metalloenediynes and demonstration of utility in an in vivo tumor model will be useful for moving these compounds into clinical trials as chemotherapeutic agents that can be systemically administered, but with little or no side effects, given the expectation that the toxicity would be predominantly localized to sites of heating.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health (NIH grant no. 1R01CA196293). We thank the Indiana Lions Cancer Control Fund and the Indiana University Melvin and Bren Simon Cancer Center Flow Cytometry Resource Facility (IUSCC FCRF) for their support. The IUSCC FCRF is funded in part by NIH grant nos. P30 CA082709 and U54 DK106846, and NIH instrumentation grant no. 1S10D012270. We thank the Indiana Center for Biological Microscopy for assistance with confocal microscopy and analysis of foci, and Quinten Carter for technical support.

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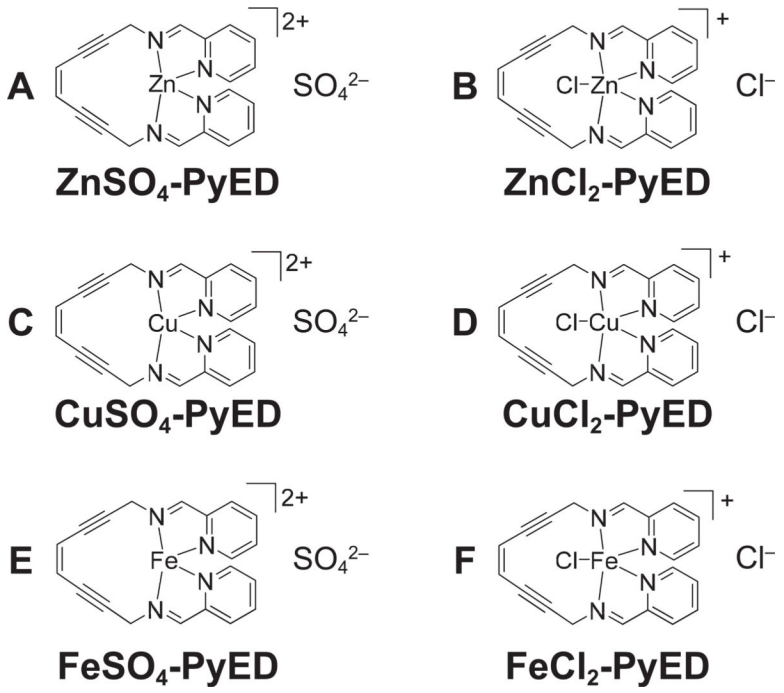

FIG. 1.

Structures of novel metallated derivatives of (*Z*)-*N*,*N*′-bis[1-pyridin-2-yl-meth-(*E*)-ylidene]oct-4-ene-2,6-diyne-1,8-diamine (PyED). Panel A: (1*E*,1′*E*)-N,N′-((*Z*)-octa-4-en-2,6-diyne-1,8-diyl)-bis(1-(pyridin-2-yl)methanimine)zinc(II) sulfate ($ZnSO_4$PyED). Panel B: (1*E*,1′*E*)-N,N′-((*Z*)-octa-4-en-2,6-diyne-1,8-diyl)bis(1-(pyridin-2-yl)methanimine)zinc(II) chloride ($ZnCl_2$PyED). Panel C: (1*E*,1′*E*)N,N′-((*Z*)-octa-4-en-2,6-diyne-1,8-diyl)bis(1-(pyridin-2-yl)methanimine)copper(II) sulfate ($CuSO_4$PyED). Panel D: (1*E*,1′*E*)-N,N′-((*Z*)-octa-4-en-2,6-diyne-1,8-diyl)bis(1-(pyridin-2-yl)methanimine)copper(II) chloride ($CuCl_2$PyED). Panel E: (1*E*,1′E)-N,N′-((*Z*)-octa-4-en-2,6-diyne-1,8-diyl)bis(1-(pyridin-2-yl)methanimine)iron(II) sulfate ($FeSO_4$PyED). Panel F: (1E,1′E)-N,N′-((*Z*)-octa-4-en-2,6-diyne-1,8-diyl)bis(1-(pyridin-2-yl)methanimine)iron(II) chloride ($FeCl_2$PyED). See Materials and Methods and Porter *et al.* (8) for syntheses of compounds. The order of diradical cyclization reactivity from the divalent complexes is as follows: Cu > Fe > Zn.

