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ORIGINAL RESEARCH

KEMTUB012-NI2, a novel potent tubulysin analog that selectively targets hypoxic cancer cells and is potentiated by cytochrome p450 reductase downregulation

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Purpose: There is an urgent need to develop effective therapies and treatment strategies to treat hypoxic tumors, which have a very poor prognosis and do not respond well to existing therapies.
Methods: A novel hypoxia-targeting agent, KEMTUB012-NI2, was synthesized by conjugating a 2-nitroimidazole hypoxia-targeting moiety to a synthetic tubulysin, a very potent antimitotic. Its hypoxic selectivity and mode of action were studied in breast cancer cell lines.
Results: KEMTUB012-NI2 exhibited a similar selectivity for hypoxic cells to that of tirapazamine, a well-established hypoxia-targeting agent, but was >1,000 times more potent in cell cytotoxicity assays. The hypoxia-targeting mechanism for both KEMTUB012-NI2 and tirapazamine was selective and mediated by one-electron reductases. However, while cytochrome p450 reductase (POR) downregulation could inhibit tirapazamine cytotoxicity, it actually sensitized hypoxic cells to KEMTUB012-NI2.
Conclusion: KEMTUB012-NI2 is a potent new agent that can selectively target hypoxic cancer cells. The hypoxia selectivity of KEMTUB012-NI2 and tirapazamine appears to be differentially activated by reductases. Since reductases are heterogeneously expressed in tumors, the different activation mechanisms will allow these agents to complement each other. Combining POR downregulation with KEMTUB012-NI2 treatment could be a new treatment strategy that maximizes efficacy toward hypoxic tumor cells while limiting systemic toxicity.
Keywords: breast cancer, tubulysin, anti-mitotic, hypoxia, cell death, reductase

Introduction

Cells that are exposed to hypoxic (low oxygen) conditions are characteristic of locally advanced solid tumors and have been described in a wide range of human malignancies.[1] Up to 50%–60% of locally advanced solid tumors exhibit regions of hypoxic and/or anoxic tissue that are heterogeneously distributed within the tumor mass. Tumors with high hypoxic volumes have a poor prognosis and an aggressive tumor phenotype and exhibit increased risk of invasion and metastasis.[2,3] It is also well established that hypoxic cells respond poorly to radiotherapy and chemotherapy. The decreased effectiveness of radiotherapy at low oxygen concentrations is primarily a result of reduced free radical formation.[1,4] The efficacy of standard chemotherapies in hypoxic tumors is affected by multiple factors as follows: 1) poor tumor vasculature can limit drug delivery to hypoxic cells; 2) the low pH of the extracellular environment can alter the ionic charge of the drug; 3) limited cell proliferation can significantly reduce the efficacy of agents that target cycling cells; 4) hypoxic cells have decreased

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susceptibility to apoptosis; and 5) hypoxic cells frequently upregulate multidrug-resistant transporters.[1,4,5] Accordingly, specific agents have been developed to overcome these issues and provide new therapeutic strategies that have the potential to selectively target hypoxic cells.

Researchers have explored the following two main strategies to develop therapeutics that selectively target hypoxic cells:[6] targeting the hypoxia inducible factor 1 (HIF1) pathway, the master regulator of the hypoxia response, and designing bioreductive prodrugs that become activated in hypoxic cells. The former approach has had limited success to date, as the prospective molecules either are not specific for hypoxic cells or produce pleiotropic effects.[6] The latter approach has yielded the following four classes of bioreductive compounds:[7,8] nitroaromatics (eg, 2-nitroimidazole-containing compounds such as nimorazole and PR104A), aromatic N-oxides (eg, tirapazamine), aliphatic N-oxides (eg, banoxantrone), and quinones (eg, apaziquone). With the exception of nimorazole that is used in Denmark to treat some hypoxic tumors, none of these bioreductive agents have been approved for clinical use, due to limited efficacy in clinical trials. This is partly because of a lack of reliable methods to stratify patients into hypoxic and nonhypoxic groups prior to therapy[4] and also reflects the limitations of the current bioreductive agents. Tirapazamine, widely considered as the leading aromatic N-oxide, is only active at micromolar concentrations in cells,[9] making it challenging to deliver an efficacious drug dose to hypoxic tumor cells. A modified version of tirapazamine (SN30000) has subsequently been developed to overcome the issue of poor tumor penetration.[10] Another leading candidate, banoxantrone, displays very limited selectivity for hypoxic cells in vitro.[11] Achieving efficacy with bioreductive agents in clinical trials is, therefore, likely to require development of more effective agents, utilizing improved treatment strategies and only using these therapies in hypoxic tumors.

Tubulysins are natural tetrapeptides that inhibit tubulin polymerization by binding to tubulin near the vinca alkaloid binding site. Synthetic tubulysin analogs have been synthesized to facilitate their development as anticancer compounds because the natural peptides produced by myxobacteria are only made in minute quantities. Tubulysins are extremely potent inhibitors of mammalian cancer cell proliferation in vitro,[12,13] block cells in the G_2/M phase of the cell cycle,[12,14,15] and induce cell death via a mechanism of action that is distinct from taxanes.[12] However, preclinical experiments indicate that they have a narrow therapeutic window,[16] and hence may be too toxic for use as traditional single agent therapeutics. A more favorable strategy could be to use them as targeted therapeutics, allowing them to be selectively directed to tumor cells and minimizing systemic toxicity. Indeed, this approach has been substantiated by conjugating tubulysin to trastuzamab;[17] the conjugated tubulysin was able to selectively target HER2-expressing cancer cells and xenografts.

In this study, we conjugated synthetic tubulysin analogs to 2-nitroimidazole to produce new hypoxia-targeted bioreductive agents. A novel derivative belonging to the synthesized compounds, namely KEMTUB012-NI2, exhibited a similar selectivity for hypoxic cells to that of tirapazamine, but was >1,000 times more potent in cell cytotoxicity assays. Moreover, it was possible to pharmacologically inhibit the hypoxia selectivity of the tubulysin analog, demonstrating that the hypoxia-targeting mechanism is selective and mediated by a one-electron flavoenzyme reductase(s). Finally, downregulation of cytochrome p450 reductase (POR) sensitized hypoxic cells to the tubulysin analog. These data demonstrate that this reductase is not required in the hypoxia-targeting mechanism and suggest that combining POR downregulation with tubulysin treatment could form the basis of a novel hypoxia-targeting therapy that could maximize efficacy toward hypoxic tumor cells while limiting systemic toxicity to cancer patients.

This article does not contain any studies with human participants or animals performed by any of the authors.

Methods

Chemistry

Tubulysin analogs KEMTUB001, KEMTUB010, and KEMTUB012 (Figure 1) were synthesized according to the previously reported procedure.[18] KEMTUB001 and KEMTUB012 were coupled via the terminal carboxylic group to compounds bearing a 2-nitroimidazole group (NI1, NI2, and NI3) to obtain tubulysin derivatives shown in Figure 1. In particular, both the tubulysin derivatives were conjugated to 2-(2-nitro-1*H*-imidazol-1-yl)ethanamine, NI1.[19] KEMTUB012 was also coupled to compounds NI2 and NI3, respectively, *N*-(3-aminopropyl)-2-(2-nitro-1*H*-imidazol-1-yl)acetamide, and N-(6-aminohexyl)-2-(2-nitro-1*H*-imidazol-1-yl)acetamide. Assembling of the final tubulysin-based conjugates KEMTUB001-NI1, KEMTUB012-NI1, KEMTUB012-NI2, and KEMTUB012-NI3 was performed by using conventional peptide synthesis in solution procedures (Figure S1). The synthetic procedures as well as data concerning the characterization of the synthesized tubulysin analogs and their derivatives bearing NI1, NI2, and NI3 groups are reported in the Supplementary materials.

