The anti-protozoan drug nifurtimox preferentially inhibits clonogenic tumor cells under hypoxic conditions

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Abstract: Tumor hypoxia is an independent prognostic indicator of tumor malignant progression and poor patient survival. Therefore, eradication of hypoxic tumor cells is of paramount importance for successful disease control. In this study, we have made a new discovery that nifurtimox, a clinically approved drug to treat Chagas disease caused by the parasitic protozoan trypanosomes, can function as a hypoxia-activated cytotoxic. We have found that nifurtimox preferentially kill clonogenic tumor cells especially under the hypoxic conditions of ≤0.1% O₂. Mechanistically, nifurtimox becomes activated after tumor cells enter into a fully hypoxic state, as shown by the stabilization of the Hypoxia-Inducible Factor 1α (HIF-1α). Nifurtimox specifically induces the formation of 53BP1 foci, a hallmark of DNA double-stranded breaks, in hypoxic tumor cells. Hypoxia-dependent activation of nifurtimox involves P450 (cytochrome) oxidoreductase. The anti-protozoan drug nifurtimox holds promise as a new hypoxia-activated cytotoxin with the potential to preferentially eliminates severely hypoxic tumor cells.

Keywords: 53BP1, clonogenic, DNA damage, hypoxia, hypoxia-activated cytotoxin, nifurtimox, nuclear foci, P450 (cytochrome) oxidoreductase

Introduction

Autonomous growth of solid tumors is accompanied by unique but chaotic tissue structures and organization characterized by abnormal angiogenesis and vascular functions among others [1-3]. These vascular deficiencies often result in insufficient oxygen (O₂) supply to the affected regions in a solid tumor, which leads to the development of hypoxia, a condition where local O₂ concentrations fall below the levels necessary for maintaining basic cell functions. Not surprisingly, hypoxia is the most common feature of tumor microenvironment in almost all types of solid tumors. Hypoxic regions are often randomly distributed in a solid tumor. Both chronically sustaining hypoxia and fluctuating hypoxia can be found in the same tumor [1, 4].

Hypoxia has the potential to exert profound impact on many critical cellular functions and intracellular pathways. Notably, hypoxia can modulate cell proliferation [5], alter cellular metabolism [6, 7], select apoptosis-resistant clones [8], promote tumor invasion and metastasis [9, 10], increase genomic instability [11, 12], and augment cancer cell stemness [13, 14]. Most importantly, clinical studies have found that hypoxia is an independent prognostic factor for advanced disease progression and poor patient survival [15-19]. In addition, tumor hypoxia poses a significant challenge to conventional cancer therapies [20].

One of the main strategies to overcome tumor hypoxia-mediated therapy resistance is to develop hypoxia-activated prodrugs or cytotoxins that preferentially kill hypoxic tumor cells, an excellent idea arising from the classical research on the bioreductive compound Mitomycin C [21]. Despite continuing efforts to search and develop new hypoxia-activated prodrugs [22, 23], there remains to be a void in the armament against hypoxic tumor cells.

In this study, we report that nifurtimox, an anti-protozoan drug used clinically to treat Chagas disease and sleeping sickness caused by trypanosomes [24-26], preferentially inhibits the
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growth of clonogenic tumor cells under hypoxic conditions, especially severely hypoxic (<0.1% O₂) tumor cells. Mechanistically, nifurtimox induces DNA damages in hypoxic tumor cells. Human P450 (cytochrome) oxidoreductase, POR, plays an important role in hypoxia-dependent activation of nifurtimox. Our findings suggest that this anti-protozoan drug has the potential to be repurposed for cancer therapy as a new drug targeting hypoxic tumor cells in solid tumors.

