

## Iodinated Nitroimidazoles as Radiosensitizers

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**Abstract.** Four different nitroimidazole derivatives, with up to two iodine atoms on the imidazole ring, were investigated for their radiosensitizing potency under hypoxic conditions, in order to test whether the introduction of iodine atoms increases the radiosensitizing potency of nitroimidazoles. Misonidazole and metronidazole were used as controls. Human colonic adenocarcinoma cells were incubated with the drugs at different concentrations and for different time-periods. Photon energies of 50 kV, 60 kV and 20 MV and total radiation doses of up to 20 Gy were used. The introduction of additional iodine atoms into the nitroimidazole derivatives resulted in a strong increase in cytotoxicity of the compounds. In parallel, there were indications that the radiosensitizing potency was also increased.

In radiation therapy and chemotherapy of tumors, hypoxia is the major cause of treatment failure. Tumor cells at low oxygen tension are highly resistant to radiation. In general, the radiosensitivity of cells increases with the partial pressure of oxygen present in the tissue. Accordingly, under anaerobic conditions, the radiation dose must be increased significantly to achieve the same degree of cytotoxicity compared to oxygenated conditions. Hypoxia can also induce the expression of specific genes and promote a more aggressive tumor phenotype. Hypoxia is often induced by the treatment itself. Potential mechanisms leading to hypoxic conditions include blood flow obstructions, abnormal or overshooting angiogenesis and excessive cellular growth, resulting in under-capacity of the capillary blood supply.

Radiosensitizers have been studied in order to increase the sensitivity of hypoxic cells towards radiation. One of the

best radiosensitizers known to date is 5-fluorouracil (5-FU). Other drugs tested include cisplatin at moderate and repeated doses, etoposide for the treatment of lung cancer (1), Paclitaxel (2), hydroxyurea and the halopyrimidines, 5-iododeoxyuridine (IUDR), 5-bromo-2'-deoxyuridine (BUDR) and 5-fluoro-2'-deoxy-beta-uridine (FUDR), which have been investigated in a number of different human tumors (3). These radiosensitizers are taken up and are metabolized only by cells synthesizing DNA, so that increased tumor proliferation should result in increased radiosensitization (4).

However, clinical experience, especially with the thymidine analogs 5-bromo- and 5-iododeoxyuridine, was disappointing because normal tissue toxicity eliminated any potential for therapeutic gain (5). Gemcitabine is an extraordinarily potent radiosensitizer in the head and neck region, but, unfortunately, it appears to have a very narrow therapeutic ratio (6). Several clinical studies have been performed with nitroimidazoles. Acharya reported on the use of metronidazole in 717 patients (7). Those receiving metronidazole plus radiation had better treatment response and more recurrence-free years than those receiving radiation and placebo. Several authors reported on the molecular design and radiosensitizing activities of nitroimidazole analogs (8-12).

In a series of two *in vitro* studies, we studied nitroimidazole derivatives with the objective of increasing their radiosensitizing efficacy by introducing additional atoms into the molecule that *per se* absorb radiation better than the nitroimidazole moiety itself. To this end, we linked iodine atoms, that are well-known absorbers of X- and gamma-rays, to nitroimidazoles and tested their *in vitro* radiosensitizing potential under hypoxic conditions in comparison to analogs without iodine. In total, we tested four new compounds; one was a nitroimidazole without iodine, two substances contained one iodine and one drug had two iodine atoms. Another objective of the study was to determine the impact of increasing hydrophilicity on the cytotoxicity of the nitroimidazole derivatives.

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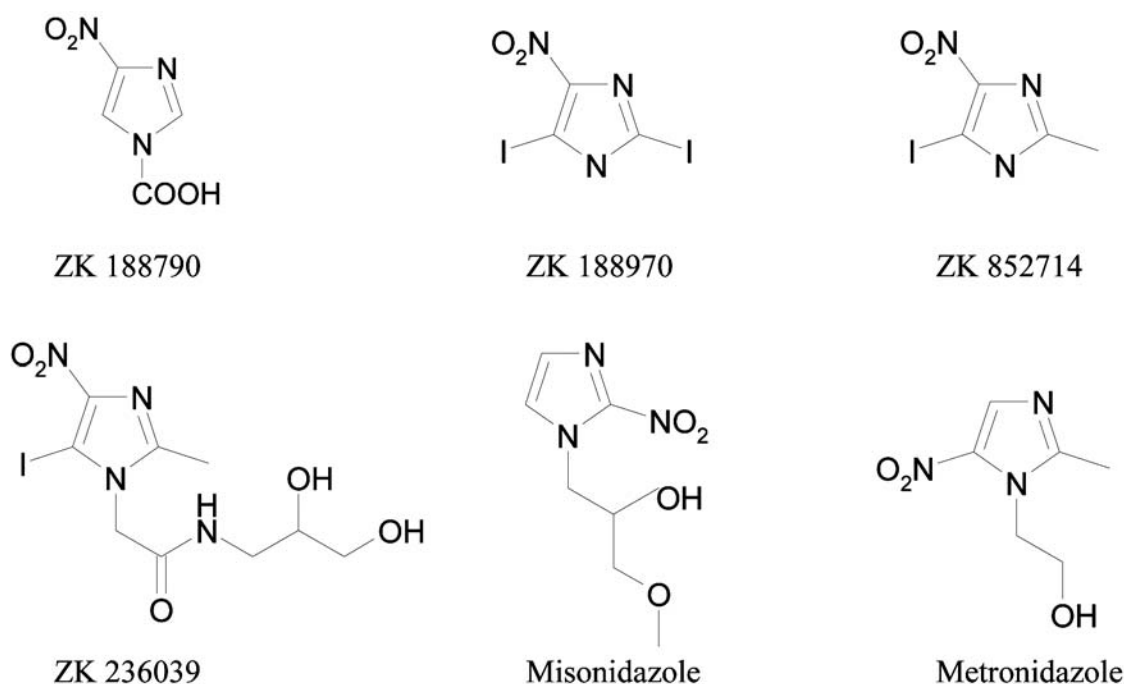