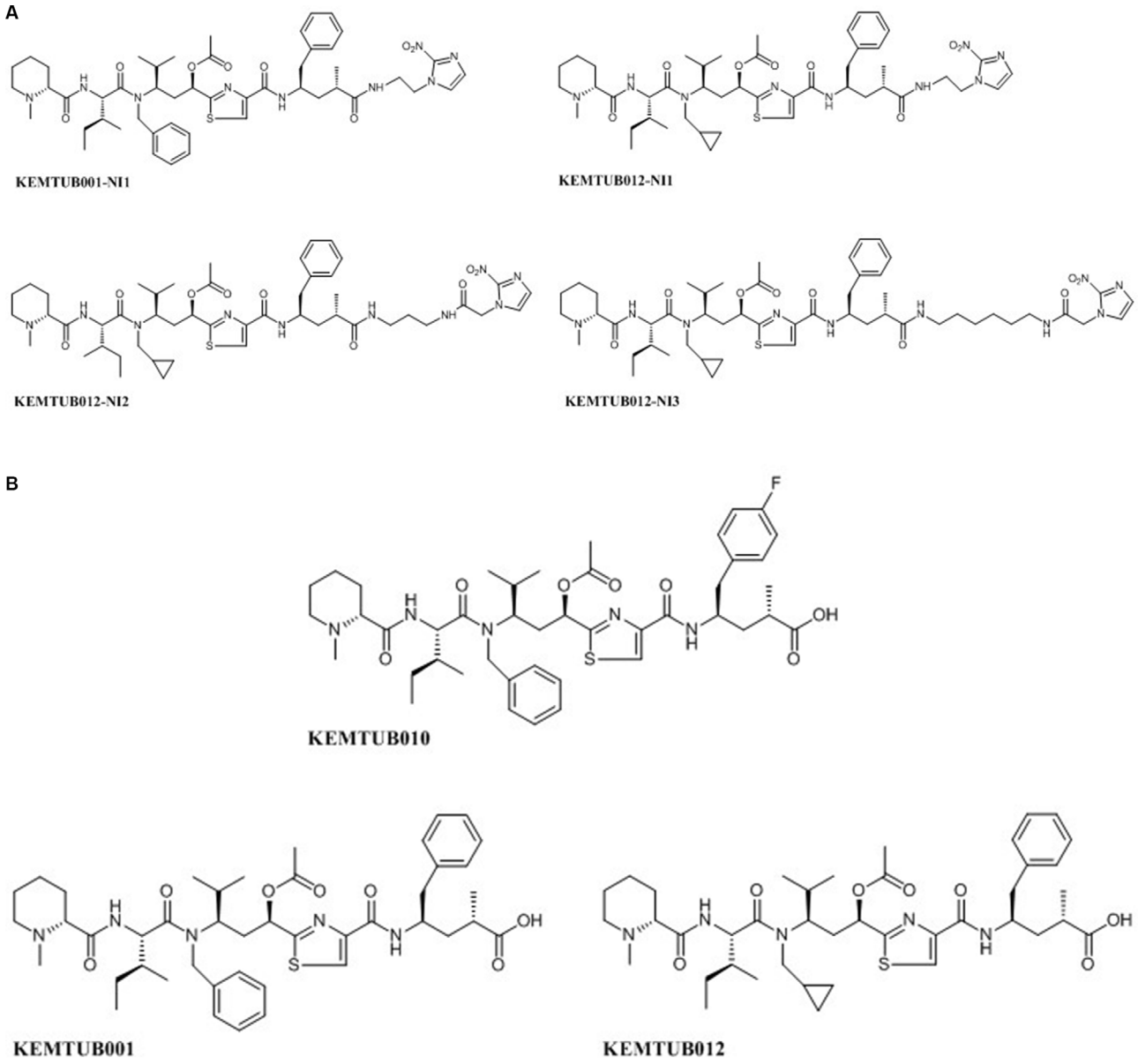

Figure 1 Chemical structures of tubulysin analogs. (**A**) Structures of nitroimidazole-tubulysin conjugates. (**B**) Structures of tubulysins.

Cell lines, cell culture, and reagents

Experiments were performed in MCF7, MDAMB231 (MDA231), and MDAMB468 (MDA468) breast cancer cells. MCF7 represents the luminal subtype, which characteristically expresses estrogen and progesterone receptors.[20] MDA231 and MDA468 represent the basal-like subtype, which typically do not contain estrogen, progesterone, or HER2 receptors and generally have a poorer prognosis than luminal tumors.

MCF7, MDA231, and MDA468 cells were purchased from American Type Culture Collection and confirmed as authentic and contamination-free. Cell cultures were maintained in Roswell Park Memorial Institute (RPMI) containing 10% (v/v) fetal calf serum, 100 Units/mL penicillin, and 100 mg/mL streptomycin and grown at 37°C in a humidified atmosphere containing 5% CO_2. All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), unless stated otherwise.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell cytotoxicity assays

For standard cytotoxicity experiments, cells were seeded in 96-well plates at 5,000 cells/well, then left to settle overnight. Stock solutions of test compounds (KemoTech Srl, Pula (CA), Italy, www.kemotech.it) were prepared in dimethyl sulfoxide (DMSO). Cells were treated with a range of drug concentrations for 24 h in triplicate wells in normoxic (21% O_2) or hypoxic conditions (1% O_2). Medium was then aspirated and replaced with fresh RPMI. Cells were left to grow for 48 h

in normoxic conditions and the number of viable cells in each well estimated by incubating cells in media containing 0.5 mg/mL MTT cell proliferation reagent for 1–2 h. Medium was aspirated, the formazan product solubilized in DMSO[21] and measured at 540 nM in a microplate reader. GraphPad Prism was used to calculate IC_{50} values.

Western blot analysis

MCF7 and MDA231 cells were seeded at 0.35×10^6/60 mm plates and left to settle overnight. Cells were incubated with DMSO or KEMTUB012-NI2 at the specified concentration for the time indicated. The protein concentration in the resulting lysates was determined by bicinchoninic acid assay. Blotting was done essentially as described previously.[22] Membranes were probed with the following antibodies: cleaved PARP (cell signaling), POR (Santa Cruz Biotechnology, Dallas, TX, USA), methionine synthase reductase (MTRR; Abcam, Cambridge, UK), diflavin oxidoreductase-1 (NDOR1), and β-actin (both Sigma-Aldrich). After 4×5 min washes in phosphate buffered saline containing 0.1% Tween20, membranes were incubated for 1 h with the appropriate infrared dye-conjugated secondary antibody (Licor, Lincoln, NE, USA) diluted 1:10,000. Images were captured with an Odyssey® CLx LI-CORE imaging system and densitometries of target proteins measured using Image studio Lite.

POR small interfering RNA (siRNA) cell transfection experiments

MCF7 and MDA231 cells were seeded in 96-well plates at 2,000 cells/well or in 60 mm plates at 0.2×10^6/60 mm plate and left to settle overnight. Next day, the cells were transfected with a random siRNA or POR siRNA (both Santa Cruz Biotechnology) using Jetprime transfection reagent (Source Bioscience, Nottingham, UK), using the protocol recommended by the manufacturer. Medium was changed the next day to remove transfection reagent. Cells were grown for another 24 h to maximize POR downregulation before any additional treatments were initiated.

Trypan blue exclusion assay to detect dead cells

Cells were seeded as described for Western blot experiments, then treated with the indicated concentrations of KEMTUB012-NI2 for the time shown. In some experiments, POR was downregulated by siRNA treatment prior to addition of KEMTUB012-NI2. Adherent cells in each plate were trypsinized and combined with floating cells collected by centrifugation (1,000 g × 4 min) from the culture medium. Cells were incubated with trypan blue for 5 min, then the proportion of blue (dead) and transparent (live) cells counted in a hemocytometer.[12,23]

Results

Synthetic tubulysins such as KEMTUB010 are very potent anticancer agents in normoxic breast cancer cells, with picomolar concentrations inhibiting cell proliferation and inducing cell death.[12] Experiments in MCF7 and MDA231 cells indicated that KEMTUB010 was similarly cytotoxic in hypoxic and normoxic conditions (Table 1). Indeed, in MCF7 cells chronic hypoxia significantly decreased the KEMTUB010 IC_{50} value, producing a normoxia:hypoxia (N:H) selectivity ratio >1, indicating enhanced cytotoxicity in hypoxic cells. This is likely a cell line-dependent effect since an equivalent decrease was not detected in MDA231 cells. In contrast, the well-established bioreductive anticancer agent tirapazamine was more effective in chronic hypoxia in

Table 1 Effect of hypoxia treatment on cell cytotoxicity of tubulysin analogs

Compound	MCF7			MDA231			MDA468		
	Normoxia (IC_{50}, nM)	Hypoxia (IC_{50}, nM)	N:H	Normoxia (IC_{50}, nM)	Hypoxia (IC_{50}, nM)	N:H	Normoxia (IC_{50}, nM)	Hypoxia (IC_{50}, nM)	N:H
KEMTUB010	0.0368±0.0038	0.0131±0.0023	2.8	0.059±0.0056	0.0587±0.0064	1.0			
KEMTUB012	0.016±0.0076	0.010±0.0052	1.6	0.0355±0.0122	0.0259±0.012	1.4	0.0321±0.027	0.0241±0.0006	1.3
KEMTUB001	0.0183±0.0083	0.01.7±0.0018	1.6	0.0412±0.0133	0.0323±0.014	1.3			
KEMTUB001-NI1	0.915±0.173	0.586±0.146	1.6	1.450±0.203	1.207±0.374	1.2			
KEMTUB012-NI1	0.854±0.336	0.433±0.386	2.0	1.466±0.250	0.943±0.234	1.6			
KEMTUB012-NI3	6.335±2.712	1.728±0.744	3.7*	10.937±2.436	4.769±1.772	2.3*	3.012±0.138	2.110±437*	1.4*
KEMTUB012-NI2	3.415±1.092	0.502±0.216	6.8*	6.050±1.946	1.904±0.856	3.2*	1.284±0.594	284±32	4.5*
Tirapazamine	36,900±9,300	3,500±900	10.6**	68,000±10.3	22,200±2,300	3.1**	4,967±2,700	500±200	9.1**

Notes: Cells were treated with a range of drug concentrations in normoxic (21% O_2) or hypoxic (1% O_2) conditions for 24 h. Medium was then aspirated and replaced with fresh RPMI and cells grown for 48 h in normoxic conditions. An MTT cell cytotoxicity assay was then performed. Results are the average ± SD of at least three independent experiments performed in triplicate. N:H ratio compares normoxic versus hypoxic IC_{50} value for each molecule. A compound with an N:H value of >1 is more effective in hypoxic conditions. Statistically significant differences between each treatment in hypoxic and normoxic conditions are shown (**P<0.01, *P<0.05).

