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Article

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Next-generation hypoxic cell radiosensitizers: nitroimidazole alkylsulfonamides

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ABSTRACT

Innovations in the field of radiotherapy such as stereotactic body radiotherapy, along with the advent of radio-immuno-oncology, herald new opportunities for classical oxygen-mimetic radiosensitizers. The role of hypoxic tumor cells in resistance to radiotherapy and in suppression of immune response continues to endorse tumor hypoxia as a *bona fide*, yet largely untapped, drug target. Only nimorazole is used clinically as a radiosensitizer and there is a dearth of new radiosensitizers in development. Here we present a survey of novel nitroimidazole alkylsulfonamides and document their cytotoxicity and ability to radiosensitize anoxic tumor cells *in vitro*. We use a phosphate prodrug approach to increase aqueous solubility and to improve tumor drug delivery. A 2-nitroimidazole and a 5-nitroimidazole analogue demonstrated marked tumor radiosensitization in either *ex vivo* assays of surviving clonogens or tumor regrowth delay.

INTRODUCTION

Hypoxia is a cardinal component of the tumor microenvironment and plays a key role in tumor cell resistance to therapy, disease progression and avoidance of immune surveillance.[1] Hypoxia contributes to resistance to therapy through a variety of mechanisms.[2-4] The clearest evidence for hypoxia-mediated resistance to therapy has been observed with radiation therapy in cervical carcinoma[5] and head and neck squamous cell carcinoma,[6-9] where patients with more hypoxic tumors fare poorly in terms of overall survival or progression free survival. Hypoxia exerts a pernicious role in disease progression with influences on angiogenesis[10], vasculogenesis,[11] the activation of a glycolytic shift in metabolism,[12,13] and enhancing invasion[14] and metastasis.[15-17] Hypoxia also promotes genomic instability through increased production of reactive oxygen species[18] and suppression of DNA repair processes,[19,20] and exerts complex and contradictory influences on immune cell responses to tumors.[21-23]

Efforts to modify hypoxia to improve treatment response have a long history.[4,24-28] Three main approaches have been explored. Hyperbaric oxygen[29] or carbogen breathing[30] have provided increased oxygen delivery to tumors. Oxygen-mimetic nitroimidazoles acted to "fix" radiation damage in hypoxic tumor cells[31-33] and these agents evolved into a third approach where hypoxic cell cytotoxins were designed to selectively kill hypoxic tumor cells.[4,34,35] While many individual trials failed to meet statistical endpoints, when taken together in a retrospective analysis, these interventions have been shown to provide benefit.[27]

Oxygen-mimetic radiosensitizers are most commonly nitroimidazoles where the electron-affinic nitro group is designed to react with DNA radicals produced by ionizing radiation in a manner similar to oxygen.[31] Formation of these adducts leads to DNA strand breaks and subsequent cell death. Early clinical trials with misonidazole (**1**, Figure 1)[36] demonstrated considerable anti-tumor activity,[33] but this was limited by delayed peripheral

neuropathies when combined with fractionated radiotherapy.[37,38] More polar analogues such as etanidazole (**2**) demonstrated improved therapeutic indices, but clinical application was again limited in combination with fractionated radiotherapy.[33,39] Doranidazole (**3**) was designed to be more polar than **1** and **2** in order to limit normal tissue exposure and underwent investigation in non-small cell lung cancer[40] and pancreatic cancer in conjunction with intra-operative RT where it displayed a small survival advantage.[41] The most successful example of a nitroimidazole radiosensitizer is nimorazole (**4**).[42,43] It is in clinical use in head and neck cancer in combination with standard fractionated radiotherapy (FRT)[24] and is undergoing further evaluation in head and neck cancer (ClinicalTrials.gov: NCT01950689, NCT01880359).[44,45] One important aim of one of the trials is the prospective validation of a hypoxic gene signature (ClinicalTrials.gov: NCT01950689) shown to predict benefit for nimorazole in a retrospective study.[43,46]

While FRT is one of the main treatments for cancer patients,[47,48] a new approach, stereotactic body radiotherapy (SBRT), is demonstrating comparable control, toxicity and efficacy profiles with improved cost and compliance, relative to daily FRT in many indications[49-51]. SBRT leverages recent advances in the accuracy and precision of radiation delivery to provide hypofractionated (1–5 doses), high dose (25–60 Gy total dose) radiation to small tumors while minimizing the effects to adjacent normal tissue. However, evidence is emerging that SBRT may accentuate the impact of hypoxia on radioresistance.[52-54] The advance of SBRT, along with the development of suitable biomarkers for assessing the extent of hypoxia in individual tumors,[55] provides new opportunities for radiosensitizers.[56]

There are few recent examples of novel oxygen-mimetic sensitizers[57-59], and these do not appear to be in clinical development. 2-Nitroimidazole radiosensitizers, such as **1**, **2** and **3**, also display hypoxia-selective cytotoxicity by virtue of their higher electron affinity. One-electron reduction by bioreductive enzymes produces a nitro radical anion, which may be

reoxidized in the presence of oxygen, or further reduced to nucleophilic species that induce cytotoxicity. This mechanism is distinct from radiosensitization and might be associated with the toxicity displayed by these compounds. One recent example of a nitroimidazole-based radiosensitizer is the sulfamide **5** which is a potent inhibitor of CAIX.[60] Although containing a metronidazole motif, **5** sensitizes tumor cells to radiation by inhibition of CAIX, through interaction of the sulfamide unit with the Zn in the catalytic center, and interruption of tumor cell pH homeostasis at drug concentrations considerably lower than those required for 5-nitroimidazole-based oxygen-mimetic radiosensitization.[61]

We recently identified a novel class of nitroimidazole sulfonamide radiosensitizer.[62,63] Here, we report our efforts to more fully characterize this class. We have explored compounds that span a wide range of electron affinity to explore the hypothesis that more electron-affinic compounds with a dual mechanism of action may be optimized for SBRT where cumulative neurotoxicity will be minimized. The contrasting premise is that we may also identify less electron-affinic compounds that are less toxic and are well tolerated with FRT. We evaluated hypoxia-selective cytotoxicity and radiosensitization *in vitro* for compounds **6**–**38** and identified lead compounds that provide substantial hypoxic cell radiosensitization *in vivo*, at least comparable to that seen with **2** and **4**.

CHEMISTRY

A series of 2-nitroimidazoles bearing sulfonamide-containing side chains were prepared using novel chemistry established in our lab.[62] Examples of neutral (**6**, **7**), basic (**8**, **9**) and acidic (**10**, **11**) side chains were prepared through condensation of halomethylsulfonyl chloride **43** with the appropriate amine to give **44**, **46**, **48**–**50**, and **52**, with subsequent alkylation of 2-nitroimidazole and deprotection if required (Scheme 1). We initially used the commercially available chloromethylsulfonyl chloride **43a** to prepare sulfonamides, but

subsequently found that the readily prepared bromomethylsulfonyl chloride **43b** gave superior yields in the synthesis of sulfonamides (e.g. **7**). Attempts to prepare sulfonamides of secondary amines (**54**, **55**) via this approach were not successful. Elaboration of the chloromethyl 2-nitroimidazole **56**[62] to the thioester **57** allowed access to the intermediate nitroimidazole methylsulfonyl chloride, and condensation with morpholine was required to prepare morpholide **12**. Higher ethylsulfonamide homologues were accessed from the 2-nitromidazole ethanol **60**, derived by deprotection of THP- or TBDMS-ethers **59** or **64**, respectively (Scheme 2). Elaboration of alcohol **60** to thioester **62** and conversion to the sulfonyl chloride *in situ* allowed condensation with amines to give **13**–**15**. Attempts to directly alkylate 2-nitroimidazole with the corresponding ethenyl sulfonamides in a Michael reaction were unsuccessful.

Examples of 4- and 5-nitroimidazole methylsulfonamides (**16**–**21**) were prepared by alkylation of 4-nitroimidazole with the appropriate halomethyl sulfonamides (**44**, **46**, **48**) (Scheme 3). These reactions gave mixtures of 4- and 5-nitro isomers in ratios of ca. 4:1 which could be separated by preparative RP-HPLC. Examples of ethylsulfonamides were accessed by alkylation of 4-nitroimidazole with bromide **63** to give a mixture of 4- and 5-nitroimidazole silyl ether isomers **68** and **69** which were separable by column chromatography (Scheme 4). Deprotection of **68** gave alcohol **70** which was converted, via thioester **71**, to the sulfonyl chloride *in situ*, with subsequent reaction with amines to give sulfonamides **22** and **72**, which was subsequently deprotected to give **24**. A similar sequence with the 4-nitroimidazole silyl ether **69** gave the sulfonamides **23** and **25**.

A series of 2-methyl-5-nitroimidazole methyl sulfonamides were prepared from metronidazole (**76**) (Scheme 5). Thioesterification of **76** gave the thioester **77** which was converted to the sulfonyl chloride *in situ* and this was reacted with a series of amines to provide sulfonamides **26**–**38**.

Oxygen-mimetic radiosensitizers need to be present at relatively high (often mM) concentrations at the time of irradiation to be effective sensitizers and so high aqueous solubility is an important prerequisite for a successful radiosensitizer. Solubility of the compounds in culture medium containing 5% fetal calf serum (FCS) was determined as a practical guide for cell culture studies (Table 1). A direct comparison of the effect of exchanging sulfonamide for carboxamide groups (i.e., **2** vs **7**) shows ca. 10-fold reduction in solubility. Other neutral nitroimidazole sulfonamides (e.g., **6**, **16**, and **17**) are considerably less soluble than either **1** or **2**, but solubility could be increased by homologation of the linker chain (e.g., **6** vs **13** and **7** vs **14**). The use of basic amine side chains also provided substantial increases in solubility over corresponding neutral compounds (e.g., **8** vs **6**, **30** vs **27**). Although the aqueous solubility was sufficient for *in vitro* experiments, concern that the relatively low aqueous solubility of the neutral alkylsulfonamides compared to **2** would limit drug delivery prompted us to consider a phosphate prodrug strategy. Phosphate prodrugs **39**–**42** of alcohols **7**, **14**, **19** and **24** were prepared via the corresponding *tert*-butyl esters **78**–**81**, respectively (Scheme 6). Although we used the traditional tetrazole-mediated phosphorylation in the preparation of **78**, we found imidazole/imidazole.HCl mediated methodology[64] to be a more convenient and efficient procedure, producing improved yields of **79**–**81**. The phosphate prodrugs **39**–**42** displayed increased aqueous solubility (220 to >600 mM) in saline containing 2 equivalents of $NaHCO_3$ (Table S1).