Materials and methods

Cell culture and hypoxia

MDA-MB-231 breast carcinoma cells, NCI-H838 non-small cell lung cancer cells, HCT116 colorectal carcinoma cells, C33A cervical carcinoma cells, LN-18 glioma cells, and FaDu head-and-neck cancer cells were from American Type Culture Collection (ATCC). KNS42 glioma cells were from RIKEN, Japan. RH-1 Ewing sarcoma cells were obtained from Dr. Nai-Kong V. Cheung of Memorial Sloan-Kettering Cancer Center (labeled as SK-N-ER). For routine culture, RH-1 cells were maintained in MEM and F12 (1:1). MDA-MB-231 and NCI-H838 cells were grown in RPMI. HCT116, LN-18, and KNS42 cells were cultured in DMEM. C33A and FaDu cells were maintained in MEM. All cell culture media were supplemented with 10% fetal bovine serum and 20 mM HEPES. The hypoxia experiments at 1%, 0.5%, or 0.1% O₂ were performed in Invivo 400 Hypoxia Workstation (Ruskinn Technology) and those at 0% O₂ were performed in Bactron Anaerobic Chamber (Sheldon Manufacturers, Inc.). Aerobic (20% O₂) experiments were carried out in a conventional CO₂ incubator with ambient air.

Drug treatment

Nifurtimox (N3415, Sigma-Aldrich) and etoposide (E1383, Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and diluted in culture media to final concentrations. Cells were incubated at different drug concentrations under different pO₂ conditions for up to 24 hrs. After the drug treatment, the monolayer cultures were washed with phosphate-buffer saline (PBS) and trypsinized to single cell suspension for the subsequent experiments. Cell viability was routinely examined using Trypan Blue.

Clonogenicity assay

Tumor cells were plated in six-well plates at a seeding density of 300 or 3,000 cells per well in complete culture media. Cells were incubated in a conventional incubator with ambient air for up to 14 days. The resultant tumor cell colonies were fixed and stained with crystal violet. The colonies (≥50 cells per colony) were counted manually under a magnifying glass. Plates with large number of colonies were examined using a digital colony counter equipped with a VRmagic camera mounted on Kaiser Copylizer eVision exe.cutive stand and ProtoCOL SR software. Plating Efficiency (PE) = #colonies formed divided by #seeded cells per well × 100%. Surviving Fraction (SF) = PE of drug-treated cells divided by PE of control cells.

RNA interference

Lentiviral vectors containing shRNA specifically against human P450 (cytochrome) oxidoreductase, POR, were purchased from Sigma-Aldrich (SHCLNG-NM_000941). We selected clones NM_000941.1-71s1c1 (shPOR24) and NM_000941.1-1867s1c1 (shPOR26) both of which have also been validated by an independent report [27]. Lentivirus was packaged according to the manufacture recommended procedure. After lentiviral transduction, stable cell lines were selected and used in subsequent experiments. Gene knockdown was validated using quantitative RT-PCR.

Western blot

The monolayer cell culture was washed in ice-cold PBS and lysed on ice in a lysis buffer containing 25 mM HEPES buffer at pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, and protease inhibitors (Complete™, Roche Diagnostics). After centrifugation, the supernatants were collected as whole cell lysates. Equal amounts of proteins were loaded per lane and separated in a SDS-polyacrylamide gel under reducing conditions. For Western blots, proteins were eletrotransferred onto nitrocellulose membranes and were probed with rabbit anti-PARP-1 monoclonal antibody (46D11, 1:1000, Cell Signaling Technology, Product Number 9532), rabbit anti-HIF-1α polyclonal antibody (1:1000, Cell Signaling Technology) or mouse monoclonal anti-β-actin (1:20,000, Sigma-Aldrich), followed by incuba-
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Immunofluorescence

Cells were seeded in 48-well plates and incubated for 24 hours before they were treated with nifurtimox or etoposide for another 24 hours. After treatment, cells were washed twice with ice-cold PBS, fixed in a solution containing 2% paraformaldehyde and 1% sucrose for 15 minutes at room temperature, and permeabilized with ice-cold methanol and acetic acid (1:1) for 20 minutes at -20°C. After being washed in PBS, the cells were incubated in a blocking buffer (4% BSA with 0.2% Triton X100 in PBS) for 30 minutes. Incubation with rabbit polyclonal anti-53BP1 antibody (1:200, Santa Cruz, sc22760) was carried out at room temperature for 1 hour or at 4°C for overnight, followed by incubation with Alexa 555-conjugated anti-rabbit IgG (1:500; Invitrogen) for 1 hour at room temperature in the dark. Nuclei were counterstained with DAPI (0.2 μg/mL, Sigma-Aldrich). Immunofluorescence were examined and pictures taken using the EVOS-FL fluorescence microscope (ThermoFisher Scientific). Nuclei with >5 foci were counted. Fraction of foci+ cells = number of foci+ cells divided by total number of cells counted.