Abbreviations: MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N:H, normoxia:hypoxia; RPMI, Roswell Park Memorial Institute; SD, standard deviation.

both cell lines tested. Acute hypoxia treatments did not have any significant effect on tirapazamine or KEMTUB010 IC_{50} values in either cell line (data not shown). These data indicate that synthetic tubulysins are at least as potent under hypoxic as normoxic conditions. Therefore, we hypothesized that conjugating tubulysin to nitroimidazole, a well-established hypoxia-targeting moiety, could create a potent anticancer agent that selectively targets hypoxic cells. For this scope, novel tubulysin derivatives bearing a 2-nitroimidazole moiety (Figure 1) were synthesized starting from synthetic tubulysins belonging to the same series of KEMTUB010.[12]

Cells were treated with each compound in both normoxic and hypoxic conditions. The MTT assays were performed in normoxic conditions to ensure that the formation of formazan by mitochondria was not influenced by low oxygen levels. The nitroimidazole group alone was not cytotoxic at the concentrations tested here (IC_{50} value >1 μM, data not shown). The tubulysins KEMTUB001 and KEMTUB012 were exceptionally potent in breast cancer cells, but hypoxic conditions had no significant effect on their IC_{50} values (Table 1). Linking a nitroimidazole group to either of these tubulysins increased the IC_{50} value by 1–2 orders of magnitude, indicating that the conjugation of the targeting moiety at the terminal carboxylic group of the tubulysin analogs moderately reduces the effectiveness of these agents. In cytotoxicity assays, KEMTUB001-NI1 generated an N:H ratio similar to KEMTUB001 in both MCF7 and MDA231 cells, indicating that the nitroimidazole moiety does not enhance its hypoxia selectivity. Likewise, the IC_{50} value of KEMTUB012 conjugate the KEMTUB012-NI1 was not noticeably influenced by hypoxia. In contrast, the KEMTUB012 conjugates KEMTUB012-NI2 and KEMTUB012-NI3 had significantly lower IC_{50} values under hypoxic conditions (Table 1), with hypoxia treatment shifting the dose curves to the left (eg, Figure 2A). KEMTUB012-NI2 displayed the highest N:H ratios in all cell lines; these were of the same order of magnitude as tirapazamine and significantly higher than banoxantrone, which displayed no significant selectivity for hypoxic MCF7 or MDA231 cells (data not shown). The lower IC_{50} values observed with KEMTUB012-NI2 and KEMTUB012-NI3 in hypoxic conditions represent decreased cell growth and/or increased cell death. KEMTUB012-NI2 was >1,000 times more potent than tirapazamine in hypoxic conditions in all cell lines tested.

The effect of oxygen concentration on the cytotoxic effect of KEMTUB012-NI2 was investigated in MCF7 and MDA231 cells (Figure 2B). In both cell lines, the N:H ratio increased as the O_2 concentration decreased from 5% to 1%. However, the cytotoxic effect was not further enhanced by decreasing the O_2 concentration to 0.1% in either cell line. To better understand this trend, we evaluated cellular uptake of a radiolabeled nitroimidazole-conjugated molecule, ^{18}F-fluoroazomycin arabinofuranoside (FAZA). FAZA uptake by MCF7 cells increased steadily as O_2 concentrations decreased from 5% to 0.1% (data not shown), confirming that cellular uptake of nitroimidazoles is not limited at low oxygen levels. Therefore, it seems likely that the absence of increased KEMTUB012-NI2 cytotoxicity between 1% and 0.1% O_2 is not due to restricted uptake of the molecule.

Since induction of cell death is an important element of the tubulysin mode of action in cancer cells,[12] we investigated whether the enhanced cytotoxic effect of KEMTUB012-NI2 in hypoxic cells could involve increased apoptosis or cell death. Initial experiments focused on induction of cPARP, a well-established biomarker of apoptosis. KEMTUB012-NI2 treatment caused a definite increase in cPARP generation in hypoxic, but not normoxic, MCF7 and MDA231 cells (Figure 2C and D). Induction of cPARP was observed with 2× IC_{50} KEMTUB012-NI2 in both cell lines, and this effect was not increased by using a higher KEMTUB012-NI2 dose. Trypan blue exclusion experiments were also performed, to determine if KEMTUB012-NI2 treatment increases the percentage of dead cells in hypoxic conditions. Indeed, 2× IC_{50} KEMTUB012-NI2 causes a significant increase in dead cells in hypoxic but not normoxic conditions in both cell lines (Figure 2E). These data confirm that at least part of the enhanced cytotoxic effect observed with KEMTUB012-NI2 in hypoxic cells is due to enhanced cell death. Higher KEMTUB012-NI2 concentrations induced cell death in both normoxic and hypoxic conditions.

Reduction of the nitroimidazole group by cellular reductases is credited with causing retention of nitroimidazole-conjugated molecules in viable hypoxic cells. However, the evidence supporting this model is very limited and the principal reductases involved in the process have not been clearly identified. Diphenyliodonium chloride (DPI) is an irreversible inhibitor of flavoenzymes that catalyze one-electron reduction reactions.[24] The reduced flavoprotein cofactor in one-electron reductases can reduce DPI, producing a phenyl radical. This can subsequently react with the flavoprotein to create a phenylated flavin, which uncompetitively inhibits the reductase. Experiments were, therefore, performed in the presence and absence of DPI to investigate the role of these enzymes in the hypoxia selectivity of KEMTUB012-NI2. Inclusion of DPI completely inhibited the hypoxia selectivity of KEMTUB012-NI2 in cytotoxic assays in both MCF7 (Figure 3A) and MDA231 cells (Figure 3B). Similar results were obtained for tirapazamine, whereas DPI had no effect

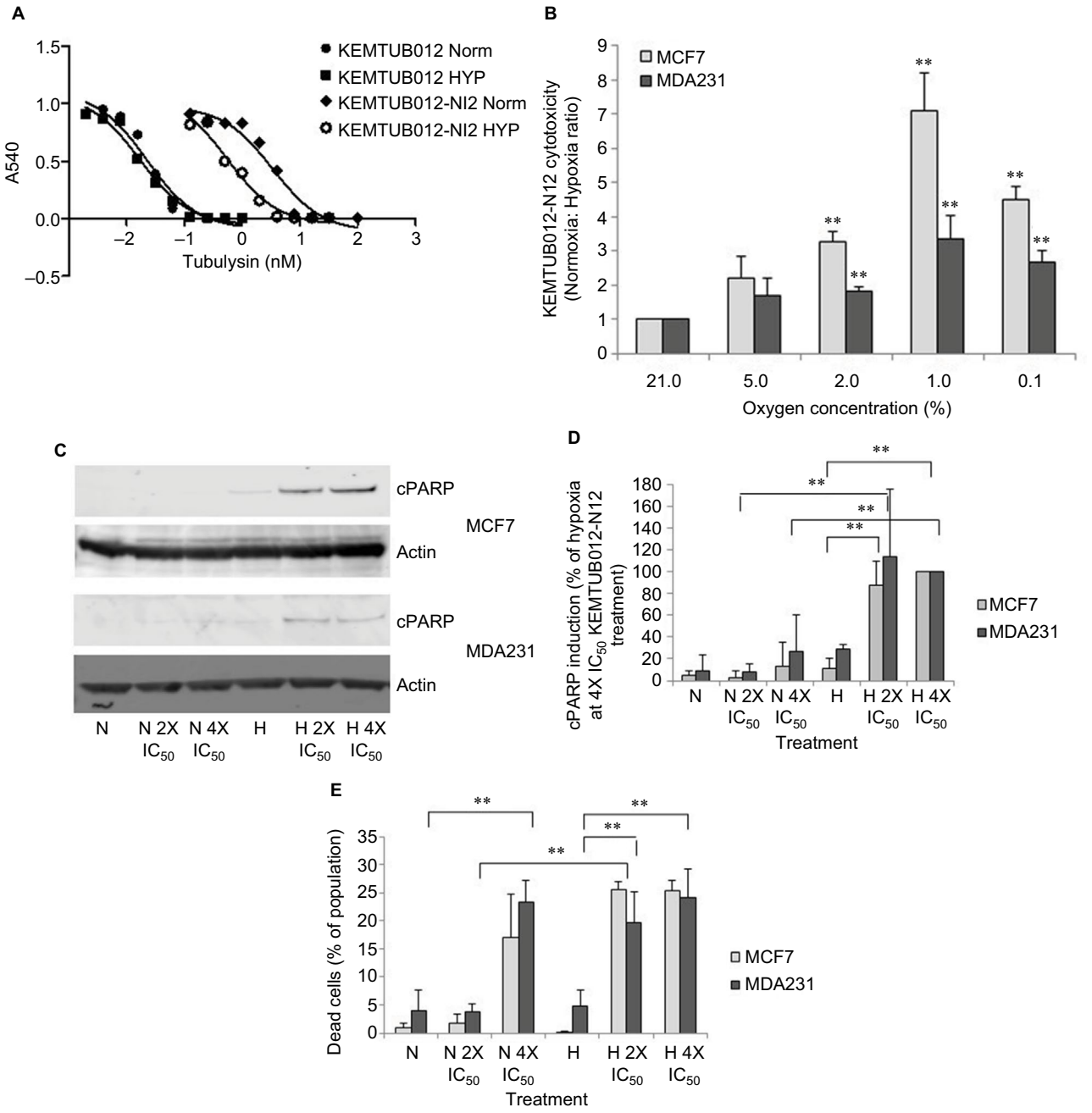

Figure 2 KEMTUB012-NI2 cytotoxicity depends on oxygen concentration and involves cell death.