The lipophilicities of the compounds were calculated as logD values at pH 7.4 (Advanced Chemistry Development, Inc. Toronto, Canada) (Table 1). The sulfonamides series spanned a range of 0.11 to -4.75 compared to -0.41 for **1** and -1.37 for **2**. Generally, the neutral analogues fell within the range bounded by **1** and **2** with the sulfonamide group providing slightly more polarity than the corresponding carboxamide (e.g., compare **7** (-1.53) with **2** (-1.37)). Compounds bearing acidic side chains were the most polar compounds (-

4.38, -4.75) with strongly basic amine side chains providing significant influence (e.g., **9**, **31**–**34**), spanning the range -1.55 to -2.21. Weaker bases such as morpholine were close to neutral in their influence, providing increased aqueous solubility, without lowering the logD below the value for **2**.

We determined the one-electron reduction potentials, $E(1)$, for representative compounds of the series since electron affinity has been defined as the key parameter in the *in vitro* structure-activity relationships (SAR) for radiosensitization[65] and toxicity for a series of nitroimidazole radiosensitizers.[66] By using each of the 2-, 4- and 5-nitroimidazole regioisomers, in combination with methyl or ethyl linker side chains to modulate the effect of the strongly electron-withdrawing sulfonamide moiety, the analogues effectively span the range of electron affinity for efficient reaction with DNA radicals (as defined by their $E(1)$ values; -538 to -342 mV) (Table 1). 2-Nitroimidazoles with a C1 linker (e.g., **6**, **7**) were more electron-affinic (-352 and -342 mV, respectively) than **2** (-388 mV), reflecting the increased electronic demand from the sulfonamide group. This effect diminished with increased side chain length (e.g., **13**, **14** at -391 mV). As expected, the 5-nitroimidazole **17** displayed lower electron affinity (-421 mV) compared to the corresponding 2-nitroimidazole analogue **6** and a further reduction in affinity (-458 mV) was observed with increasing side chain length, (e.g., **22**). The electron-donating influence of the 2-methyl substituent further reduced electron affinity (e.g., -500 mV for **26**) although this could be countered somewhat by the presence of a positive charge on the side chain (e.g., -475 mV for **34**). The 4-nitroimidazole analogues (**16** and **23**) were less electron-affinic than their corresponding 5-nitroimidazole analogues (**17** and **22**).

RESULTS AND DISCUSSION

We have prepared a novel series of nitroimidazole sulfonamides that span a wide range of aqueous solubility, lipophilicity and electron affinity and have examined their *in vitro* hypoxic selectivity and radiosensitization properties to identify lead analogues suitable for in-depth preclinical investigation.

***In vitro* cytotoxicity.** We determined the cytotoxicity (as IC_{50}) of each compound using a sulforhodamine B (SRB) proliferation assay in HCT116 human colorectal carcinoma cells following a 5 day regrowth after 4 h drug exposure under oxic or anoxic conditions. The hypoxic cytotoxicity ratio (HCR) was determined as $HCR = IC_{50(oxic)}/IC_{50(anoxic)}$.

The 2-nitroimidazoles **6**–**15** displayed oxic cytotoxicity in the low mM range (IC_{50} values 0.38–4.8 mM) and anoxic potency of these compounds spanned a range from ca. 10 μM to ca. 4 mM (Table 1 and Figure 2). The relatively high anoxic potency displayed by **6**–**9** reflects the increased electron affinity due to the strong electron-withdrawing effect from the side chain with the *E*(1) value for **7** being 46 mV higher than for **2**. 2-Nitroimidazoles **6**–**9** with neutral and basic side chains displayed considerable hypoxic selectivities (HCR 40–96) while examples with acidic side chains (**10**, **11**) displayed lower hypoxic selectivity, due to decreased anoxic potency as a result of reduced electronic withdrawal due to the presence of the anionic side chain (Table 1 and Figure 2). Extension of the side chain by one methylene unit (**13**–**15**) also reduced hypoxic selectivity (HCR 5–10) through decreased anoxic potency, reflecting the reduced influence of the sulfonamide group on electron affinity [*E*(1) for **14** = -391 mV].

The 4- and 5-nitroimidazoles (**16**–**38**) showed similar oxic potency to the 2-nitroimidazoles **6**–**15**, but all of these displayed little to no hypoxic selectivity. As expected, the electron affinity was considerably lower for the 5-nitroimidazole analogues with *E*(1) for **17** being 69 mV lower than for the corresponding 2-nitroimidazole isomer **6**. This resulted in a substantial drop in anoxic potency and hypoxic selectivity (HCR 1.4–3.7) for compounds

19 and **21** compared to **7** and **8**, respectively. Curiously, **17** displayed unexpected oxic cytotoxicity, leading to a loss in hypoxic selectivity. Homologation of the side chain led to a reduction of 29 mV in *E*(1) for **22** compared to **17** and this was accompanied by a loss of anoxic potency and low HCR values. Similarly, addition of an electron-donating methyl substituent at the 2- position further reduced the electron affinity of analogues **26**–**38** [*E*(1) for **22**: -450 mV; for **34**: -475 mV], resulting in anoxic cytotoxicity in the low mM range and low HCR values. The 4-nitroimidazoles **16**, **18** and **20** had slightly less electron affinity to the 2-methyl-5-nitroimidazole analogues [*E*(1) for **16**: -503 mV] and homologation (**23**, **25**) resulted in a further drop in electron affinity [*E*(1) for **23**: -538 mV]. It is clearly evident that analogues with *E*(1) values below ca. -420 mV do not display hypoxic selectivity (Figure 2). This difference in hypoxic selectivity reveals two broad categories of compound. In the first, the more electron-affinic 2-nitroimidazole analogues (**6**–**15**) are likely to deliver both radiosensitization and bioreductive enzyme-mediated cytotoxicity in hypoxic cells as seen for **1** and **2**. In contrast, 4- and 5-nitroimidazole analogues with lower electron affinity, reflected by *E*(1) values below ca. -420 mV, show little hypoxic selectivity and are expected to be pure radiosensitizers, similarly to **4**.

Assigning *E*(1) values to all compounds based on the measured *E*(1) for each chemical subclass allowed examination of SAR by multiple linear regression. Anoxic IC_{50} was found to be related to the physiochemical properties (*E*(1), pKa and logP), while no relationship was found for the oxic IC_{50}:

$$log(IC_{50}) = a + b\,E1 + c\,pKa + d\,logP \quad (1)$$

where a = -3.08 ± 0.79 (p = 0.00056), b = -0.0083 ± 0.0017, (p = 2.9e-05), c = -0.109 ± 0.052673 (p = 0.045) and d = -0.348 ± 0.165 (p = 0.044280). R^2 = 0.4859.

A stronger relationship was found for the HCR:

$$\log(HCR) = a + b\,E1 + c\,pKa \quad (2)$$

where a = 3.32 ± 0.51 (p = 5.12e-07), b = 0.00782 ± 0.00111 (p = 1.32e-07) and c = 0.0822 ± 0.0366 (p = 0.0328), R^2 = 0.6499.

***In vitro* radiosensitization: Survival ratios.** We evaluated radiosensitization by the compounds by measuring clonogenic survival in HCT116 cells under anoxic conditions (Table 1). HCT116 cells were treated with compounds at a single non-toxic drug concentration and a single radiation dose (15 Gy) in a custom-designed metal anoxic chamber apparatus.[62,63,67] The drug concentration equivalent to the anoxic IC_{50} value was used in this assay and was non-toxic due to the shorter drug exposure time (1 h) and the exposure at room temperature (21 °C) rather than 37 °C for the IC_{50} assay (Figure S1). Cells were exposed to drug for 1 h, irradiated at 15 Gy in 96-well plates and plated for clonogenic survival. Survival ratios at 15 Gy (SR_{15}) were calculated as: SR_{15} = (cell survival with radiation)/(cell survival with drug + radiation).

SR_{15} is a measure of the efficacy of the radiosensitizer at a single high dose of radiation and provides a relatively simple screening assessment of hypoxic cell radiosensitization and allows triage of compounds at this stage.[63] The appropriate radiation dose was determined empirically for HCT116 cells as 15 Gy, which alone induces ca. 1 log of cell kill (Figure S1), and we used the anoxic IC_{50} value to compare the compounds at an equitoxic drug concentration, rather than compare compounds at an equimolar concentration. No significant drug-alone toxicity was observed across the compound set (p = 0.39, one-way ANOVA) at these drug concentrations (see Figure S1). This approach allowed us to quickly evaluate radiosensitization for compounds with widely varying solubility and hypoxic cytotoxicities. It became clear that in trying to make an equitoxic comparison the SR_{15} values were strongly influenced by the drug concentration in the assay. For example, the weakly electron-affinic **4** appears to be a good radiosensitizer compared to both **1** and **2**, but was used at ca. 10- or 20-

fold higher concentration than **2** and **1**, respectively. This cytotoxicity/radiosensitization conundrum underpins any consideration of radiosensitizers with mixed mechanisms of action and can only be resolved by consideration of the achievable concentrations at the target site in efficacy studies *in vivo*. It is clear that a number of the more electron-affinic compounds (e.g., **6**-**9**, **12**, **17**) have high molar potencies as radiosensitizers, and the lack of drug-alone toxicity (Figure S1) suggests this was due to radiosensitization alone. We had previously reported a steep concentration dependence of radiosensitization for the strongly electron-affinic **6**.[63] This observation encouraged us to advance **6** and similar electron-affinic compounds, alongside representative compounds from each nitroimidazole subclass, to a full radiation dose-response assay in comparison to **1**, **2** and **4**. For each subclass of radiosensitizer, the SR_{15} was related to the drug concentration and HCR:

$$\log(SR_{15}) = a + b + c\,Conc + d\,HCR \quad (3)$$

where a = 0.3123 ± 0.1497 (p = 0.046), b = 1.0855 ± 0.177 ((for 5-nitroimidazoles **17**, **19**, **21**, **22** and **24**), p = 1.14×10^{-6}), or b = 0.350 ± 0.154 ((for 2-Me 5-nitroimidazoles **26**–**38**), p = 0.031), c = 0.519 ± 0.103 (p = 2.11×10^{-5}), d = 0.583, (p = 0.000352), R^2 = 0.720. The relationship to HCR is stronger than to nominal *E*(1) and HCR provides a rapid continuous measure of electron affinity in drug screening.