Statistical analysis

Two group comparison were analyzed by two-tailed, unpaired Student’s t-test. Significant difference was declared if P<0.05.

Results

Nifurtimox preferentially inhibits clonogenic growth of tumor cells under hypoxic conditions

Nifurtimox, (RS)-3-methyl-N-[(1E)-5-nitro-2-fur-yl]-methylene]-thiomorpholin-4-amine 1,1-dioxide, is a nitrofuran derivative and becomes an active cytotoxic agent upon biological reduction mediated by nitroreductases [28-30]. Upon one-electron reduction, a nitro anion free radical is formed, but it can be re-oxidized back to its original form in the presence of molecular oxygen [28-30]. We therefore hypothesized that hypoxia may enhance the cytotoxic effects of nifurtimox. First, we determined whether nifurtimox could synergize with hypoxia to kill hypoxic cells using the clonogenic assay for stringent assessment of clonogenic potentials.

Figure 1. Nifurtimox specifically inhibits clonogenic growth of hypoxic tumor cells. RH-1 Ewing sarcoma cells (A & B) and MDA-MB-231 breast cancer cells (C, D) were incubated with 50 μM nifurtimox (NFMX) or DMSO under aerobic (20% O2) and anoxic (0% O2) conditions, respectively. Treated cells were seeded in triplicates at 300 and 3000 cells per well, respectively, in six-well plates for clonogenic growth. (A, C) Representative images of colonies. (B, D) Plating efficiencies (mean ± S.D., n = 3). These experiments were independently confirmed more than three times.
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Figure 2. Nifurtimox inhibits clonogenic growth of multiple tumor types under hypoxia. Various human tumor cell lines were incubated with nifurtimox (NFMX) or DMSO at normoxia (20% O₂) or anoxia (0% O₂). A. Images of colony growth for tumor cells plated at 3,000 cells/well in six-well plates to demonstrate the synergistic effects of NFMX and hypoxia. B. Relative surviving fraction (mean ± S.D., n = 3) was calculated using data from the plates seeded at clonal density (300 cells/well). The data from DMSO-treated cells at 20% O₂ were used as control. These results were validated by at least two independent experiments.
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RH-1 Ewing sarcoma cells and MDA-MB-231 breast cancer cells were first treated with 50 µM nifurtimox for 24 hours either at normoxia (20% O₂) or severe hypoxia (approximately 0% O₂). The incubation with nifurtimox did not induce significant cell death based on Trypan Blue staining, which was consistent with the lack of proteolytic cleavage of poly ADP ribose polymerase 1 (PARP1) under the same treatment conditions (Figure 5).

For the clonogenicity assay, the drug-treated cells were washed in PBS to remove nifurtimox and then re-plated in 6-well plates at 3,000 and 300 cells/well, respectively, for continued incubation under the conventional tissue culture condition. These two cell-seeding densities represent a clonal density at 300 cells/well or approximately 0.3 cells/mm² [31] and above clonal density at 3,000 cells/well, or approximately 3 cells/mm². The seeding at the high density was to determine whether nifurtimox was capable of killing clonogenic tumor cells under hypoxic conditions even when paracrine communication among cells were not a limiting factor.

As clearly shown in Figure 1, tumor cells treated by nifurtimox under the hypoxic condition almost completely lost their clonogenic potential whereas tumor cells treated by nifurtimox at the same concentration under the normoxic condition by and large maintained their clonogenic potential. For comparison, the nifurtimox concentration of 50 µM used in this experiment is similar to the concentrations at which nifurtimox induces a loss of 50% cell viability in several neural tumor cell lines after 48-72 hrs of incubation under normoxic culture conditions [32-34]. Our data clearly demonstrate that the ability to inhibit clonogenic growth is a much more stringent test than the cell viability test for anti-cancer drugs. Furthermore, our data have uncovered a new function for this anti-trypanosomiasis drug and demonstrate that nifurtimox can function as a hypoxia-activated cytotoxin to specifically eliminate clonogenic tumor cells under hypoxic conditions without significant toxicity toward non-hypoxic cells, which is important for reducing drug-related toxicity in normal tissues.