Notes: (**A**) Representative MTT IC_{50} curves after MCF7 cells were treated with KEMTUB012 or KEMTUB012-NI2 in normoxic (norm) and hypoxic (hyp) conditions. *X*-axis is a log scale. (**B**) The KEMTUB012-NI2 IC_{50} value was measured in MCF7 and MDA231 cells treated at the indicated oxygen concentrations and data expressed as a normoxia:hypoxia ratio. Statistically significant differences between each hypoxic and normoxic treatment are shown in graph (**$P<0.01$). (**C–E**) Cells were treated with 2× or 4× IC_{50} KEMTUB012-NI2 as indicated in normoxic (N) (21% O_2) or hypoxic (H) (1% O_2) conditions. (**C**) Representative cPARP blot in MCF7 and MDA231 lysates. (**D**) Quantitation of cPARP data. (**E**) Trypan blue exclusion assays in MCF7 and MDA231 cells. (**B, D**, and **E**) Each data set is the average ± SD of at least three independent experiments. Statistically significant comparisons in cPARP quantitation and trypan blue exclusion experiments are shown in graphs (**$P<0.01$).

Abbreviations: MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; cPARP, cleaved PARP; SD, standard deviation.

on the cytotoxicity of banoxantrone (data not shown). DPI also completely abrogated the KEMTUB012-NI2-induced increase in dead MCF7 (Figure 3C) and MDA231 cells (Figure 3D) and the hypoxia-induced increase in cPARP (Figure 3E and F). These results clearly demonstrate that the hypoxia-selective cytotoxicity of KEMTUB012-NI2 is mediated via a specific process, and a reductase(s) that catalyzes one-electron reduction reactions is involved.

It has been reported that the one-electron reductase POR is involved in activating SN30000[10,25] and PR104A[26] under anoxic conditions, but that other unidentified reductases also make important contributions.[10,25,26] Western blot analysis established that POR and several other one-electron reductases are expressed in a range of breast cancer cell lines (Figure 4A). Novel diflavin oxidoreductase (NDOR1) was expressed at similar levels in all cell lines tested. In

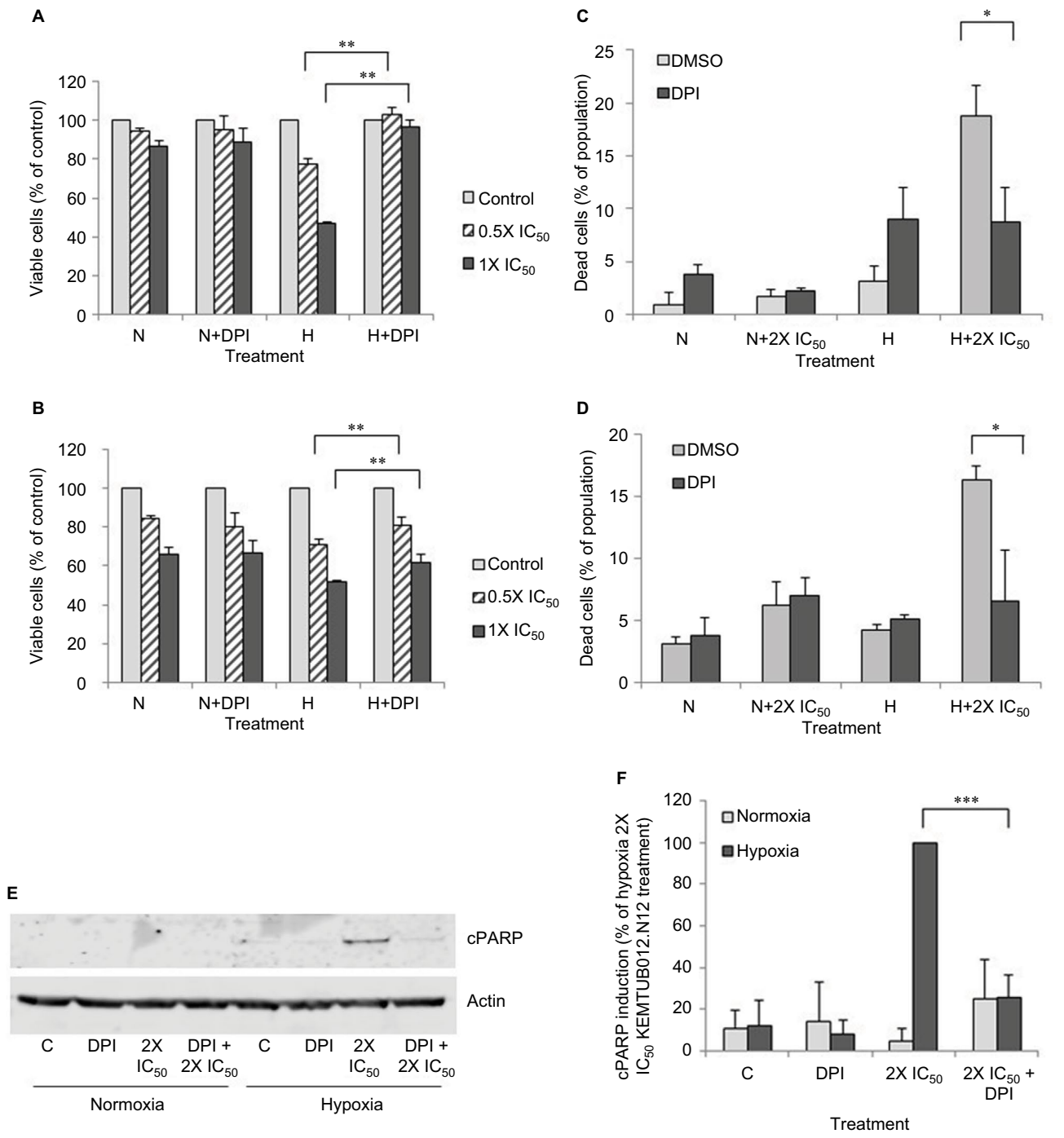

Figure 3 The hypoxia selectivity of KEMTUB012-NI2 involves reductase(s) that catalyze one-electron reductions.

Notes: Cells were treated with 0.5×, 1×, or 2× IC_{50} KEMTUB012-NI2 as indicated in normoxic (N) (21% O_2) or hypoxic (H) (1% O_2) conditions. Some cells were treated with 2 μM DPI. Cell cytotoxicity assay in MCF7 (**A**) and MDA231 (**B**) cells. Trypan blue exclusion assays in MCF7 (**C**) and MDA231 (**D**) cells. (**E**) Representative cPARP blot in MCF7 lysates. (**F**) Quantitation of MCF7 cPARP data. All data sets (except **E**) are the average ± SD of at least three independent experiments. Statistically significant comparisons of hypoxic KEMTUB012-NI2 treatment in the presence versus absence of DPI are shown in graphs (*P<0.05, **P<0.01, ***P<0.001).

Abbreviations: DMSO, dimethyl sulfoxide; DPI, diphenyliodonium chloride.

contrast, MTRR expression (upper band – Figure 4B and C) was relatively low in MCF7 and BT474 cells, whereas POR expression ranged from relatively low in MDA231 cells to high in MCF7, BT474, and SkBr3.

A POR-specific siRNA was used to investigate the role of this reductase in activation of bioreductases in the cell line models used here. POR expression was consistently decreased by ~80%–90% after 48 h (Figures 4 and 5A). In normoxic conditions, POR downregulation had no significant effect on cell cytotoxicity in MCF7 or MDA231 cells in the presence or absence of tirapazamine or KEMTUB012-NI2 (Figure 5B and C). Likewise, under hypoxic conditions POR

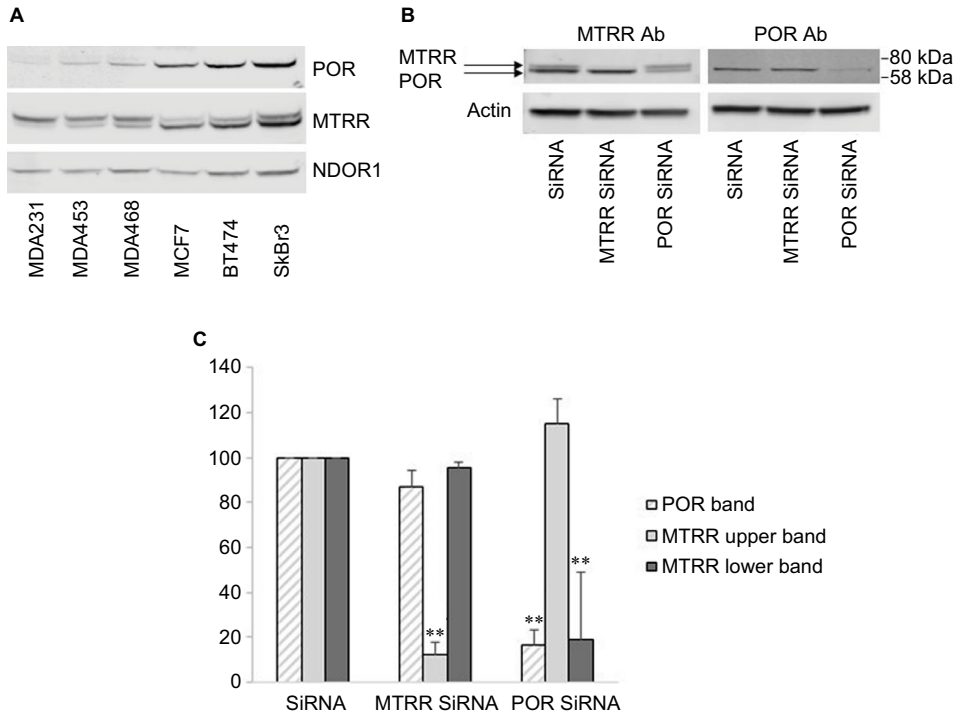

Figure 4 Breast cancer cell lines express multiple one-electron reductases.