***In vitro* radiosensitization: Sensitizer enhancement ratios.** We determined radiosensitization for selected compounds at concentrations equivalent to the anoxic IC_{50} as above, but using a lead wedge above the anoxic chamber in order to generate a range of radiation doses (6–29 Gy) in the 96-well plates.[63,67] HCT116 cells were exposed to drug for 1 h as above, irradiated, and then dissociated and plated for clonogenic survival. Sensitizer enhancement ratios (SER) were calculated as the ratio of radiation dose for 1% survival without or with the drug (See Figure S2). Each radiosensitization experiment included **1** as a control and the SER data were normalized (SER_{norm}) against the activity of **1** (Table 2). All

compounds demonstrated radiosensitization with **4**, **19**, **24** and **30** showing clearly increased sensitization compared to **1**.

SER correlated well with the $\log SR_{15}$ ($R^2 = 0.683$, $p = 6 \times 10^{-4}$, Figure 4b) while the logSR from the SER experiments was also highly correlated with the $\log SR_{15}$ ($R^2 = 0.764$, $p = 6 \times 10^{-5}$, Figure 4a). This correlation could be improved using multiple regression to normalize for the effects of cell kill by radiation alone (SF_{rad}), since SR is radiation dose dependent, whereas the SER is usually considered constant for a defined drug concentration. This allowed prediction of the SER at the IC_{50} drug concentration, from data collected in the screening process. The correlation was also improved by the addition of SER_{MISO} to the multiple regression allowing for experimental variability with a final R^2 of 0.802.

$$SER = a\log(SR_{15}) + b\log(SF_{Rad}) + c\,SER_{MISO} \quad (4)$$

where a = -0.335 ± 0.060, ($p = 2.46 \times 10^{-5}$), b = 0.6588 ± 0.157 (p = 0.000545) and c = 0.994 ± 0.40263 (p = 0.0238); SF (surviving fraction). The theoretical relationship between SR and SER is:

$$-\log(SR) = \alpha SERD + \beta SER^2D^2 - \alpha D - \beta D^2$$

However, as the SR data were determined at a constant dose of 15 Gy, the relationship is approximately linear. SER was primarily determined by the radiosensitizer concentration (though the SR) and a single term involving SF_{rad}. This provides evidence that the appropriately determined SR, at a non-toxic concentration, can be used as a measure of radiosensitization for screening compounds.

Tumor radiosensitization. A maximum tolerated dose (MTD) was determined in non-tumor bearing mice. In those cases where an MTD could not be determined because of solubility limitations a maximum achievable dose (MAD) was defined. Drug plasma and tumor pharmacokinetic profiles were determined following single i.v. doses at the MTD or MAD to determine achievable drug concentrations and the optimal time for irradiation (T_{max})

(Table 3). Oxygen-mimetic radiosensitizers act at the time of radiation damage and so maximizing the drug concentration in hypoxic tumor cells at the time of irradiation is critical. Compounds were compared with **2** and **4** for *in vivo* radiosensitization (12.5 Gy) of hypoxic cells in HCT116 human tumor xenografts using tumor excision 18 h later and clonogenic survival as the endpoint. Radiosensitization was determined as the ratio of surviving fraction (SF) after radiation without and with radiosensitizer present (the *in vivo* SR). The advantage of this *in vivo*/*ex vivo* assay is that it integrates radiosensitizer tumor micro-pharmacokinetics and extravascular transport to the radio-resistant hypoxic fraction, and explicitly assesses impact on clonogenic cell survival.

We had previously explored the activity of **6** and **7** in combination with radiation against HCT116 colorectal tumor xenografts in comparison with **2**.[63] We had found that **2** was well tolerated with a MAD of 2.20 mmol/kg leading to plasma and tumor drug concentrations of 3.64 mM and 0.80 mM, respectively (Table 3). As expected, **2** was active demonstrating an *in vivo* SR of 4.0. Plasma and tumor concentrations of **6** and **7** were 6–10 fold lower than for **2** as a result of poorer solubility and greater toxicity, with **7** showing an *in vivo* SR of 2.4 (Table 3 and Figure 5). We had explored the potential of lipophilic amines that showed increased aqueous solubility, with **15** achieving a higher MAD and plasma and tumor concentrations than **6** and **7**, but this did not translate into significant radiosensitization (*in vivo* SR 2, P = 0.096).[63]

In the present study, we confirmed the activity of **2** as an effective radiosensitizer (*in vivo* SR 8) (Table 3, Figure 5). We also explored the activity of **4** in the HCT116 xenograft model since **4** is the clinical benchmark, although it is used orally in patients. In our hands we found **4** to be relatively toxic when given i.v. and could only achieve a MTD of 1.24 mmol/kg. When given at 75% of MTD **4** did demonstrate significant radiosensitization with an *in vivo* SR of 4.

We sought to increase aqueous solubility and improve drug delivery through the use of phosphate prodrugs. We prepared the phosphate prodrug **39** of alcohol **7** and observed a substantial increase in aqueous solubility (e.g., solubility of **39** in culture medium is 185 mM compared to 18 mM for **7**). Phosphate **39** was not toxic when administered at 2.20 mmol/kg (equimolar with **2**) and was rapidly converted to alcohol **7** *in vivo* (Figure S3), leading to a ca. 2- and 4-fold increase in plasma and tumor drug concentrations, respectively, of the alcohol **7** compared to direct administration of **7** (Table 3). This increase resulted in significant radiosensitization with an *in vivo* SR of 10. Expansion of the phosphate prodrug approach led to the preparation of phosphates **40**–**42** based on the corresponding alcohols **14**, **19** and **24**, respectively. Although **14** displayed modest sensitization *in vitro* (SER_{norm} 0.92), both **19** and **24** were good sensitizers (SER_{norm} 1.28 and 1.23, respectively) (Table 2). Moreover, the three alcohols along with **7** represented structural subclasses explored in this study. Phosphates **40**–**42** all demonstrated excellent aqueous solubility (Table S1) and were able to be administered without any observable toxicity at 2.20 mmol/kg (equimolar with **2**). Phosphates **40** and **41** were also rapidly converted to the corresponding alcohols **14** and **19** achieving plasma and tumor concentrations similar to those of **7** when administered as **39** (Table 3 and Figure S3). The radiosensitization demonstrated by **40** and **42** was modest, whereas **41** provided the largest effect with an *in vivo* SR of 13.

The antitumor activity of phosphates **39** and **41** in combination with a single dose of radiation (12.5 Gy) was also compared with **2** and **4** using tumor growth inhibition as an endpoint. All compounds were well tolerated at their MAD (2.20 mmol/kg for **2**, **39** and **41**) or 75% of MTD (0.93 mmol/kg for **4**) with minimal body weight loss (Figure 6a). Compounds **39** and **41** showed similar radiosensitization activity to **2** and **4** at equitoxic doses, appearing to delay the time taken for HCT116 tumors to triple in size (RTV^3, relative tumour volume 3×) compared to radiation alone, although these differences did not reach

statistical significance for any of the treatments (Log-Rank with Holm-Sidak multiple comparison analysis) (Figure 6b).

CONCLUSIONS

We have identified two novel radiosensitizers **7** and **19** with significant *in vivo* activity against hypoxic tumor cells when delivered as the phosphate prodrugs **39** and **41**, respectively. The phosphate prodrug strategy improved aqueous solubility to allow equimolar comparison with etanidazole (**2**) and improved drug delivery, resulting in increased peak tumor drug concentrations, compared to administration of the corresponding alcohols. Although these peak tumor drug concentrations were still lower than for **2**, both **39** and **41** demonstrated substantial radiosensitization of hypoxic tumor cells *in vivo* and were equally active as either **2** or **4** at inhibiting tumor growth in combination with radiation. The 2-nitroimidazole **7** is similar to **2** in so far as it is highly electron-affinic and displays hypoxia-selective cytotoxicity in addition to radiosensitization *in vitro*. Etanidazole (**2**) was an effective radiosensitizer preclinically,[68] but failed to provide benefit in cancer patients.[39, 69] Although designed to be more polar and more readily cleared than misonidazole **1**, the toxicity was still limiting in a FRT setting[70-72] and could be associated with bioreductive mechanisms. Compound **7** is similar in polarity to **2** (logD 1.53 and 1.37, respectively), but is more electron-affinic and displays increased hypoxia-selective cytotoxicity compared to **2**, identifying **7** (as its prodrug **39**) as a compound more suited to use in a SBRT setting. In contrast, compound **19** is more akin to nimorazole (**4**) in its properties. It is slightly more electron-affinic than **4** with an $E(1)$ likely to be around -420 mV and displays little hypoxic cytotoxicity (HCR 4) suggesting potential for prodrug **41** in the context of FRT. We plan to further explore the potential of both **39** and **41** as representatives of each end of the mechanistic spectrum of these nitroimidazole oxygen-mimetic radiosensitizers.