We further determined whether the hypoxia-dependent cytotoxic effects of nifurtimox could
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Data clearly demonstrate that the preferential killing of clonogenic tumor cells by nifurtimox depends primarily on hypoxia but independent of tumor types. Clonogenic inhibition by nifurtimox is inversely related to oxygen concentrations. Data on cell lines include RH-1 Ewing sarcoma cells, MDA-MB-231 breast cancer cells, HCT116 colon cancer cells, NCI-H838 lung cancer cells, C33A cervical cancer cells, LN-18 glioma cells, KNS42 glioma cells, and FaDu head-and-neck cancer cells. Except FaDu cells being extremely sensitive to severe hypoxia, each of the other five cell lines significantly lost its clonogenicity upon incubation with nifurtimox under hypoxic but not normoxic conditions. However, KNS42 and FaDu cells appear to be more sensitive to nifurtimox under normoxia than the other four tumor cell lines are (Figure 2). This is likely due to cell type-dependent differences in basal oxidoreductive potentials. Nevertheless, these data clearly demonstrate that the preferential killing of clonogenic tumor cells by nifurtimox depends primarily on hypoxia but independent of tumor types.

Clonogenic inhibition by nifurtimox is inversely related to oxygen concentrations.

$O_2$ concentrations within solid tumor microenvironment are highly variable, ranging from anoxia in necrosis to physiological tissue-level oxygen concentrations [35]. Mean $pO_2$ values in several human tumors are around 9 mmHg or 1.2% $O_2$ [35], at which the Hypoxia-Inducible Factor 1α (HIF-1α) and/or 2a (HIF-2α) proteins...
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Figure 5. Nifurtimox treatment does not activate the PARP-1 proapoptosis pathway under hypoxia. RH-1 Ewing sarcoma cells (A) and MDA-MB-231 breast cancer cells (B) were incubated for 24 hours with nifurtimox (abbreviated as N, 50 µM) or dimethyl sulfoxide (abbreviated as D) under aerobic (20% O₂) and anoxic (0% O₂) conditions, respectively. For the re-oxygenation treatment, the hypoxia-treated cells were placed in the aerobic incubator for 24 hours. Etoposide (ETOP, 10 µM) was used as positive control. PARP-1 cleavage was examined by Western blot analysis with β-actin as the internal loading control. These results were confirmed by more than three independent experiments.

become robustly stabilized [36], a canonical mechanism of cellular response to hypoxia [37]. We therefore examined the ability of nifurtimox to inhibit clonogenic growth of RH-1 and MDA-MB-231 cells at normoxia (20% O₂ or tissue culture condition) and different levels of hypoxia (1, 0.5, 0.1 and 0% O₂, respectively). As shown in Figure 3, the cytotoxic effects of nifurtimox increased only slightly over the dose range of 0-100 µM at 20% O₂. However, the cytotoxic activity of nifurtimox increased more dramatically with decreasing pO₂ with the strongest inhibition of clonogenic growth occurring at <0.1% O₂ (Figure 3). At 0% O₂, there were no surviving clonogenic tumor cells for RH-1 cells treated with ≥50 µM nifurtimox or MDA-MB-231 cells treated with ≥75 µM nifurtimox. The non-zero data points (1 × 10⁻⁴) of the survival fraction were chosen for compatibility with the log scale Y-axis. Nevertheless, these results strongly suggest that nifurtimox has the potential to preferentially kill clonogenic tumor cells residing in the severely hypoxic tumor microenvironment where these tumor cells are likely to be highly resistant to conventional therapies [20, 23]. Because normal tissue O₂ concentrations are often ≥2% O₂ [35], these data also suggest that the cytotoxic effects of nifurtimox in normal tissues are likely to be very limited.