Notes: (**A**) Expression of endogenous reductases in breast cancer cell lines. (**B** and **C**) MCF7 cells were treated with random siRNA (siRNA), 20 nM MTRR siRNA (MTRR siRNA) or 25 nM POR siRNA (POR siRNA) and grown for 48 h. Cells were harvested and lysates analyzed by Western blotting with MTRR (left panel) or POR (right panel) antibody (**B**). (**C**) Quantification of POR, MTRR upper, and MTRR lower bands. Results are average ± SD of three independent experiments. Statistically significant decreases in band intensities are shown in graphs (**$P<0.01$). The data demonstrate that the upper band in the MTRR blot represents MTRR and the lower band represents POR. The high homology between these proteins likely permits the MTRR antibody used in this study to also detect POR.

Abbreviations: ab, antibody; siRNA, small interfering RNA; MTRR, methionine synthase reductase; POR, cytochrome p450 reductase; NDOR1, diflavin oxidoreductase-1.

siRNA treatment alone did not have a significant effect on cell cytotoxicity in either cell line. POR downregulation did, however, significantly decrease the cytotoxicity of tirapazamine in hypoxic MCF7 cells, but enhanced tirapazamine cytotoxicity in MDA231 cells (Figure 5B). These results imply that POR makes an important contribution to tirapazamine activation in MCF7 cells, but not in MDA231 cells, which express low POR levels (Figure 4A). In the presence of KEMTUB012-NI2, POR siRNA treatment caused a significant increase in cell cytotoxicity in hypoxic MCF7 and MDA231 cells (Figure 5C). POR downregulation also increased the proportion of dead cells (Figure 5D and E) in KEMTUB012-NI2-treated hypoxic cells. Therefore, since POR downregulation sensitizes hypoxic cells to KEMTUB012-NI2, combining these treatments could be an effective strategy to maximize the hypoxia selectivity of KEMTUB012-NI2.

Discussion

Development of effective treatments for hypoxic tumor cells has been a priority ever since it was recognized that hypoxic tumors are associated with a poor outcome for cancer patients[2,3] and are resilient to established radiotherapy and chemotherapy treatments. Chemotherapies that specifically target hypoxic tumor cells offer an opportunity to selectively kill hypoxic cells with limited effect to normoxic cells, thereby minimizing systemic toxicity for patients. However, research activities have not yet translated into routine clinical treatments. Several distinct bioreductive agents have produced promising results in vitro, yet have disappointed in clinical trials.[7,8] This is partly a result of not preselecting patients with hypoxic tumors for bioreductive agent therapy; in two clinical studies where hypoxic tumors were identified using Positron Emission Tomography imaging, it was demonstrated that only patients with hypoxic tumors benefited from bioreductive drugs.[27,28] The limitation of the prevailing bioreductases is also a contributing factor. Well-documented issues include the limited tumor penetration of tirapazamine[10] and the lack of selectivity of banoxantrone for hypoxic cancer cells.[11] New hypoxia-selective agents and/or alternative treatment strategies are required to advance hypoxia-targeted therapies into routine treatments that are consistently effective in hypoxic tumors.

We report here a novel tubulysin analog, KEMTUB012-NI2, that selectively targets hypoxic cancer cells. Selectivity was demonstrated in multiple breast cancer cell lines (Table 1) and required the nitroimidazole targeting moiety,

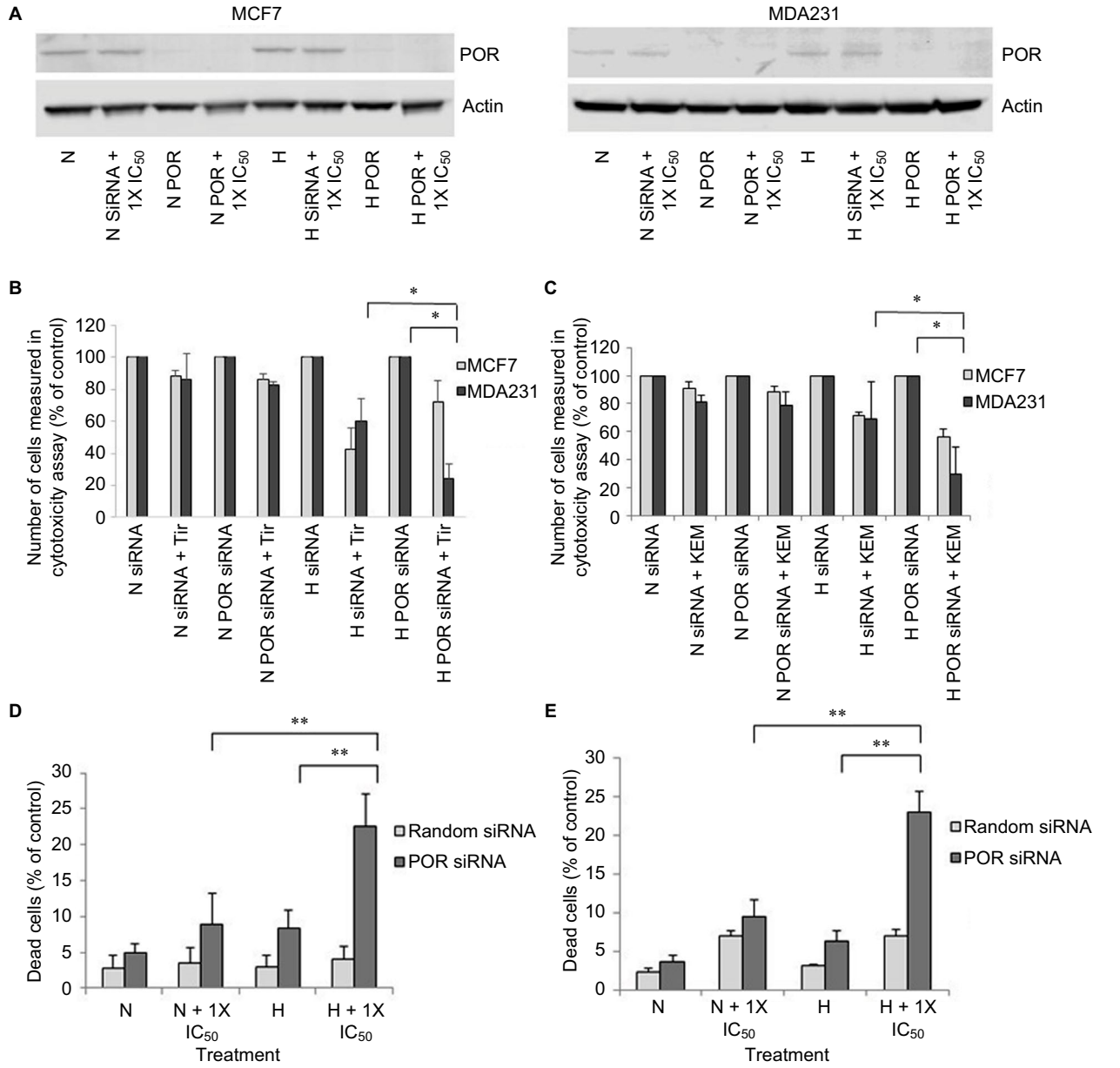

Figure 5 Combination of POR downregulation with tirapazamine or KEMTUB012-NI2 has differential effects on cell cytotoxicity in hypoxic conditions.
Notes: MCF7 and MDA231 cells were pretreated with either random siRNA (siRNA) or POR siRNA (POR), prior to treatment with 1× IC_{50} tirapazamine (Tir) or 1× IC_{50} KEMTUB012-NI2 (KEM) in normoxic (N) and hypoxic (H) conditions. (**A**) Representative Western blots of POR expression in cell lysates after treatment. Cell cytotoxicity assays with tirapazamine (**B**) or KEMTUB012-NI2 (**C**). Trypan blue exclusion assays in MCF7 (**D**) and MDA231 (**E**) cells. Data are representative (**A**) or the average ± SD (**B–E**) of at least three independent experiments. Statistically significant differences in cell cytotoxicity are observed in both cell lines when tirapazamine or KEMTUB012-NI2 treatment is combined with POR siRNA (*P<0.05), and there was a significant increase in dead cells when KEMTUB012-NI2 is combined with POR siRNA (**P<0.01).
Abbreviations: siRNA, small interfering RNA; POR, cytochrome p450 reductase.

as unconjugated tubulysin did not selectively target hypoxic cells. In hypoxic cells, KEMTUB012-NI2 induced the characteristic increase in cytotoxicity (Table 1) and cell death (Figure 2) previously described for tubulysin analogs.[12] Under moderate (1% O_2) hypoxic conditions, KEMTUB012-NI2 exhibited a similar selectivity for hypoxic cells to that of tirapazamine and was superior to banoxantrone. However, KEMTUB012-NI2 efficacy was not further increased at lower oxygen concentrations (Figure 2B), presumably because any potential benefit of improved KEMTUB012-NI2 uptake is counterbalanced by the very low cell cycling rate, which will limit the effectiveness of the tubulysin. KEMTUB012-NI2 exhibited an IC_{50} concentration in the low nanomolar range, which compares favorably with both tirapazamine and banoxantrone, which are both efficacious at micromolar concentrations (Table 1).[9] A much lower dose of KEMTUB012-NI2

will therefore be required to impact hypoxic cells, making it less likely to be limited by poor tumor penetration.