Figure 1. Structures of the investigated nitroimidazole derivatives.

## Materials and Methods

**Drug substances.** In total, we synthesized four nitroimidazole derivatives, the structures of which are illustrated in Figure 1. For comparison we used misonidazole (Hoffmann-La Roche, Basle, Switzerland) and metronidazole (Aldrich, Milwaukee, MI, USA). The four substances differed in the number of iodine atoms (0, 1, 2) and their hydrophilicity (high, medium, low).

The drugs were dissolved in water. The final concentrations used for cell incubation were 1.0 mM and 0.2 mM for the first series and 0.05, 0.1, 0.2, 1.0 and 2.0 mM for cytotoxicity testing and 0.2 mM for irradiation experiments in the second series.

**Cells and media.** Cells of a human colonic adenocarcinoma cell line (WiDr, CCL 218) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA) and were used after 319 (ZK 188790 and ZK 188970) and 300 passages (ZK 236039 and ZK 852714), respectively. The culture medium was RPMI 1640 with 10% fetal bovine serum albumin and 1.0 mM sodium pyruvate. Due to a pH change during hypoxic conditions, the buffer was exchanged for RPMI 1640 with HEPES buffer at the time of radiosensitizer addition (4 h and 24 h). After radiation exposure, the medium was substituted for the original medium. Pre-incubation of the carcinoma cells was performed under 5% CO<sub>2</sub>, 3% O<sub>2</sub>, 100% H<sub>2</sub>O in order to adapt the cells to hypoxia. After treatment, the cells were incubated under 5% CO<sub>2</sub>, 21% O<sub>2</sub> and 100% H<sub>2</sub>O.

**Testing methods.** For testing cytotoxicity and radiosensitization efficacy, a colony formation assay was used according to Puck *et al.* (13). Twenty-four h before treatment, the cells were seeded from the routine pre-incubation culture into 25-ml culture flasks, such that

200-600 colonies were obtained per flask after 10 days. The cells were incubated with the radiosensitizers for 24 h and 4 h (series 1), and 4 h (series 2), respectively. After 10 days, the cells were fixed and stained (2.5 g/100 ml crystal violet and 1 g/100 ml ammonium oxalate). Counting of the surviving colonies was performed with an automatic counter (Artek model 880, BioSys GmbH, Karben, Germany), which was manually calibrated on a regular basis. Survival following hypoxia, drug treatment and radiation, respectively, was calculated on the basis of the plating efficiency under hypoxic conditions with the drug added but without radiation (=100% survival). Plating efficiency was between 15 and 65%, depending on the drug and its concentration. Cytotoxicity was tested in series 1 and 2 at 0.2, 0.5, 1.0, 2.0 and 5.0 mM with incubation times of 24 h (series 1) and of 4, 24 and 48 h (series 2), respectively.

**Establishment of hypoxic conditions.** Using gas-tight 25-ml culture flasks with 19G cannulas, the colonies were gassed with nitrogen (2.5 ml/min). The oxygen concentration of the medium was determined using an oxygen probe (WTW Oxi 325, Weilheim, Germany) before the start of the main experiment and the conditions of hypoxia were optimized accordingly. For all tests, the oxygen concentration was  $0.1 \pm 0.03$  mg/l. As a control, using the  $\beta$  parameters of the Linear Quadratic (LQ) Model, the oxygen enhancement ratio (OER) was determined as an indicator of the survival of the cells under oxic and hypoxic conditions. An OER of 4.7 for oxic *versus* hypoxic conditions was in good agreement with the literature data (14).