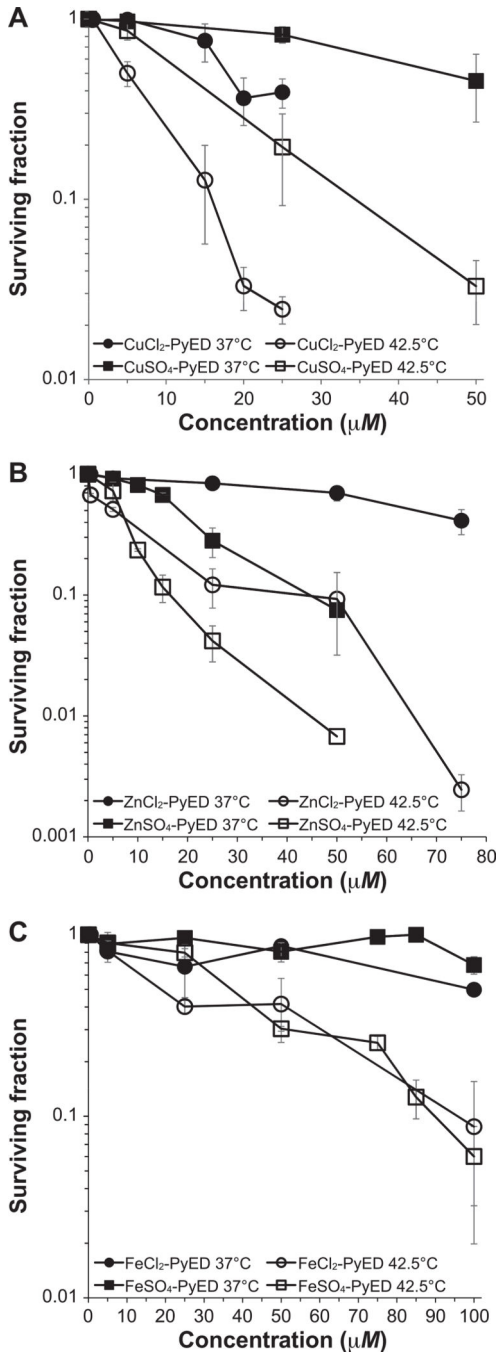

FIG. 2.

Hyperthermia enhances the cytotoxicity of six novel copper, iron or zinc metalloenediynes. U-1 melanoma cells were treated with various concentrations of (panel A) $CuCl_2$-PyED or $CuSO_4$-PyED, (panel B) $ZnCl_2$-PyED or $ZnSO_4$-PyED or (panel C) $FeCl_2$-PyED or $FeSO_4$-PyED for 1 h at 37°C or 42.5°C, and then plated for assessment of clonogenic survival and determination of surviving fraction. Surviving fractions were normalized to the vehicle treatment at each of the respective temperatures. Error bars represent the standard error of the mean (SEM) from 2–4 experiments per compound.