Identifying the reductase(s) involved in bioreductase activation/tumor uptake is essential to utilize these agents to their maximum potential and enable predictions about whether an agent is likely to be effective in a particular tumor. The data presented here indicate that KEMTUB012-NI2, tirapazamine, and banoxantrone are differentially activated by reductases. Banoxantrone was not affected by DPI, consistent with the literature that suggests that it utilizes two-electron reductases such as cytochrome P450 enzymes[29] or inducible nitric oxide synthase.[30] In contrast, the hypoxia selectivity of KEMTUB012-NI2 and tirapazamine were both abrogated by DPI (Figure 3), implying a role for one-electron reductases. Tirapazamine appears to be activated by multiple reductases; POR is a key activator in MCF7 cells, but other reductases also contribute, as POR downregulation only partially decreased its cytotoxicity in MCF7 cells and did not decrease cytotoxicity in MDA231 cells (Figure 5B). These data are broadly consistent with previous studies that identified POR as a major determinant of sensitivity to hypoxia-activated agents, such as SN30000[10,25] and PR104A.[26] Yet other reductases clearly also make important contributions, and their relative importance appears to be cell line dependent. It is not yet apparent which reductase(s) activates KEMTUB012-NI2, since POR downregulation actually enhanced the effect of KEMTUB012-NI2 in MCF7 and MDA231 cells (Figure 5C). These data indicate that POR is not necessary for KEMTUB012-NI2 activation, but actually may play an important role in cell survival under hypoxic conditions. To our knowledge, this is the first report of cytotoxic synergy between a hypoxia-selective agent and POR downregulation and could form the basis of a therapeutic mechanism to sensitize cells to this agent. The fact that a corresponding effect was observed for tirapazamine in MDA231 cells (Figure 5B) indicates that this effect is not restricted to KEMTUB012-NI2 and may be representative of a more general mechanism. An equivalent effect was not observed in MCF7 cells (Figure 5B), presumably due to a requirement for POR in its activation mechanism.

Combination of POR downregulation with KEMTUB012-NI2 treatment in hypoxic cells also resulted in a synergistic increase in cell death (Figure 5D and E). The fact that this effect was only observed in hypoxic conditions using POR siRNA treatment but not with random siRNA argues that it is not a result of toxicity during the transfection process. Indeed, as the effect was only observed under hypoxic conditions, it appears as if POR downregulation can sensitize cells to the hypoxia-targeted tubulysin, equivalent to a synergistic drug combination treatment. POR plays a critical role as the principal redox partner for the microsomal p450 enzymes responsible for drug and xenobiotic metabolism, cholesterol and bile synthesis, and heme breakdown.[31] Therefore, there are multiple potential mechanisms that could account for the observed increase in cytotoxicity and cell death when KEMTUB012-NI2 is combined with POR downregulation.

KEMTUB012-NI2 is an exciting prospect for further development as a hypoxia-targeting agent. It has comparable selectivity for hypoxic cancer cells as tirapazamine under moderate hypoxia, yet is >1,000 times more potent. KEMTUB012-NI2 has a distinct mode of action to tirapazamine and banoxantrone and is apparently activated by different reductases in cells. This could be advantageous as POR, a key reductase implicated in tirapazamine activation, is apparently only expressed in ~21% of tumors.[26] Indeed, as downregulation of POR appears to sensitize cells to KEMTUB012-NI2, additional experiments are required to determine whether downregulation of POR makes cells more sensitive to KEMTUB012-NI2 or whether cells that naturally express low POR levels are also intrinsically more sensitive to KEMTUB012-NI2. Further experiments with KEMTUB012-NI2 are warranted to better understand the attributes and limitations of this new agent.

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Disclosure

M Spiga is current employee of KemoTech s.r.l., Furthermore, M Sani, P Lazzari, and M Zanda are cofounders of KemoTech s.r.l. (www.kemotech.it). IN Fleming has no conflicts of interest to disclose regarding this study. The authors report no other conflicts of interest in this work.

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Supplementary materials

Methods

Chemistry

Data concerning the characterization of the synthetic tubulysins KEMTUB001, KEMTUB010, and KEMTUB012, and their 2-nitroimidazole derivatives are reported in the following sheets.

KEMTUB001: (2*S*,4*R*)-4-(2-((1*R*,3*R*)-1-acetoxy-3-((2*S*,3*S*)-*N*-benzyl-3-methyl-2-((*R*)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methylpentyl) thiazole-4-carboxamido)-2-methyl-5-phenylpentanoic acid

R_f = 0.21 (dichloromethane/methanol 95:5). ^{1}H nuclear magnetic resonance (NMR) (400 MHz, CD_3OD) δ: 8.07 (s, 1H), 7.47 (d, J = 7.3 Hz, 2H), 7.39–7.08 (m, 8H), 5.90 (t, J = 6.8 Hz, 1H), 5.07 (d, J = 17.1 Hz, 1H), 4.74 (d, J = 8.8 Hz, 1H), 4.46 (d, J = 16.6 Hz, 1H), 4.42–4.29 (m, 2H), 3.14–3.04 (m, 1H), 3.00–2.82 (m, 3H), 2.61–2.50 (m, 1H), 2.38 (s, 3H), 2.37–2.27 (m, 3H), 2.18 (s, 3H), 2.13–1.55 (m, 10H), 1.54–1.26 (m, 3H), 1.19 (d, J = 6.8 Hz, 3H), 1.06 (d, J = 6.4 Hz, 3H), 0.86 (d, J = 6.8 Hz, 3H), 0.78 (t, J = 7.4 Hz, 3H), 0.72 (d, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ: 175.9, 173.5, 171.8, 171.3, 162.7, 151.0, 139.6, 130.6, 130.1, 129.4, 129.3, 129.1, 127.5, 71.4, 69.8, 59.7, 56.5, 55.5, 51.1, 44.5, 42.0, 39.6, 38.0, 36.4, 32.0, 31.3, 25.6, 25.4, 23.8, 21.1, 21.0, 20.8, 18.9, 16.4, 11.0. Mass spectrometry (MS) electrospray ionisation (ESI) *m/z*: 804.4 $[M+H]^+$, 826.4 $[M+Na]^+$.

KEMTUB010: (2*S*,4*R*)-4-(2-((1*R*,3*R*)-1-acetoxy-3-((2*S*,3*S*)-*N*-benzyl-3-methyl-2-((*R*)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methylpentyl) thiazole-4-carboxamido)-5-(4-fluorophenyl)-2-methylpentanoic acid

R_f = 0.25 (dichloromethane/methanol 95:5). ^{1}H NMR (400 MHz, CD_3OD) δ: 8.06 (s, 1H), 7.47 (d, J = 7.1 Hz, 2H), 7.39–7.08 (m, 5H), 6.90 (t, J = 8.8 Hz, 2H), 5.88 (t, J = 6.6 Hz, 1H), 5.05 (d, J = 16.6 Hz, 1H), 4.72 (d, J = 9.0 Hz, 1H), 4.46 (d, J = 16.9 Hz, 2H), 4.42–4.24 (m, 2H), 3.23–3.05 (m, 2H), 2.95–2.83 (m, 2H), 2.62–2.51 (m, 2H), 2.47 (s, 3H), 2.38–2.28 (m, 2H), 2.16 (s, 3H), 2.10–1.98 (m, 2H), 1.95–1.28 (m, 10H), 1.22–1.16 (m, 3H), 1.05 (d, J = 6.4 Hz, 3H), 0.85 (d, J = 6.6 Hz, 3H), 0.75 (t, J = 7.3 Hz, 3H), 0.69 (d, J = 6.6 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ: 181.6, 176.0, 173.5, 171.8, 171.4, 162.7, 161.9, 151.0, 139.6, 135.6, 132.3, 132.2, 130.0, 129.3, 129.1, 125.2, 116.1, 115.9, 71.4, 69.9, 59.7, 56.5, 55.5, 51.1, 44.5, 41.2, 39.5, 39.0, 38.0, 36.5, 32.0, 31.3, 25.6, 25.4, 23.8, 21.1, 21.0, 20.8, 19.0, 16.4, 10.9; ^{19}F NMR (376.45 MHz, CD_3OD) δ: –119.0 (s, 1F). MS (ESI) m/z: 822.4 [M+H]+, 844.4 [M+Na]+.