EXPERIMENTAL SECTION

Chemistry. General experimental details are described in the Supporting Information. All final products were analyzed by RP-HPLC (Altima C18 5 μm column, 150 mm × 3.2 mm; Alltech Associated, Inc., Deerfield, IL) using an Agilent HP1100 equipped with a diode-array detector. Mobile phases were gradients of 80% MeCN/20% H_2O (v/v) in 45 mM ammonium formate at pH 3.5 and 0.5 mL/min. Purity was determined by monitoring at 330 (± 50 nm) and was >95% with the exception of compound **10** (90.4%) (Table S1). The solubility of the final products was determined by HPLC analysis after incubation of saturated solutions of drug in αMEM containing 5% FCS and 1% DMSO at 37 °C to mimic cell culture conditions (Table S1). Stability of the same solutions after 24 h incubation at 37 °C was measured by HPLC analysis and was found to be >95% (Table S1).

Example of Synthetic Methods. See Supporting Information for full experimental details.

Method A. Preparation of halomethyl sulfonamides. A solution of alkyl sulfonyl chloride (1.0 eq.) in dry DCM (2 mL/mmol) was added drop wise to a stirred solution of amine (1.2 eq.) and iPr_2NEt (1.3) in dry DCM (10 mL/mmol) at 0 °C and the solution stirred at 20 °C for 16 h. The solution was diluted with DCM (50 mL/mmol) and washed with water (3 × 20 mL/mmol), washed with brine (20 mL/mmol), dried and the solvent evaporated. The residue was purified by chromatography, eluting with appropriate mixtures of EtOAc/pet. ether, to give the sulfonamide.

Method B. Alkylation of nitroimidazoles. A mixture of nitroimidazole (1.5 eq.), haloalkyl sulfonamide (1.0 eq.), Cs_2CO_3 (1.1 eq.) and NaI (1.0 eq.) in dry DMF (10 mL/mmol) was stirred at 80 °C for 3 h. The solvent was evaporated and the residue suspended in EtOAc (100 mL/mmol) and washed with water (3 × 30 mL/mmol), washed with

brine (20 mL/mmol), dried and the solvent evaporated. The residue was purified by chromatography, eluting with appropriate mixtures of EtOAc/pet. ether, to give the nitroimidazole sulfonamide.

Method C. Deprotection of nitroimidazole sulfonamides. A solution of silyl ether (1.0 eq.) in HOAc/water/THF (3:1:1, 10 mL/mmol) was stirred at 20 °C for 48 h. The solvent was evaporated and the residue suspended in toluene (3 × 20 mL) and the solvent evaporated. The residue was triturated with pet. ether or purified by chromatography, eluting with appropriate mixtures of EtOAc/pet. ether, to give the alcohol.

Method D. Preparation of nitroimidazole thioesters. Potassium thioacetate (1.5 eq.) was added to a stirred solution of nitroimidazole alkyl chloride or mesylate (1.0 eq.) in anhydrous DMF (5 mL/mmol), and the mixture was stirred at 20 °C for 16 h, then heated at 80 °C for 4 h. The mixture was cooled and partitioned between EtOAc (30 mL/mmol) and water (30 mL/mmol). The organic layer was washed with water (3 × 10 mL/mmol) then washed with brine (10 mL/mmol), dried and the solvent was evaporated. The residue could be purified by column chromatography, eluting with a gradient (50–100%) of EtOAc/pet. ether, to give thioacetate or used directly as the crude product.

Method E. Conversion of thioesters to sulfonamides. A solution of thioacetate (1 eq.) in CH_3CN (2 mL/mmol) was added drop wise to a mixture of NCS (4.0 eq.) in aqueous HCl (2 M, 12 mL/mmol) and CH_3CN (3 mL/mmol) at 10 °C. The mixture was stirred at 10–15 °C for 30 min, and allowed to warm to 20 °C over 10 min. The mixture was partitioned between EtOAc (50 mL/mmol) and aq $NaHCO_3$ (25 mL/mmol). The organic phase was washed with aqueous NaCl (3 × 25 mL/mmol), washed with brine (20 mL/mmol), dried and the solvent was evaporated to give the crude sulfonyl chloride which could be used without further purification. Amine (1.1 eq.) and iPr_2NEt (1.3 eq.) were successively added to a solution of the crude sulfonyl chloride in anhydrous DCM (10 mL/mmol) at 0 °C. The

mixture was allowed to warm to 20 °C and stirred for 16 h. The mixture was diluted with DCM (30 mL/mmol), washed with water (3 × 15 mL/mmol), washed with brine (15 mL/mmol), dried and the solvent was evaporated. The residue was purified by column chromatography, eluting with appropriate mixtures of EtOAc/pet. ether, to give the sulfonamide.

Method F. Preparation of phosphate prodrugs. $iPr_2NP(OtBu)_2$ (1.5 eq.) was added drop wise to a stirred solution of alcohol (1.0 eq.), imidazole (1.0 eq.) and imidazole hydrochloride (1.5 eq.) in DMF (5 mL/mmol) at 20 °C and the solution was stirred at 20 °C for 1 h. The solution was cooled to 0 °C and aqueous H_2O_2 solution (30%, 2.5 eq.) was added drop wise. The mixture was allowed to warm to 20 °C over 1 h. The mixture was diluted with EtOAc (100 mL/mmol) and washed with cold water (3 × 50 mL/mmol), washed with cold brine (50 mL/mmol), dried and the solvent evaporated. The residue was purified by chromatography, eluting with a gradient (0–6%) of MeOH/DCM, to give the phosphate ester. A solution of HCl in MeOH (1.25 M, 10 eq.) was added drop wise to a stirred solution of the phosphate ester (1.0 eq.) in DCM (20 mL/mmol) and the solution was stirred at 20 °C for 3 days. The solvent was evaporated and the residue triturated with DCM (3 × 10 mL/mmol) and dried to produce a white soft solid. The residue was dissolved in water (5 mL/mmol) and freeze dried to give the phosphate.

One-electron reduction potentials. Pulse radiolysis studies were carried out using the University of Auckland's Dynaray 4 (4 MeV) linear accelerator (200 ns pulse length with a custom-built optical radical detection system) as previously described.[73] The one-electron reduction potentials of the compounds, $E(A/A^{\bullet-})$, vs. NHE, were determined at pH 7.0 (2.5 mM phosphate buffer) by establishing redox equilibria within 20 μs between three mixtures of the one-electron reduced compounds and the reference compounds benzylviologen ($E(BV^{2+}/BV^{+})$ = -380 ± 10 mV), methylviologen ($E(MV^{2+}/MV^{\bullet+})$ = -447 ± 7 mV) or triquat

($E(TQ^{2+}/TQ^{\bullet+})$ = -548 ± 7 mV) and calculating ΔE values from the equilibrium constants, K_e, using the Nernst equation.[74] Data were obtained at three concentration ratios at room temperature (22 ± 2 °C).

***In vitro* cytotoxicity testing.** HCT116 cells were authenticated by short tandem repeat profiling at DNA Diagnostics Ltd, Auckland, NZ. Cells were grown in αMEM medium with 5% fetal calf serum (FCS), without antibiotics, and were confirmed mycoplasma-free using a PCR-ELISA method (Roche Diagnostics). Log-phase cells were seeded into 96-well plates at 700 cells/well, allowed to attach for 2 h, then test compounds were added by dilution from DMSO stocks to give a top concentration in <1% DMSO before serial 3-fold dilution in the plates. After 4 h, cultures were washed 3 times with fresh medium and grown for a further 5 days before staining with sulforhodamine B to determine IC_{50} values as previously.[75] For hypoxic exposure to compounds, cells were pelleted by centrifugation, transferred to a Pd-catalyst anaerobic chamber (Bactron-II, Shel Lab), resuspended in anoxic medium and exposed to drugs as above, but using medium and plates that had been equilibrated in the chamber for at least 3 days. After drug washout, cells were grown and stained as for the oxic IC_{50} assays. The hypoxic cytotoxicity ratio HCR was determined as $HCR = IC_{50(oxic)}/IC_{50(anoxic)}$.

Radiosensitization studies. Cells were seeded onto 96-well plates at 10^5 cells per well and allowed to attach for 2 hours. Replicates were treated with compound at the respective anoxic IC_{50} concentration 30 min prior to irradiation. For anoxic irradiation, plates were transferred to a custom-built, air-tight, stainless steel, portable box (13 × 16.5 × 3 cm) within the anaerobic chamber, then sealed and transported to the radiation machine. The plates were irradiated (Eldorado 78 ^{60}Co teletherapy unit, ~2 Gy/min) with 15 Gy under anoxia at room temperature for SR experiments. A metal wedge placed on the top of the metal chamber was used to achieve a graduated radiation dose across the plate varying from 6

to 29 Gy, calibrated by Fricke dosimetry with ammonium thiocyanate as previously described[63,67] for SER experiments. The control plate (compound alone, no radiation) was left inside the anaerobic chamber at room temperature during the irradiation period. After treatment, the cells were trypsinized and suspended in αMEM + 5% FCS + 1% penicillin-streptomycin, and 10-fold serial dilutions were plated for clonogenic survival. After 10 days plates were stained with methylene blue (2 g/L in EtOH:H_2O, 1:1 v/v) and colonies with more than 50 cells were counted. The surviving fraction (SF) was determined as: SF = $PE_{(irradiated)}$ / $PE_{(control)}$ where the plating efficiency (PE) = (No. of colonies) / (No. of cells plated). SF was plotted against radiation dose. Survival ratios (SR) were calculated: SR = (cell survival with radiation) / (cell survival with drug + radiation). Sensitizer enhancement ratios (SER) were calculated: SER = (radiation dose for 1% survival without compound) / (radiation dose for 1% survival with compound). Misonidazole (**1**) at its hypoxic IC_{50} (0.5 mM) was used as an intra-experiment control.

***In vivo* toxicity.** A maximum tolerated dose (MTD) was determined for each compound in non-tumor bearing specific pathogen-free NIH-III female mice (~20 g). Freshly prepared solutions of compounds were given i.v. to mice at 10 mL/kg body weight, using 1.33-fold dose increments. The MTD was defined as the highest dose causing no drug-related deaths, body weight loss of more than 15%, or severe morbidity in a group of three to six animals, with an observation time of up to 14 days. A maximum achievable dose (MAD) was defined as the maximum dose able to be dissolved in the injection volume. Drugs were administered as solutions in 0.9% saline containing 1% DMSO or for **39**–**42** as solutions in 0.9% saline containing 2 molar equivalents of $NaHCO_3$.