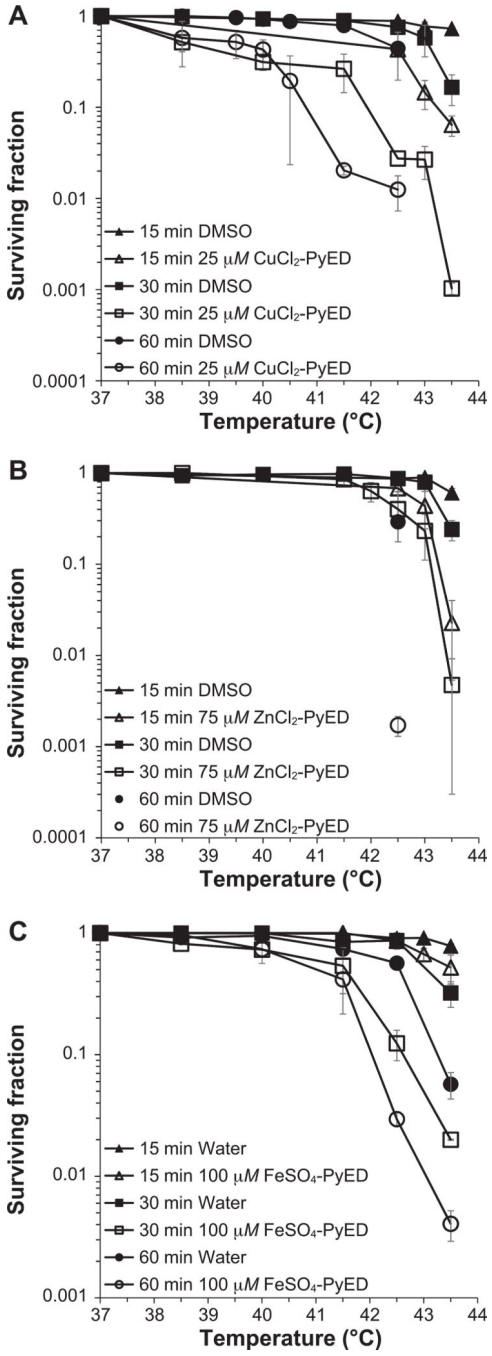

FIG. 3.

Time-temperature dependence of potentiation of metalloenediyne cytotoxicity. U-1 cells were heated at various temperatures (38.5–43.5°C) during treatment for 15, 30 or 60 min with either vehicle (1.5% DMSO or water) or (panel A) 25 μ*M* $CuCl_2$-PyED, (panel B) 75 μ*M* $ZnCl_2$-PyED or (panel C) 100 μ*M* $FeSO_4$-PyED. Surviving fractions were normalized to the respective drug or vehicle treatment at 37°C. Error bars represent the SEM from 2–4 experiments (however, the following time points represent data from one experiment:

$FeSO_4$-PyED 15 min at 41.5°C; $FeSO_4$-PyED 30 min at 38.5°C and 40°C; $CuCl_2$-PyED 30 min at 42.5°C).