KEMTUB012: (2*S*,4*R*)-4-(2-((1*R*,3*R*)-1-acetoxy-3-((2*S*,3*S*)-*N*-(cyclopropylmethyl)-3-methyl-2-((*R*)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methylpentyl) thiazole-4-carboxamido)-2-methyl-5-phenylpentanoic acid

R_f = 0.27 (dichloromethane/methanol 90:10). ^{1}H NMR (400 MHz, CD_3OD) δ: 8.09 (s, 1H), 7.44–6.98 (m, 5H), 5.90 (d, J = 13.2 Hz, 1H), 4.87–4.79 (m, 1H), 4.44–4.28 (m, 1H), 3.84 (dd, J = 16.5, 5.5 Hz, 1H), 3.15–3.03 (m, 1H), 3.02–2.85 (m, 4H), 2.63–2.38 (m, 3H), 2.35 (s, 3H), 2.17 (s, 3H), 2.08–1.84 (m, 4H), 1.83–1.50 (m, 6H), 1.49–1.20 (m, 4H), 1.16 (d, J = 7.0 Hz, 3H), 1.14–1.08 (m, 1H), 1.05 (d, J = 6.4 Hz, 3H), 1.00 (d, J = 6.8 Hz, 3H), 0.94 (t, J = 7.2 Hz, 3H), 0.88–0.78 (m, 3H), 0.77–0.55 (m, 2H), 0.54–0.39 (m, 1H), 0.38–0.25 (m, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ: 181.4, 173.0, 172.1, 171.8, 162.9, 151.0, 139.7, 130.7, 129.5, 127.5, 125.2, 71.6, 69.7, 56.5, 55.6, 51.1, 44.3, 42.2, 39.4, 39.8, 38.7, 38.1, 36.7, 31.9, 31.2, 25.6, 25.5, 23.6, 21.1, 21.0, 20.7, 18.8, 16.7, 13.2, 11.2, 6.6, 6.4. MS (ESI) m/z: 768.4 [M+H]+, 790.4 [M+Na]+.

The tubulysin-based conjugates KEMTUB001-NI1, KEMTUB012-NI1, KEMTUB012-NI2, and KEMTUB012-NI3 were synthesized according to the following procedures.

KEMTUB001-NI1: (1*R*,3*R*)-3-((2*S*,3*R*)-*N*-benzyl-3-methyl-2-((*R*)-1-methylpiperidine-2-carboxamido) pentanamido)-4-methyl-1-(4-(((2*R*,4*S*)-4-methyl-5-((2-(2-nitro-1H-imidazol-1-yl)ethyl)amino)-5-oxo-1-phenylpentan-2-yl)carbamoyl)thiazol-2-yl)pentyl acetate

To a solution of KEMTUB001 (0.037 mmol) in dichloromethane (3 mL), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (0.041 mmol), 1-hydroxy-7-azabenzotriazole (HOAt) (0.041 mmol), 2-(2-nitro-1*H*-imidazol-1-yl)ethanamine (0.056 mmol), and triethylamine (0.078 mmol) were added. The reaction mixture was stirred for 4 h and then washed with water (5 mL). The organic phase was extracted with dichloromethane (3×5 mL), and the combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. The crude product thus obtained was purified by flash chromatography (dichloromethane/methanol, 90:10), affording 34.3 mg of KEMTUB001-NI1 as a yellow foam (yield: 98%).

R_f: 0.31 (dichloromethane/methanol 90:10). ^{1}H NMR (400 MHz, CD_3OD) δ: 8.07 (s, 1H), 7.52–7.43 (m, 3H), 7.36–7.09 (m, 9H), 7.06 (d, J = 1.2 Hz, 1H), 5.91 (dd, J = 11.1, J = 2.3 Hz, 1H), 5.09 (d, J = 17.1 Hz, 1H), 4.69 (d, J = 9.0 Hz, 1H), 4.62–4.42 (m, 4H), 4.26–4.14 (m, 1H), 3.69–3.55 (m, 2H), 3.01–2.92 (m, 1H), 2.90–2.75 (m, 2H), 2.65 (dd, J = 11.1, J = 2.8 Hz, 1H), 2.45–2.32 (m, 2H), 2.24

(s, 3H), 2.17 (s, 3H), 2.15–2.08 (m, 1H), 2.06–1.72 (m, 5H), 1.71–1.24 (m, 9H), 1.14–0.92 (m, 9H), 0.87 (d, J = 6.6 Hz, 3H), 0.73 (t, J = 7.5 Hz, 3H), 0.65 (d, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ: 179.3, 176.4, 175.1, 171.9, 171.4, 162.9, 150.9, 139.9, 139.3, 130.6, 130.1, 129.5, 129.4, 129.2, 129.0, 128.6, 127.6, 125.4, 71.3, 70.6, 56.7, 55.4, 51.0, 50.6, 45.0, 42.6, 40.1, 39.9, 39.0, 38.0, 36.5, 32.0, 31.8, 26.2, 25.4, 24.4, 21.1, 20.9, 20.7, 19.5, 16.5, 10.9; MS (ESI) *m/z*: 942.5 (M^++H^+), 964.4 (M^++Na).

KEMTUB012-NI1: (1*R*,3*R*)-3-((2*S*,3*R*)-*N*-(cyclopropylmethyl)-3-methyl-2-((*R*)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methyl-1-(4-(((2*R*,4*S*)-4-methyl-5-((2-(2-nitro-1*H*-imidazol-1-yl)ethyl)amino)-5-oxo-1-phenylpentan-2-yl)carbamoyl)thiazol-2-yl)pentyl acetate

To a solution of KEMTUB012 (0.065 mmol) in dichloromethane (4 mL), HATU (0.071 mmol), HOAt (0.071 mmol), 2-(2-nitro-1*H*-imidazol-1-yl)ethanamine (0.097 mmol), and triethylamine (0.14 mmol) were added. The reaction mixture was stirred for 4 h and then washed with water (10 mL). The organic phase was extracted with dichloromethane (3×10 mL) and the combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. The crude product thus obtained was purified by flash chromatography (chloroform/methanol 95:5), affording 58.5 mg of KEMTUB012-NI1 as a white foam (yield: 99%).

R_f: 0.37 (dichloromethane/methanol 90:10). ^{1}H NMR (400 MHz, CD_3OD) δ: 8.11 (s, 1H), 7.49–7.43 (m, 1H), 7.30–7.12 (m, 5H), 7.07 (d, J = 1.2 Hz, 1H), 5.92 (dd, J = 11.2, J = 2.4 Hz, 1H), 4.91–4.83 (m, 1H), 4.62–4.46 (m, 2H), 4.29–4.16 (m, 1H), 3.90 (dd, J = 15.8, J = 5.3 Hz, 1H), 3.70–3.54 (m, 2H), 3.04–2.83 (m, 4H), 2.60 (dd, J = 11.0, J = 2.9 Hz, 1H), 2.50–2.25 (m, 3H), 2.19 (s, 3H), 2.17 (s, 3H), 2.14–2.04 (td, J = 11.7, J = 3.1 Hz, 1H), 2.03–1.84 (m, 3H), 1.83–1.71 (m, 2H), 1.71–1.45 (m, 5H), 1.38–1.15 (m, 3H), 1.15–0.98 (m, 10H), 0.97–0.86 (m, 3H), 0.80 (d, J = 6.6, 3H), 0.76–0.57 (m, 2H), 0.55–0.41 (dd, J = 9.7, J = 4.5 Hz, 1H), 0.40–0.26 (m, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ: 177.9, 174.4, 173.9, 170.6, 170.5, 161.6, 149.5, 138.1, 129.3, 128.1, 128.0, 127.2, 126.2, 124.1, 70.0, 69.2, 55.3, 53.8, 49.6, 49.2, 43.6, 41.2, 38.7, 38.4, 37.6, 36.8, 35.4, 30.4, 30.4, 24.9, 24.3, 23.0, 19.7, 19.6, 19.2, 18.1, 15.4, 12.1, 9.8, 5.5, 5.2; MS (ESI) *m/z*: 906.5 (M^++H^+), 928.5 (M^++Na).

KEMTUB012-NI2: (1*R*,3*R*)-3-((2*S*,3*R*)-*N*-(cyclopropylmethyl)-3-methyl-2-((*R*)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methyl-1-(4-(((2*R*,4*S*)-4-methyl-5-((3-(2-(2-nitro-1*H*-imidazol-1-yl)acetamido)propyl)amino)-5-oxo-1-phenylpentan-2-yl)carbamoyl)thiazol-2-yl)pentyl acetate

To a solution of KEMTUB012 (0.052 mmol) in N,N-dimethyl formamide (DMF) (3 mL), HATU (0.057 mmol), HOAt (0.057 mmol), *N*-(3-aminopropyl)-2-(2-nitro-1*H*-imidazol-1-yl)acetamide (0.062 mmol), and triethylamine (0.11 mmol) were added. The reaction mixture was stirred for 4 h and then washed with water (10 mL). The organic phase was extracted with dichloromethane (3×10 mL), and the combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. The crude product thus obtained was purified by flash chromatography (chloroform/methanol 95:5), affording 42.4 mg of KEMTUB012-NI2 as a white foam (yield: 83%).