Pharmacokinetics. Female NIH-III mice were inoculated subcutaneously with 10^6 HCT116 cells in 100 µl media. Once tumor size reached ca. 300 mm^3 mice were injected with a single i.v. dose at the MAD or 75% of the MTD for each compound. At multiple time

points after dosing, blood and tumor tissue were sampled by cardiac puncture and tumor excision under terminal CO_2 anaesthesia. Blood was immediately centrifuged (3,000 × g, 3 minutes), and plasma and tumor stored at −80 °C for HPLC. Tumor samples were prepared and analysed by HPLC using standard protocols established in our laboratory.[63,76,77] Noncompartmental PK parameters were determined by Phoenix WinNonLin v 6.2 (Pharsight Corp.).

Tumor radiosensitization: *ex vivo* clonogenic assay. Compounds were evaluated for *in vivo* radiosensitization of hypoxic cells in HCT116 human tumor xenografts using clonogenic survival as an endpoint using previously report methods.[78] HCT116 tumors were grown subcutaneously on the flanks of NIH-III mice by injecting 10^6 cells. Once tumors reached a volume of approximately 300 mm^3, mice were randomly assigned to 4 treatment groups and treated with: A) vehicle control; (B) 12.5 Gy RAD only; (C) sensitizer only; (D) sensitizer given before 12.5 Gy RAD. Drugs were administered as a single i.v. dose at the MAD or 75% of the MTD. Mice were injected with drug and irradiated (Eldorado 78 ^{60}Co teletherapy unit, ~2 Gy/min, whole body irradiation at 12.5 Gy) 10 minutes later. Tumors were excised 18 h after treatment, and tumor cells were dissociated enzymatically and plated to determine the number of surviving (clonogenic) cells per gram of tumor tissue.[78] SF and SR were calculated as described above. Statistical significance of drug effects was tested using one-way ANOVA with Holm-Sidak test for multiple comparisons vs radiation alone (Table 3).

Tumor radiosensitization: tumor regrowth delay. Compounds were evaluated for their ability to inhibit tumor growth in combination with a single dose of radiation (12.5 Gy) in HCT116 tumor xenografts on the backs of NIH-III mice. Mice were randomly assigned to 4 treatment groups and treated with: A) vehicle control; (B) 12.5 Gy RAD only; (C) sensitizer only; (D) sensitizer given 10 min before 12.5 Gy RAD. Drugs were administered as a single

i.v. dose at the MAD or 75% of the MTD. Treatment commenced when the average tumor size reached (300 to 400 mm^3) and tumor size was measured until study endpoint. Mice were placed in a custom-built lead-shielded jig that allowed irradiation of the whole tumor, but shielded the mouse from radiation. Tumor growth inhibition was presented as the time taken for tumors to increase in relative tumor volume to 3× tumor size at the time of treatment (RTV^3). The significance of differences in activity between the groups was assessed by Log-Rank with Holm-Sidak multiple comparison analysis. All animal experiments were conducted under the auspices of the University of Auckland Animal Ethics Committee (Approval 1781).

ASSOCIATED CONTENT

Supporting information. . The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.

Experimental details and characterization data for compounds **6**–**42** and intermediates **43**–**81**. Tables of *in vitro* data for compounds **6**–**42** and associated figures. ^1H, ^{13}C NMR and HPLC spectra for compounds **6**–**42.** Molecular formula strings (CSV).

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Notes

The authors declare no competing interests.

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ABBREVIATIONS USED

DCM, dichloromethane; *E*(1), one-electron reduction potential; FCS, foetal calf serum; FRT, fractionated radiotherapy; HCR, hypoxic cytotoxicity ratio, αMEM, minimum essential media; NCS, *N*-chlorosuccinimide; SAR, structure-activity relationship; SBRT, stereotactic body radiotherapy; SF, surviving fraction; SR, survival ratio; SER, sensitizer enhancement ratio.

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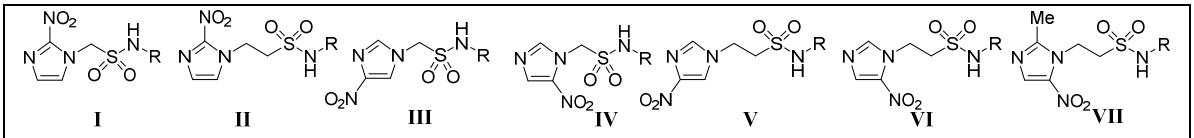
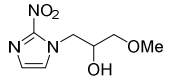
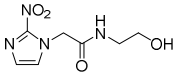
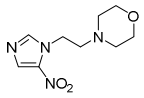
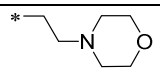
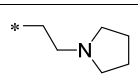
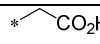
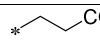
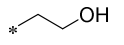
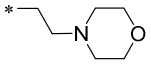
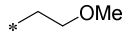
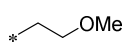
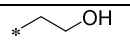
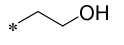
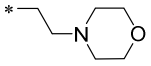
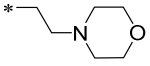
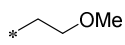
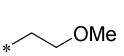
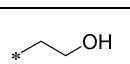
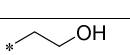
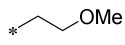
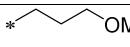
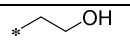
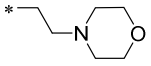
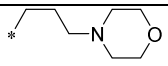
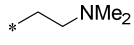
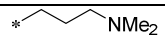
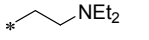
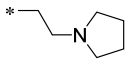
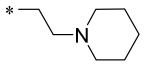
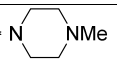
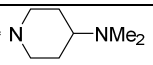
Table 1. Physicochemical and *in vitro* cytotoxicity and radiosensitization data for compounds 1–38.



I II III IV V VI VII

No	Structure		Purity % [a]	Solubility mM [b]	$LogD_{7.4}$ [c]	E(1) mV	$IC_{50anoxic}$ HCT116 ± SE (mM)	HCR [d] HCT116 ± SE	SR_{15} [e] ± SE
1	 misonidazole			>100	-0.41	-389 [f]	0.53 ± 0.09	>62.44	7.1 ± 0.7
2	 etanidazole			>200	-1.37	-388 [f]	1.0 ± 0.3	19 ±11	7 ± 1
4	 nimorazole			>100	-0.22	-457 [f]	9.8 ± 2.8	0.8 ± 0.1	37 ± 9
6 [g]	I		99.7	6.45	-1.19	-352 ± 10	0.08 ± 0.04	46 ± 2	6.3 ± 0.3
7 [g]	I		100.0	18.14	-1.53	-342 ± 8	0.11 ± 0.06	40 ± 33	2.6 ± 0.9
8	I		99.9	450	-1.18		0.08 ±0.01	50 ± 11	10.5
9	I		98.7	16.1	-2.21		0.011 ± 0.005	96 ± 34	1.7 ± 0.5
10	I		90.4	59.3	-4.75		0.9 ± 0.6	10 ± 14	4 ± 1
11	I		99.3	72.1	-4.38		4.4 ± 1.3	1 ± 1	2.0 ± 0.5
12	I		94.3	1.08	-0.17		0.03 ± 0.01	11 ± 4	3.8 ± 0.9
13	II		99.8	59.4	-1.03	-391 ±8	0.4 ± 0.2	5 ± 2	6.4 ± 0.1

14	II		100	75.3	-1.37	-391 ±7	0.8 ± 0.1	8 ± 3	12 ± 4
15	II		99.7	>163	-1.02		0.40 ± 0.08	10 ± 2	6 ± 1
16[g]	III		99.9	23.5	-0.83	-503 ± 7	0.5 ± 0.3	1.1 ± 0.5	2.4 ± 0.5
17[g]	IV		100	>26.8	-0.64	-421 ± 8	0.07 ± 0.02	1.8 ± 0.7	1.5 ± 0.3
18	III		100	ND	-1.16		>6	<0.5	
19	IV		97.3	23.7	-0.98		2.3 ± 0.8	4 ± 3	181 ± 80
20	III		100	ND	-0.81		3.3 ± 1.4	1.3 ± 0.4	7 ± 3
21	IV		98.5	ND	-0.63		2.1 ± 0.4	1.4 ± 0.3	60 ± 17
22	VI		99.5	24.4	-0.80	-450 ± 7	4.4 ± 0.5	2.5	122 ± 50
23	V		98.6	16	-1.05	-538 ± 7	>5.3	ND	9 ± 1
24	VI		98.9	69.3	-1.14		5.3 ± 0.2	2.0 ± 0.1	282 ± 100
25	VII		97.7	>100	-1.38		10.9 ± 2.3	1.0 ± 0.5	6.3 ± 0.3
26[g]	VII		99.6	29.6	-0.84	-500 ± 8	2.9 ± 0.4	1.62 ± 0.05	5.3 ± 0.2
27	VII		96.5	17	-0.28		3.7 ± 0.3	1.2 ± 0.4	5 ± 1
28	VII		98.1	>49.3	-1.17		0.4 ± 0.4	1.3 ± 0.8	3.4 ± 0.3
29	VII		99.4	44.1	-0.83		0.8 ± 0.2	1.4 ± 0.2	2.71 ± 0.03
30	VII		99.6	>55.2	-0.85		2.8 ± 1.0	0.8 ± 0.3	8 ± 2
31	VII		96.3	46.8	-1.82		0.8 ± 0.4	6 ± 6	11 ± 4

32	VII		99.7	>53.8	-1.71		1.2 ± 0.3	3 ± 1	8 ± 4
33	VII		96.1	45.7	-1.55		1.3 ± 0.3	2.0 ± 0.4	9 ± 3
34[g]	VII		99.2	61.7	-1.87	-475 ± 8	0.7 ± 0.3	1.8 ± 0.7	20.3 ± 0.1
35[g]	VII		98.2	>51.0	-0.39		1.3 ± 0.7	2 ± 2	15 ± 5
36	VII		100	2.77	0.02		0.038 ± 0.004	1.5 ± 0.2	1.33 ± 0.03
37	VII		98.3	14.5	0.11		1.6 ± 0.3	0.6 ± 0.2	1.8 ± 0.3
38	VII		100	46.5	-0.80		0.7 ± 0.3	2.9 ± 0.6	12 ± 5

Notes. [a] Determined by HPLC analysis of DMSO stock solutions. [b] Determined by HPLC analysis of saturated solutions in αMEM containing 5% FCS and 1%DMSO. [c] Calculated using ACD software (Advanced Chemistry Development, Inc. Toronto, Canada). [d] HCR = IC_{50}(oxic)/IC_{50}(anoxic). [e] SR_{15} = (cell survival with 15 Gy radiation)/(cell survival with drug + 15 Gy radiation). [f] From Reference 74. [g] From Reference 62.