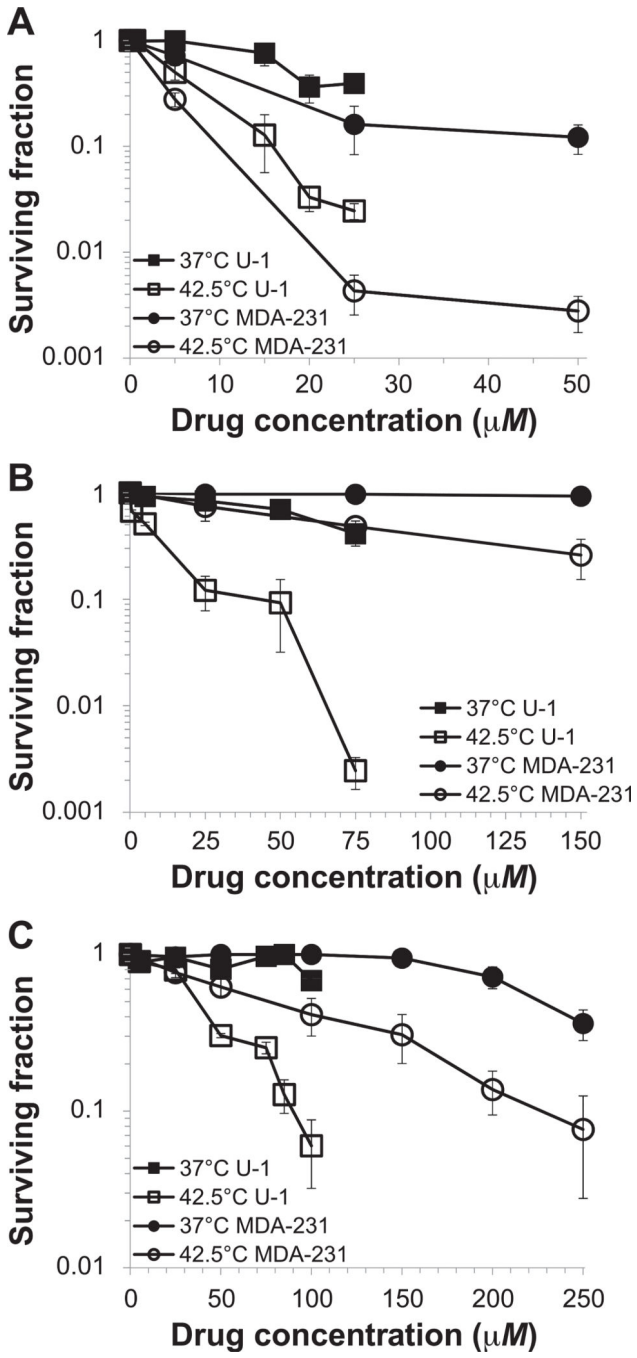

FIG. 4.

A comparison of metalloenediyne cytotoxicity at 37°C and enhancement of cytotoxicity by hyperthermia in two different cell lines. U-1 melanoma or MDA-231 breast cancer cells were treated with various concentrations of (panel A) $CuCl_2$-PyED, (panel B) $ZnCl_2$-PyED or (panel C) $FeSO_4$-PyED, or vehicle (1.5% DMSO or water) for 1 h at either 37° or 42.5°C, and then plated for assessment of clonogenic survival and determination of surviving fraction. Surviving fraction was normalized to the surviving fraction of cells exposed to

vehicle only at each respective temperature. Error bars represent the SEM from 2–4 experiments.

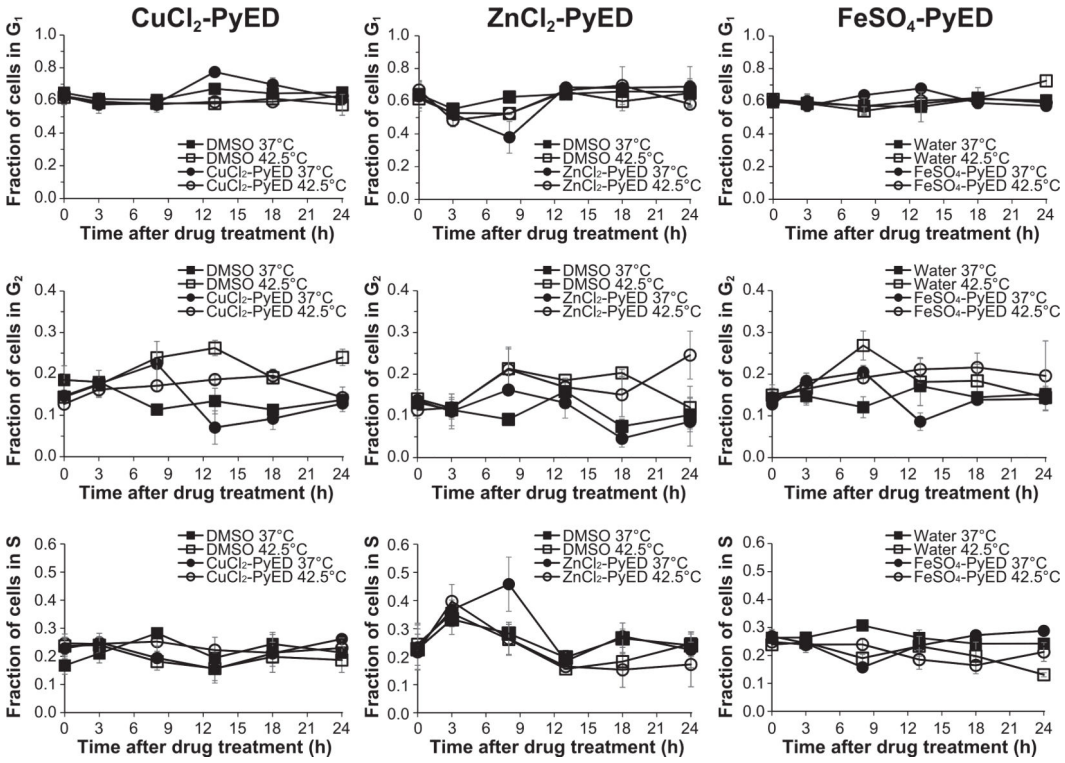

FIG. 5.