We further found that the cytotoxic effects of nifurtimox become more prominent after 6-8 hr incubation under hypoxia (Figure 4A, 4D). Incidentally, the stabilization of the hypoxia-inducible factor 1α (HIF-1α) protein, a canonical event of hypoxia response [37], occurred after 1-2 hr of exposure to hypoxia (Figure 4B-F). These observations suggest that nifurtimox becomes activated after cells fully enter into a hypoxic state, which is consistent with the activation of hypoxia-activated prodrugs [23].

Nifurtimox induces DNA double-strand breaks but does not immediately activate proapoptosis pathway

To understand the mechanisms of nifurtimox-mediated clonogenic inhibition under hypoxic conditions, we first asked whether nifurtimox treatment could induce apoptosis. As shown in Figure 5, nifurtimox did not induce proteolytic cleavage of PARP 1, a characteristic result of activation of cell death proteases [38], under either normoxia or hypoxia. Nonetheless, PARP cleavage did occur in tumor cells treated with etoposide, a DNA-damaging anticancer agent, suggesting that the PARP cleavage pathway remains intact and functional in both cell types (lane 7, Figure 5). Furthermore, nifurtimox-treated RH-1 cells, but not MDA-MB-231 cells or DMSO-treated cells, exhibited PARP cleavage during reoxygenation (lanes 5-6, Figure 5), suggesting that the PARP cleavage pathway is regulated in a cell- and stress-dependent manner. Nevertheless, these observations collectively suggest that the nifurtimox-mediated clonogenic inhibition of hypoxic tumor cells does not directly involve acute cell death. Consistently, we found no significant cell death by Trypan Blue staining of tumor cells after 24 hrs incubation with nifurtimox under hypoxia or normoxia.

As a main mechanism of cytotoxicity, hypoxia-activated drugs or cytotoxins induce DNA damages via free radical formed under hypoxic conditions [22, 23]. We performed the nuclear 53BP1 foci assay [39, 40] to assess nifurtimox-induced DNA double-strand breaks, using etoposide as a positive control. Under severe hypoxia, nifurtimox significantly induced 53BP1 foci formation in both RH-1 cells and MDA-MB-231 cells compared to DMSO-treated cells.
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(Figure 6). In contrast, nifurtimox did not significantly induce 53BP1 foci formation under normoxia. The ability of etoposide to induce DNA double-strand breaks was not affected by pO₂. These results suggest that, consistent with other hypoxia-activated cytotoxins, nifurtimox is capable of inducing DNA double-strand breaks upon bioreductive activation under hypoxia.

*P450 (cytochrome) oxidoreductase, POR, is involved in hypoxia-dependent activation of Nifurtimox*

Nifurtimox becomes activated by bacterial-like nitroreductases in *Trypanosoma cruzi* [28-30]. However, it is still not clear which reductases are involved in its activation under hypoxic conditions in mammalian cells. A recent study using genome-wide shRNA screens has found that human P450 (cytochrome) oxidoreductase, POR, plays an important role in activation of several hypoxia-activated prodrugs [27]. Using two independent targeting shRNA sequences, shPOR24 and shPOR26, we found that suppression of POR expression partially rescued the nifurtimox-mediated inhibition of clonogenic survival of RH-1 Ewing sarcoma cells under anoxia (Figure 7A). In contrast, both shPOR24 and shPOR26 showed no deleterious effects on clonogenic survival of RH-1 cells under normoxia. The shPOR24 construct exhibited better rescue than the shPOR26 construct did, which by and large correlated with more efficient gene knockdown by shPOR24 (Figure 7A). We obtained similar results using shPOR24
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Figure 7. P450 (cytochrome) oxidoreductase, POR, is involved in hypoxia-dependent activation of nifurtimox. Stable RH-1 Ewing sarcoma cells (A) and MDA-MB-231 breast cancer cells (B) were selected to express shRNA against human POR (shPOR24 or shPOR26) or shRNA against GFP (shGFP) as control. The RNAi efficiency was assessed using quantitative RT-PCR (****P<0.0001 versus shGFP, n = 5 each group). The lentivirus-transduced tumor cells were incubated with 75 µM nifurtimox for 24 hr either at normoxia (20% O2) or anoxia (0% O2) and then plated for clonogenic survival under the ambient tissue culture condition (****P<0.0001 versus shGFP, n = 6 each group). The results shown are from one of three independent experiments.

in MDA-MB-231 breast cancer cells (Figure 7B). These results demonstrate that P450 (cytochrome) oxidoreductase is mechanistically involved in metabolic activation of nifurtimox under hypoxic conditions.