Table 2. *In vitro* radiosensitization data for selected compounds.

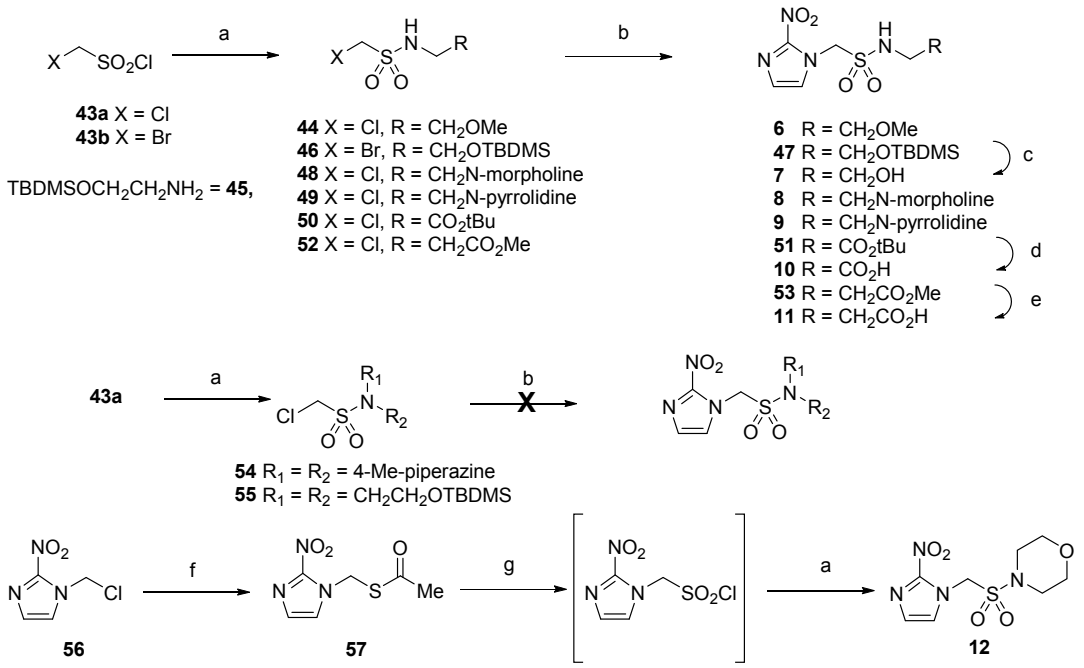
No	Drug SER [a] ± SE	[Drug] mM [b]	MISO SER ± SE	SER_{norm} [c]	Drug SR [d] ± SE	MISO SR ± SE	SR_{norm} [e]
1	1.40 ±0.02[f]	0.5[f]	1.40 ± 0.02[f]	1.00[f]	6.8 ± 0.7	6.8 ± 0.7	1.00
2	1.339 ± 0.002	1.0	1.41 ± 0.02	0.95	4.6	6.2 ± 0.6	0.75
4	1.80 ± 0.01	9.8	1.41 ±0.02	1.27	44.9 ± 0.8	6.2 ± 0.6	7.28
6	1.20[f]	0.08[f]	1.28 ±0.01[f]	0.94[f]	2.9	3.73 ± 0.08	0.78
7	1.11 ± 0.02[f]	0.11[f]	1.28 ±0.01[f]	0.87[f]	1.8 ± 0.3	3.73 ±0.08	0.47
8	1.28 ± 0.04	0.08	1.44 ± 0.05	0.89	3.9 ± 0.7	8 ± 2	0.51
12	1.11 ± 0.01	0.03	1.28±0.01	0.87	2.0 ± 0.2	3.5 ± 0.2	0.58
14	1.27 ±0.01	0.81	1.38 ± 0.01	0.92	4.2 ± 0.3	7.7 ± 0.7	0.54
15	1.357 ±0.005	0.40	1.47 ± 0.04	0.92	5.0 ± 0.2	9 ± 1	0.57
16	1.105 ± 0.002[f]	0.52[f]	1.27 ± 0.02[f]	0.87[f]	1.3 ± 0.1	3.1 ± 0.2	0.44
17	1.10 ± 0.01[f]	0.11[f]	1.29 ±0.01[f]	0.85[f]	1.9 ± 0.2	4.3 ± 0.5	0.44
19	1.81 ± 0.04	2.32	1.41 ±0.02	1.28	114 ± 49	8 ± 2	14.8
21	1.43	2.10	1.41 ±0.02	1.01	8.2		ND
22	1.56 ± 0.02	4.39	1.45 ± 0.01	1.08	26 ± 4	12 ± 1	2.23
24	1.81 ± 0.04	5.0	1.47 ± 0.02	1.23	163 ± 16	13.4 ± 0.4	12.1
26	1.34 ± 0.01[f]	2.86[f]	1.47 ± 0.02[f]	0.91[f]	4.8 ± 0.3	8.3	0.58
28	1.176 ±0.001	0.68	1.43 ± 0.05	0.82	1.616 ±0.003	4.5 ± 0.9	0.36
30	1.68 ±0.04	2.76	1.41 ± 0.01	1.20	28 ± 2	7.1 ± 0.2	3.96
31	1.57 ± .01	0.82	1.48 ± 0.02	1.06	14.9 ± 0.3	10 ± 1	1.51
34	1.54 ± 0.02[f]	0.70[f]	1.48 ± 0.02[f]	1.04[f]	13.6 ± 0.8	9.0 ± 0.1	1.51
35	1.71 ± 0.02[f]	1.34[f]	1.51 ± 0.01[f]	1.13[f]	24 ± 4	10 ± 2	2.36
38	1.67 ± 0.03	0.68	1.45 ± 0.03	1.15	25 ± 1	10.9 ± 0.6	2.30

Notes. [a] SER = (radiation dose for 1% survival – compound)/(radiation dose for 1% survival + compound). [b] [Drug] is the drug concentration used in SER experiments. [c] SER_{norm} = SER_{Drug}/SER_{Miso}. [d] SR = (cell survival with radiation)/(cell survival with drug + radiation) interpolated from the radiation dose response curves at 15Gy. [e] $SR_{norm} = SR_{Drug}/SR_{Miso}$. [f] From reference 62.

Table 3. *In vivo* toxicity, pharmacokinetic and radiosensitization data.

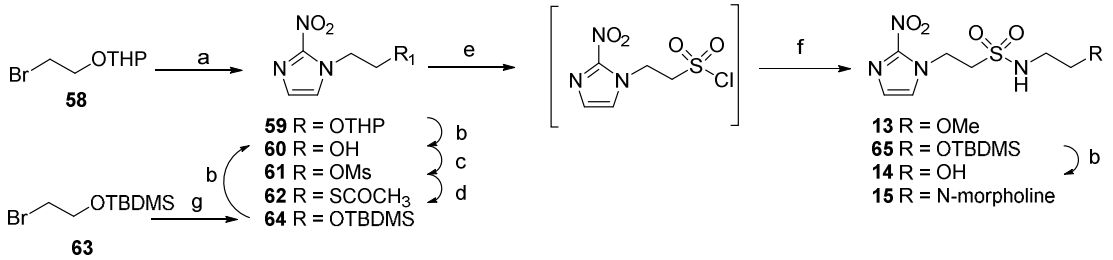
No.	MAD or MTD[a] mmol/kg	C_{max} plasma mM ± SEM	T_{max} plasma min	C_{max} tumor mM ± SEM	T_{max} tumor min	*in vivo* SR[b]	P[c] RAD vs RAD + drug
2	2.20	3.64 ±0.07	5	0.80 ±0.08	5	8 ± 3	<0.001
4	1.24[a]	1.21 ± 0.04	5	0.091 ± 0.02	10	4.0 ± 0.6	<0.001
7[d]	0.60	0.57 ±0.04	5	0.094 ± 0.002	10	2.4 ± 0.1	0.001
15[d]	1.12	1.04 ±0.08	5	0.41 ±0.01	10	2 ± 1	0.096
39	2.20	0.9 ±0.1 [e]	10	0.320 ± 0.009 [d]	10	10 ± 3	<0.001
40	2.20	1.14 ± 0.04 [e]	10	0.39 ± 0.15 [d]	10	2.9 ± 0.9	0.030
41	2.20	1.10 ± 0.09 [e]	10	0.14 ± 0.01 [d]	30	13 ± 5	<0.001
42	2.20	ND	ND	ND	ND	2.6 ± 0.4	0.040

Notes. [a] Maximum tolerated dose. [b] *In vivo* SR = (Surviving $Fraction_{RAD}$) / (Surviving $Fraction_{RAD+drug}$). [c] One-way ANOVA with Holm-Sidak test for multiple comparisons vs radiation alone. [d] Data from Reference 63. [e] Determined as the corresponding alcohols.