Changes in cell cycle progression after exposure of U-1 melanoma cells to metalloenediynes and/or hyperthermia. U-1 cells were treated with a metalloenediyne (25 μ*M* $CuCl_2$-PyED, 75 μ*M* $ZnCl_2$-PyED or 100 μM $FeSO_4$-PyED) or vehicle (1.5% DMSO or water) for 1 h at 37°C or 42.5°C. After treatment, cells were incubated at 37°C in fresh media and allowed to progress through the cell cycle for various lengths of time. Cells were then fixed, stained and analyzed for DNA content by flow cytometry to determine the fraction of cells in each phase of the cell cycle as a function of time after treatment. Error bars represent the SEM from two experiments.

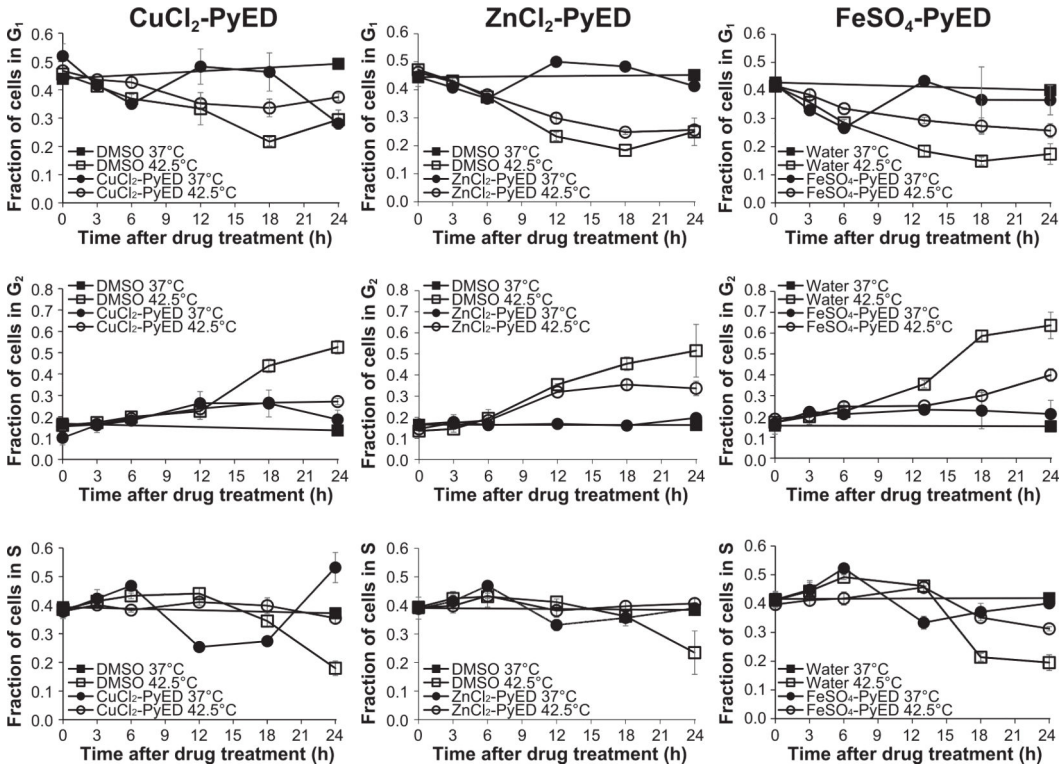

FIG. 6.