R_f: 0.22 (dichloromethane/methanol 90:10). ^{1}H NMR (400 MHz, CD_3OD) δ: 8.09 (s, 1H), 7.46 (d, J = 1.2 Hz, 1H), 7.29–7.19 (m, 4H), 7.18–7.13 (m, 2H), 5.93 (dd, J = 11.2, J = 2.2 Hz, 1H), 5.17 (s, 2H), 4.91–4.83 (m, 1H), 4.34–4.22 (m, 1H), 3.90 (dd, J = 15.9, J = 5.4 Hz, 1H), 3.31–3.20 (m, 2H), 3.19–3.06 (m, 1H), 3.02–2.80 (m, 4H), 2.58 (dd, J = 11.1, J = 2.80 Hz, 1H), 2.53–2.27 (m, 3H), 2.18 (s, 3H), 2.17 (s, 3H), 2.14–1.84 (m, 4H), 1.83–1.47 (m, 9H), 1.43–1.17 (m, 3H), 1.15–1.05 (m, 7H), 1.04–0.99 (m, 3H), 0.98–0.91 (m, 3H), 0.81(d, J = 6.6, 3H), 0.77–0.59 (m, 2H), 0.55–0.44 (m, 1H), 0.40–0.27 (m, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ: 178.8, 175.8, 175.4, 172.0, 171.7, 171.5, 168.2, 163.0, 150.9, 139.5, 130.7, 129.5, 128.5, 127.6, 125.5, 71.4, 70.7, 56.7, 55.2, 53.0, 50.7, 45.0, 42.7, 40.0, 39.1, 38.3, 38.2, 37.7, 36.7, 31.8, 31.8, 30.1, 26.3, 25.7, 24.4, 21.1, 21.0, 20.6, 19.4, 16.8, 13.5, 11.2, 6.9, 6.6; MS (ESI) *m/z*: 977.3 (M^++H^+), 1,000.3 (M^++Na).

KEMTUB012-NI3: (1*R*,3*R*)-3-((2*S*,3*R*)-*N*-(cyclopropylmethyl)-3-methyl-2-((*R*)-1-methylpiperidine-2-carboxamido)pentanamido)-4-methyl-1-(4-(((2*R*,4*S*)-4-methyl-5-((6-(2-(2-nitro-1*H*-imidazol-1-yl)acetamido)hexyl)amino)-5-oxo-1-phenylpentan-2-yl)carbamoyl)thiazol-2-yl)pentyl acetate

To a solution of KEMTUB012 (0.065 mmol) in DMF (3 mL), HATU (0.071 mmol), HOAt (0.071 mmol), *N*-(6-aminohexyl)-2-(2-nitro-1*H*-imidazol-1-yl)acetamide (0.08 mmol), and triethylamine (0.14 mmol) were added. The reaction mixture was stirred for 4 h and then washed with water (10 mL). The organic phase was extracted with dichloromethane (3×10 mL), and the combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. The crude product thus obtained was purified by flash chromatography (chloroform/methanol 95:5), affording 66.0 mg of KEMTUB012-NI3 as a yellow oil (yield: quantitative).

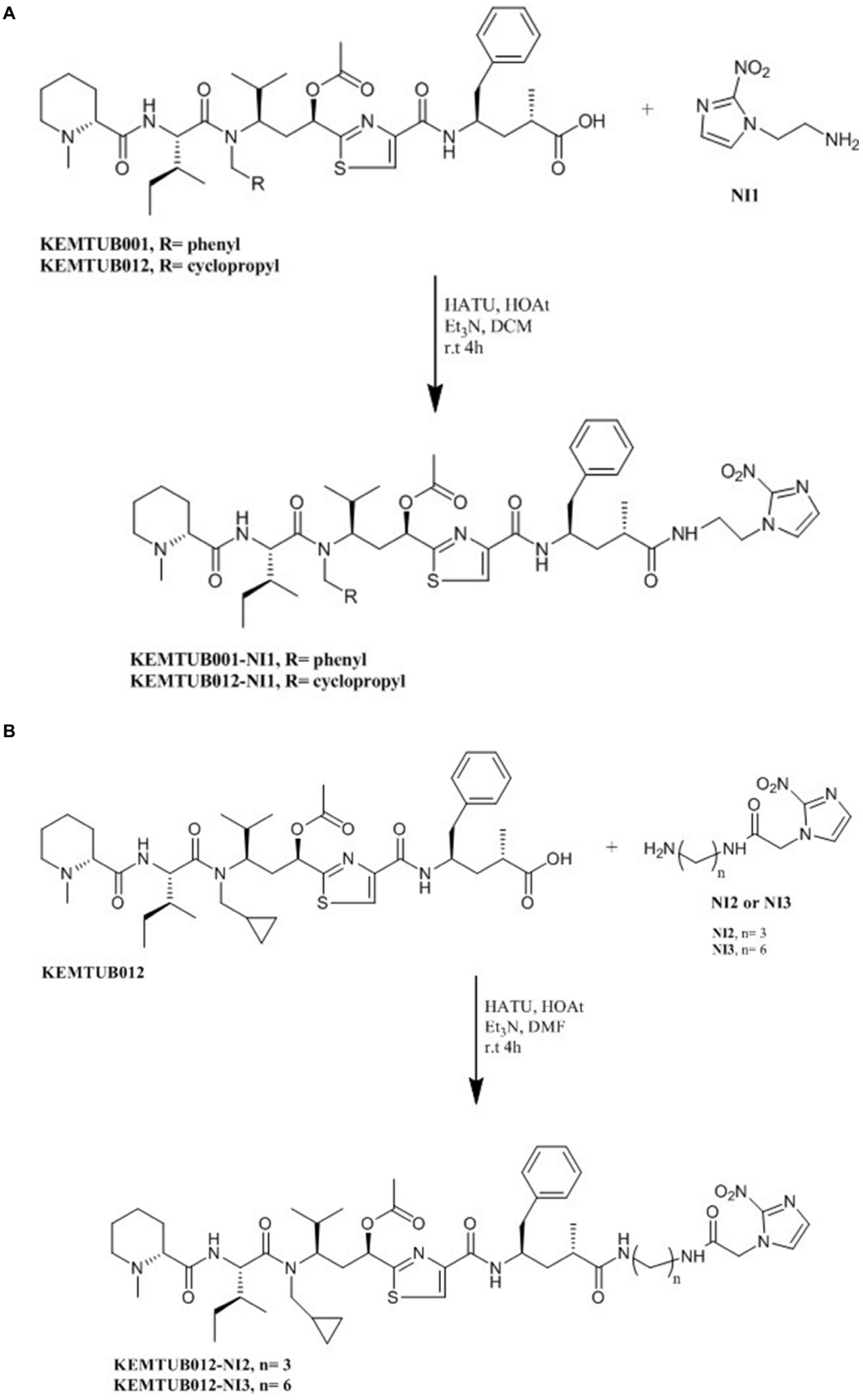

Figure S1 Scheme demonstrating synthesizes of tubulysin analogs. (**A**) Synthesis of NI1 tubulysins. (**B**) Synthesis of NI2 and NI3 tubulysins.

R_f: 0.23 (dichloromethane/methanol 90:10). ^{1}H NMR (400 MHz, CD_3OD) δ: 8.11 (s, 1H), 7.44 (d, J = 1.2 Hz, 1H), 7.28–7.19 (m, 4H), 7.18–7.12 (m, 2H), 5.93 (dd, J = 11.2, J = 2.2 Hz, 1H), 5.12 (s, 2H), 4.93–4.83 (m, 1H), 4.33–4.23 (m, 1H), 3.89 (dd, J = 15.7, J = 5.4 Hz, 1H), 3.29–3.17 (m, 3H), 3.16–3.07 (m, 2H), 3.03–2.92 (m, 2H), 2.91–2.85 (m, 2H), 2.67 (dd, J = 11.0, J = 2.69 Hz, 1H), 2.53–2.28 (m, 3H), 2.22 (s, 3H), 2.17 (s, 3H), 2.15–2.09 (m, 1H), 2.07–1.94 (m, 2H), 1.85–1.71 (m, 2H), 1.71–1.45 (m, 9H), 1.44–1.25 (m, 9H), 1.15–1.05 (m, 7H), 1.04–0.99 (m, 3H), 0.98–0.89 (m, 3H), 0.81 (d, J = 6.6, 3H), 0.77–0.60 (m, 2H), 0.54–0.42 (m, 1H), 0.42–0.27 (m, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ: 178.5, 174.9, 172.0, 171.8, 167.9, 163.0, 150.9, 139.5, 130.6, 129.5, 129.5, 128.5, 127.6, 125.5, 71.4, 70.5, 56.7, 55.2, 53.0, 50.9, 44.8, 42.5, 40.6, 40.3, 40.1, 39.1, 38.2, 36.8, 33.2, 31.8, 31.7, 30.4, 30.3, 27.6, 27.5, 26.1, 25.7, 24.3, 21.1, 21.0, 20.6, 19.4, 18.5, 16.7, 13.5, 11.2, 6.9, 6.6; MS (ESI) m/z: 1,019.5 (M^++H^+), 1,042.4 (M^++Na).