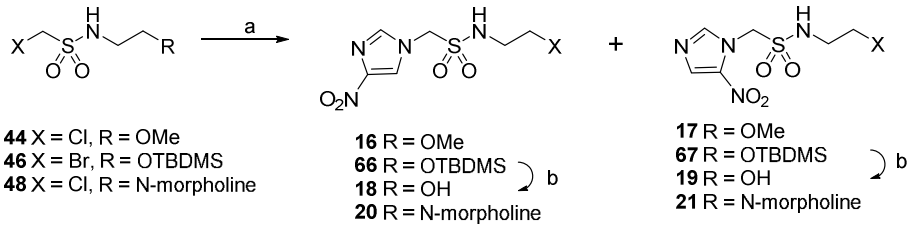
Scheme 1. Synthesis of 2-nitroimidazole methylsulfonamides[a]

[a]Reagents and conditions: (a) Amine, Et_3N or iPr_2NEt, DCM, 20 °C; (b) 2-Nitroimidazole, Cs_2CO_3, NaI, DMF, 80 °C; (c) $HOAc/H_2O$/THF, 20 °C; (d) CF_3CO_2H, DCM, 20 °C; (e) LiOH, $MeOH/H_2O$, 20 °C; (f) KSAc, DMF, 20 °C; (g) NCS, 2 M aq. HCl, MeCN, 10–20 °C.



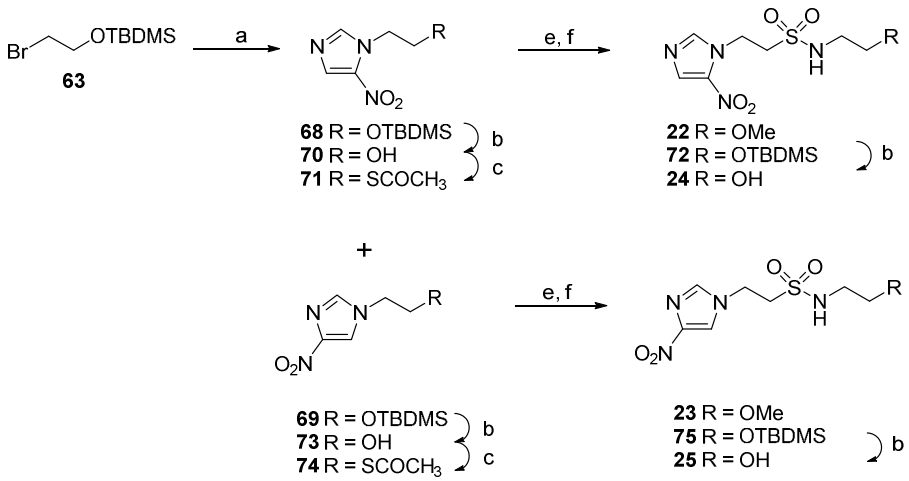
Scheme 2. Synthesis of 2-nitroimidazole ethylsulfonamides[a]

[a]Reagents and conditions: (a) 2-Nitroimidazole, Cs_2CO_3, DMF, 20 °C; (b) HOAc/H_2O/THF, 20 °C; (c) MsCl, iPr_2NEt, DMAP, DCM, 0–20 °C; (d) KSAc, DMF, 20 °C; (e) NCS, 2 M aq. HCl, MeCN, 10–20 °C; (f) Amine, Et_3N, DCM, 20 °C; (g) 2-Nitroimidazole, Cs_2CO_3, NaI, DMF, 80 °C.



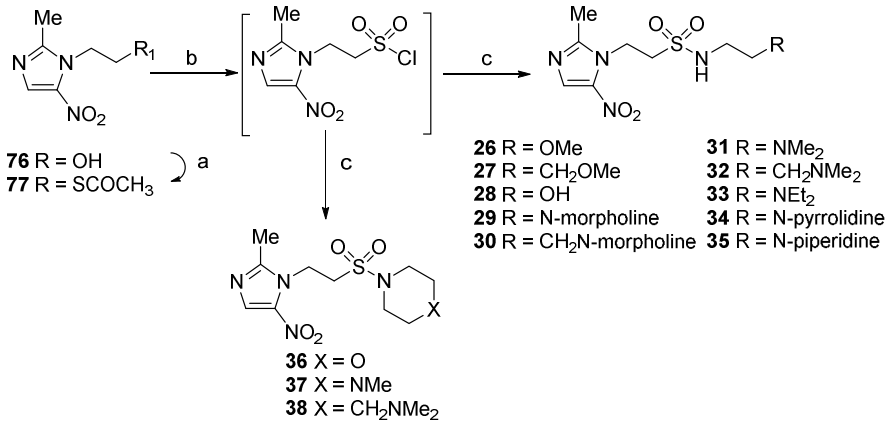
Scheme 3. Synthesis of 4- and 5-nitroimidazole methylsulfonamides[a]

[a]Reagents and conditions: (a) 4-Nitroimidazole, Cs_2CO_3, NaI, DMF, 80 °C; (b) HOAc/H_2O/THF, 20 °C.



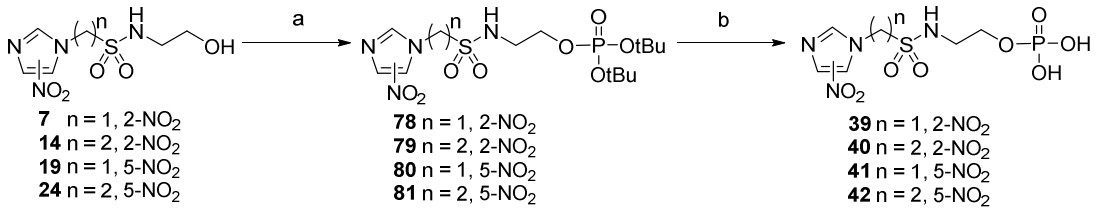
Scheme 4. Synthesis of 4- and 5-nitroimidazole ethylsulfonamides[a]

[a]Reagents and conditions: (a) 4-Nitroimidazole, Cs_2CO_3, NaI, DMF, 80 °C; (b) HOAc/H_2O/THF, 20 °C; (c) MsCl, iPr_2NEt, DMAP, DCM, 0–20 °C; (d) KSAc, DMF, 20 °C; (e) NCS, 2 M aq. HCl, MeCN, 10–20 °C; (f) Amine, Et_3N, DCM, 20 °C.



Scheme 5. Synthesis of 2-methyl-5-nitroimidazole ethylsulfonamides[a]

[a]Reagents and conditions: (a) MsCl, Et_3N, DMAP, DCM, 0–20 °C; (b) KSAc, DMF, 20 °C; (c) NCS, 2 M aq. HCl, MeCN, 10–20 °C; (d) Amine, Et_3N, DCM, 20 °C.



Scheme 6. Synthesis of phosphate prodrugs of nitroimidazole alkylsulfonamides[a]

[a]Reagents and conditions: (a) $iPr_2NP(O)(OtBu)_2$, tetrazole, MeCN/THF; then H_2O_2, 0–20 °C; or $iPr_2NP(O)(OtBu)_2$, imidazole, imidazole.HCl, DMF; then H_2O_2, 0–20 °C.

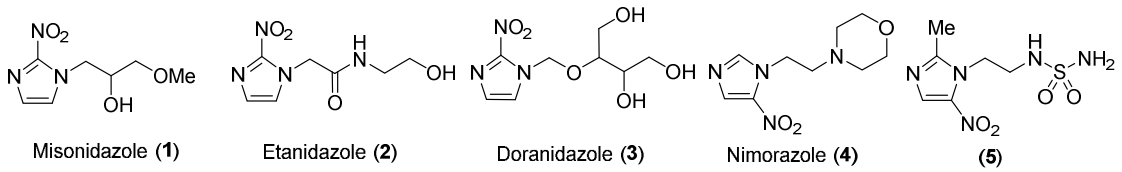


Figure 1. Nitroimidazole radiosensitizers

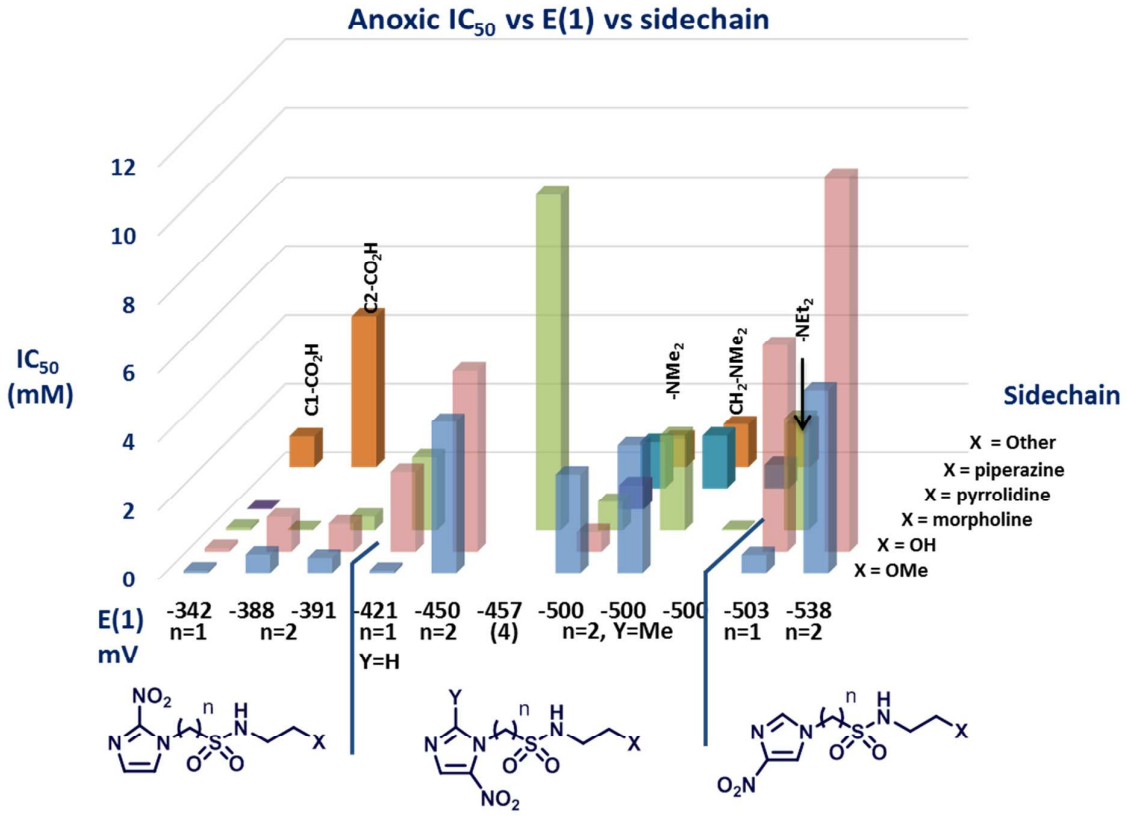


Figure 2. Anoxic cytotoxicity categorized by structural features for compounds in HCT116 colorectal carcinoma cells.