Changes in cell cycle progression after exposure of MDA-231 breast cancer cells to metalloenediynes and/or hyperthermia. MDA-231 cells were treated with a metalloenediyne (25 μ*M* $CuCl_2$-PyED, 150 μ*M* $ZnCl_2$-PyED 150 m FeSO4-PyED) or vehicle (1.5% DMSO or water) for 1 h at 37°C or 42.5°C. After treatment, cellswere incubated at 37°C in fresh media and allowed to progress through the cell cycle for various lengths of time. Cells were then fixed, stained and analyzed for DNA content by flow cytometry to determine the fraction of cells in each phase of the cell cycle as a function of time after treatment. Error bars represent the SEM from 2–3 experiments.

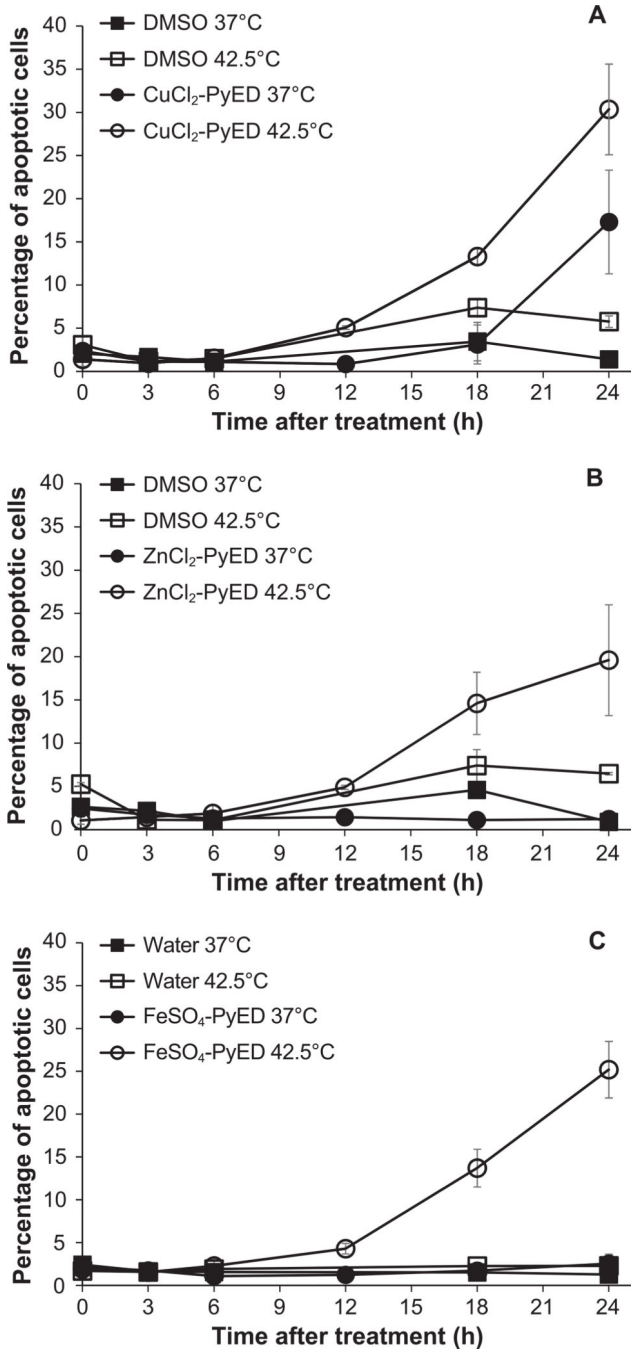

FIG. 7.

Heating during treatment with metalloenediynes alters the mode of cell death. U-1 melanoma cells were incubated with 25 μ*M* $CuCl_2$-PyED, 75 μ*M* $ZnCl_2$-PyED, 100 μ*M* $FeSO_4$-PyED or vehicle (1.5% DMSO or water) for 1 h at 37°C or 42.5°C. After treatment, cells were then incubated at 37°C with fresh drug-free media for various lengths of time before being stained with Annexin V-EGFP and PI. Apoptotic fractions were obtained from bivariate plots (not shown) after flow cytometric analysis, and were plotted as a function of

time after metalloenediyne treatment. Error bars represent SEM for 2–3 experiments. Panel A: $CuCl_2$-PyED. Panel B: $ZnCl_2$-PyED. Panel C: $FeSO_4$-PyED.