Discussion

Nifurtimox has been used clinically to treat American Trypanosomiasis or the Chagas disease caused by the protozoan parasite Trypanosoma cruzi [24, 25]. Nifurtimox is metabolized and activated by reductases to produce cytotoxic intermediates including free radicals and nitrile compounds [28-30]. A number of studies have also explored the use of nifurtimox as an anticancer agent especially in pediatric neuronal cancers. Saulnier Scholler et al. observed that nifurtimox exhibited antitumor activity in a pediatric patient with neuroblastoma [41] and was capable of inducing apoptosis in neuroblastoma cell lines [34]. Others have shown that nifurtimox can reduce N-myc expression in neuroblastoma cell lines [42]. Nifurtimox is also cytotoxic to other neural tumor cells [32]. However, the mechanisms of nifurtimox activation and cytotoxic function remains to be understood.

In the current study, we have made a novel observation that nifurtimox exhibits preferential inhibition of clonogenic growth of a wide variety of human cancer cells under hypoxic conditions, especially under near anoxic conditions. Within the effective dose range of 50-100 µM, nifurtimox does not show significant clonogenic inhibition at or above 1% O2, suggesting slight cytotoxicity against clonogenic tumor cells located in normoxic microenvironment but also insignificant toxicity against normal tissues. In contrast to the widely used cell growth assay, cell viability assay, and apoptosis assay, the clonogenic assay, a gold standard assay used in the field of radiation biology and stem cell biology, offers a more robust assessment of the clonogenic tumor cells under very stringent conditions by seeding cells at clonal density of 1-2 cells/mm² or less [31]. The ability of nifurtimox to inhibit clonogenic growth under hypoxia suggests that it has potential to effectively control the tumor-initiating or stem-like cells.

The biochemical mechanism of activation of nifurtimox involves biological reduction at its nitrofuran moiety [28-30], which shares significant similarities to that of other hypoxia-activat-
ed cytotoxins [22, 23]. However, it remains to be determined whether and which oxidoreductases are involved in activation of nifurtimox in mammalian cells. Here we have found, for the first time, that hypoxia-induced activation of nifurtimox in human tumor cells involves, at least in part, the P450 (cytochrome) oxidoreductase POR. Our results are consistent with the recent report showing that POR is involved in activation of several hypoxia-activated prodrugs [27]. However, it is highly possible that multiple oxidoreductases are potentially involved in hypoxia-dependent activation of nifurtimox and/or other hypoxia-activated cytotoxins/prodrugs.

In addition to the reduced nitrile intermediates, the free radicals generated by the one-electron reduction have the potential to form adducts to a wide range of cellular macromolecules including proteins and DNA. One of the profound effects of hypoxia-activated cytotoxins is DNA double-strand breaks, which can lead to cell death [22, 23]. Here, we have found that nifurtimox can also induce DNA double-strand breaks preferentially under hypoxic conditions, as shown by the formation of 53BP1-positive nuclear foci. Although we did not observe activation of apoptosis based on PARP cleavage, we could not rule out the possibilities that the reduced nifurtimox products could trigger other forms or pathways of cell death.

Tumor hypoxia not only exists extensively in solid tumors, but also poses a significant challenge to cancer treatment because hypoxic tumor cells are highly aggressive and resistant to all conventional therapies. Discovery and development of a drug that specifically target hypoxic tumor cells will undoubtedly make a significant impact on cancer therapy. Nifurtimox holds great hope in this regard because it has been used clinically for more than 40 years, albeit, to treat the Chagas disease and a clinical trial was also attempted in neuroblastoma patients [43]. Since nifurtimox preferentially targets clonogenic or tumorigenic cells in the hypoxic regions of solid tumors, the clinical values of nifurtimox as an anticancer drug could potentially be fully realized in a combination therapy with standard chemotherapy and/or radiation therapy.

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Disclosure of conflict of interest

None.

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