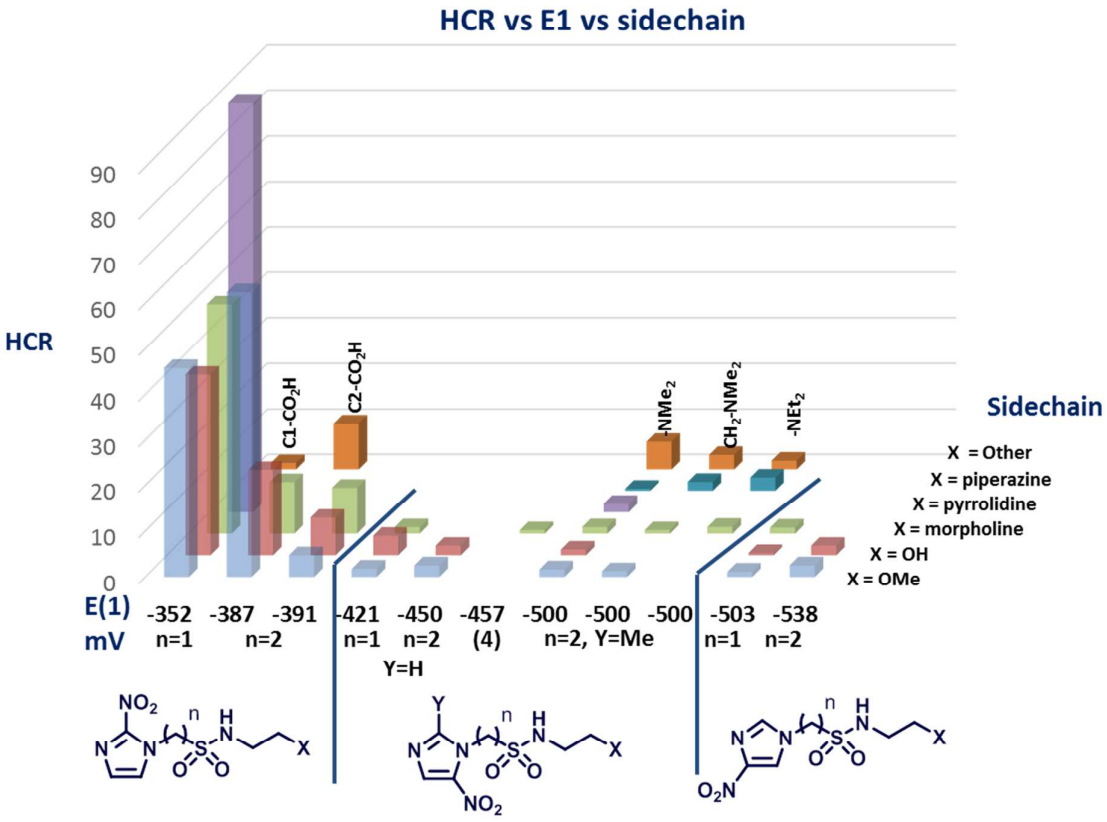


Figure 3. Hypoxic selectivity categorized by structural features for compounds in HCT116 colorectal carcinoma cells.

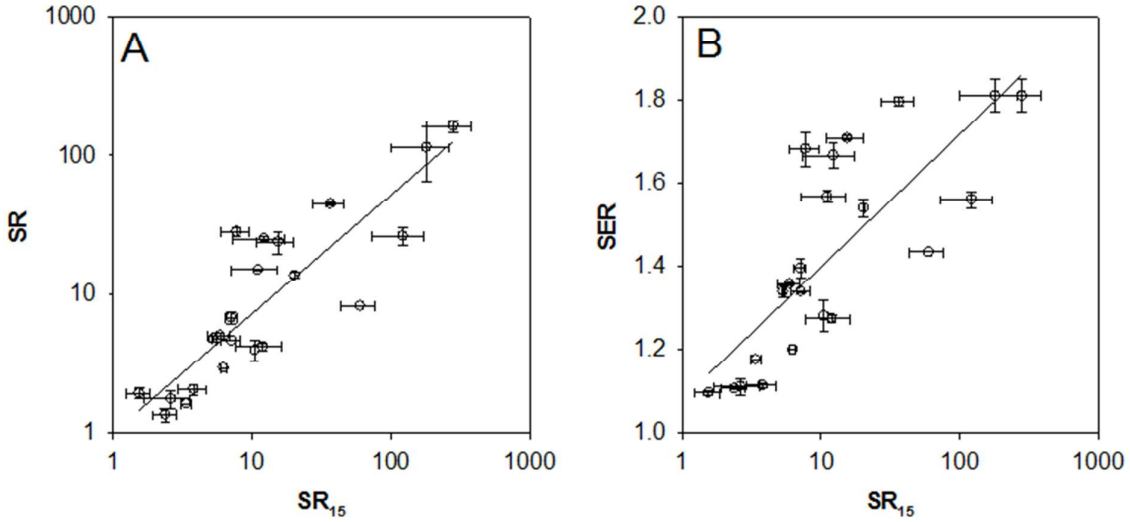


Figure 4. A) Correlation between SR_{15} determined in 15Gy radiation sensitization experiments and SR determined in SER dose response experiments. B) Correlation between SR_{15} and SER determined in the dose response experiments.

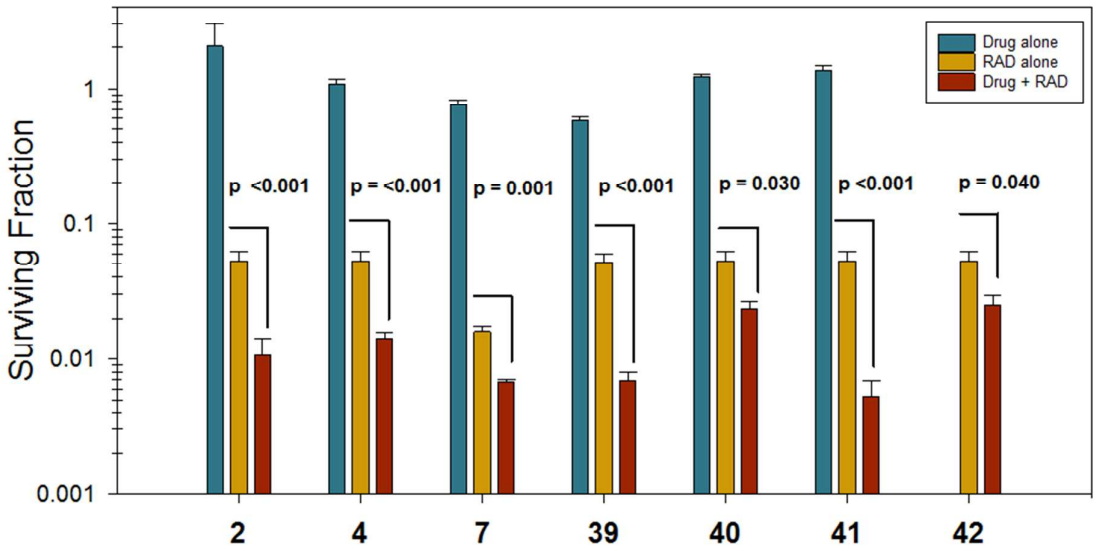


Figure 5. Evaluation of *in vivo* radiosensitization of hypoxic cells in HCT116 tumor xenografts by *ex vivo* clonogenic assay. NIH-III mice with HCT116 tumors were treated with drug alone (see Table 3 for doses), 12.5 Gy radiation alone, or 12.5 Gy radiation combined with drug (n = 6–8). Tumors were excised 18 h after treatment; tumor cells were dissociated enzymatically and plated to determine the number of surviving (clonogenic) cells per gram of tumor tissue. SF = $PE_{(irradiated)}$ / $PE_{(control)}$ where the plating efficiency (PE) = (No. of colonies) / (No. of cells plated).). Data for **7** from Reference 63.

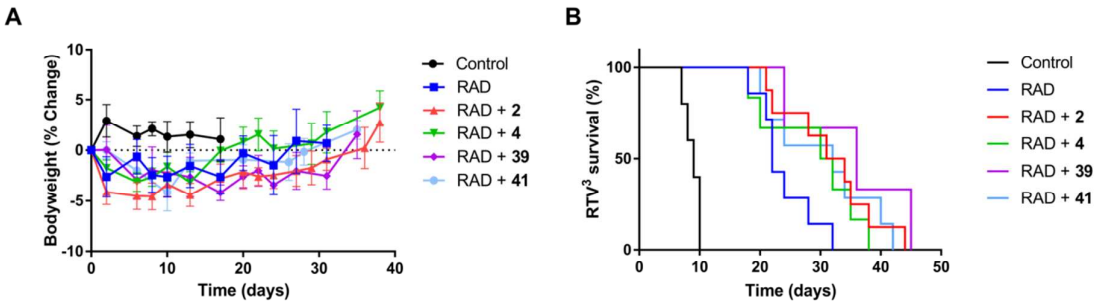


Figure 6. Tumor growth inhibition in HCT116 xenografts. A) Bodyweight change (mean ± SEM) and B) RTV^3 survival data for NIH-III mice with HCT116 tumors treated with control vehicle, 12.5 Gy radiation alone, or 12.5 Gy radiation combined with 2.20 mmol/kg **2**, 0.93 mmol/kg **4**, 2.20 mmol/kg **39** or 2.20 mmol/kg **41** (n = 6–8).

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