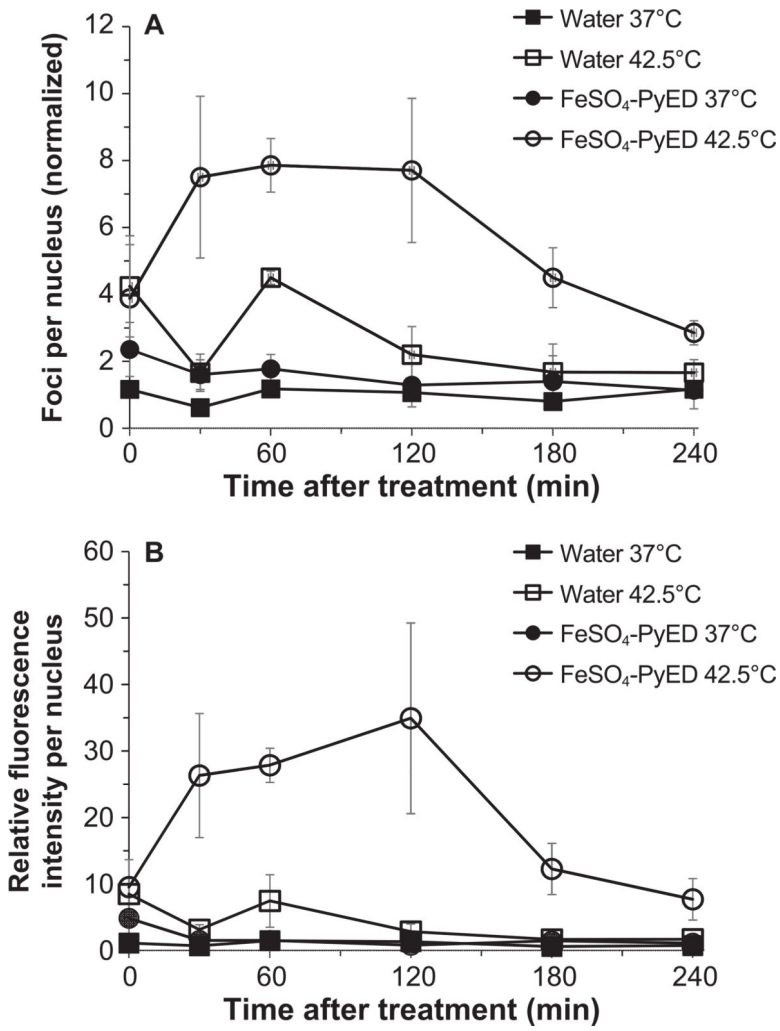

FIG. 8.

Treatment of cells with $FeSO_4$-PyED at supraphysiological temperature enhances double-strand break (DSB) formation and inhibits repair. U-1 cells were treated with 100 μ*M* of $FeSO_4$-PyED or vehicle (1.5% DMSO or water) for 1 h at either 37°C or 42.5°C, and then incubated with fresh media at 37°C for various times before fixation and staining for γ-H2AX. Number of fluorescent γ-H2AX foci and γ-H2AX fluorescence were measured in each nucleus. An average of 33 nuclei per treatment were analyzed for each experiment and each data point represents the mean from 2–3 experiments. In panel A, the average number of γ-H2AX foci per nucleus (total number of foci divided by the number of nuclei analyzed for each respective treatment) are plotted as a function of time after each treatment. In panel B, average γ-H2AX fluorescence per nucleus (total fluorescence of γ-H2AX-stained nuclei was divided by the number of nuclei) is plotted as a function of time after treatment. Note: Due to differences in background staining between experiments, in each individual experiment the value for each time point (average number of foci per nucleus or fluorescence per nucleus) was normalized to the average value for cells treated with vehicle-only at 37°C for all time points before the means from each experiment were combined; we

chose to average all of the time points for the “vehicle-only at 37°C” time points to eliminate effects of the variability seen in those non-treated samples at different time points.