**Irradiation.** The cells were irradiated in 25-ml flasks suspended in 5 ml medium on a 30-mm phantom using a 20-MV Linear Accelerator (Mevatron, Siemens, Erlangen, Germany). The time

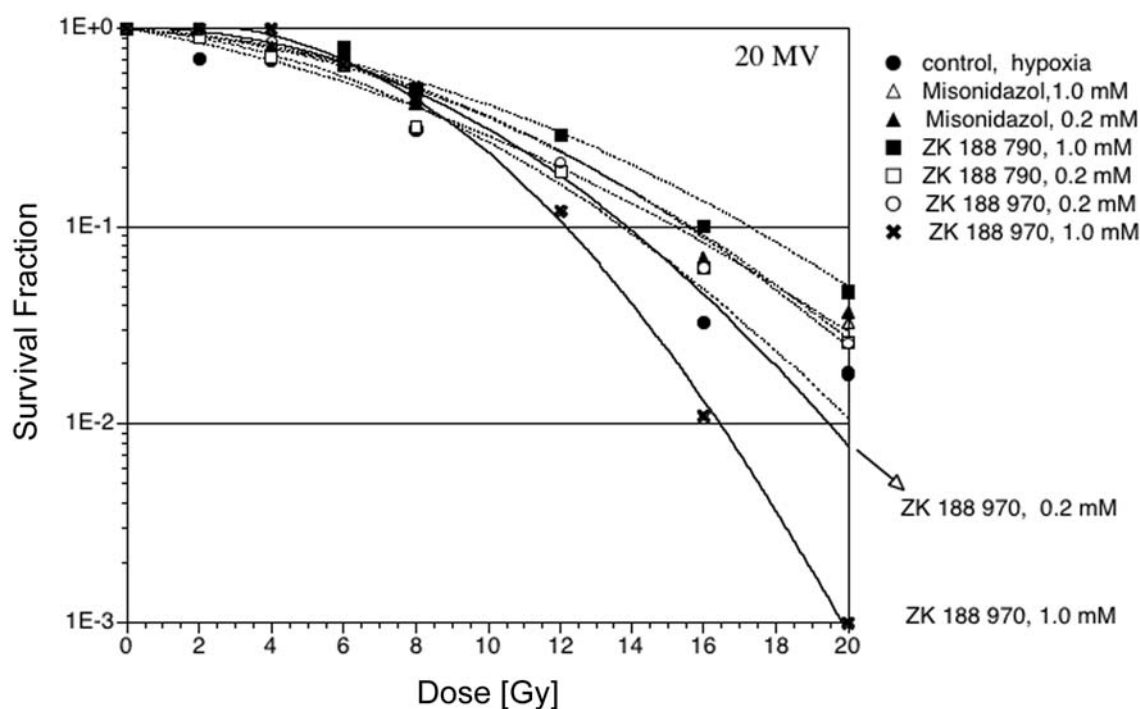


Figure 2. Radiosensitizing efficacy of ZK 188790 and ZK 188970 in comparison to misonidazole and hypoxic control. Incubation time was 4 h. Concentrations were 0.2 and 1.0 mM. Photon energy was 20 MV, total dose was 20 Gy.

of irradiation was 3.8 min and the total dose was 20 Gy. Alternatively, a 60-kV X-ray machine with a dose rate of 0.07 Gy/min was used (Monopon 60, Siemens, Erlangen, Germany). Due to the low dose rate, only total doses of 2, 4 and 6 Gy were studied. For this type of irradiation, a special tube was constructed that allows the production of a homogeneous field with a diameter of 115 mm. Field homogeneity was verified using film dosimetry.

In addition to the 20 MV Linear Accelerator, experiments were performed using a 150 kV X-ray machine (Müller MG 150, Philipps, Hamburg, Germany) with a dose rate of 1.99 Gy/min (50 kV, 20 mA, Be window, no filters). Doses of 2, 4, 6, 8, 12, 16 and 20 Gy were verified by dosimetry.

**Evaluation.** Mean values of the surviving fractions of the cells were used to determine the dose-effect curves. Following linear regression calculation using the Linear Quadratic (LQ) Model, parameters  $\alpha$  and  $\beta$  of the dose-response curves were determined. From the ratios of the  $\alpha$ - and  $\beta$ -values of the dose-response curves with and without radiosensitizer, the dose-modification factors (DMF) were calculated. For misonidazole, a DMF of approx. 1.5 was in good agreement with the literature data. The experiments were performed 2-3 times.

## Results

**Cytotoxicity.** ZK 188790 and ZK 188970 showed considerable differences in cytotoxicity upon incubation for 24 h. Whereas for the former drug no toxicity was observed in the concentration range up to 5.0 mM, ZK 188970 exhibited cytotoxicity starting at 1.0 mM.

With a 4-h incubation period, ZK 236039 exhibited the same degree of cytotoxicity as did metronidazole. With longer incubation periods (24 and 48 h), ZK 236039 was slightly more cytotoxic than metronidazole. ZK 852714 was more cytotoxic than the other drugs, particularly at concentrations higher than 1.0 mM.

**Radiosensitizing efficacy.** For 20 MV photon energy, concentrations of 0.2 and 1.0 mM and a 4-h incubation period, the radiosensitizing efficacies of ZK 188790 and ZK 188970 in comparison to misonidazole and control were determined, as illustrated in Figure 2.

Data for ZK 236039 and ZK 852714 in comparison to metronidazole at photon energies of 20 MV and 50 KV, a drug concentration of 0.2 mM and a 4-h incubation period, are illustrated in Figure 3. The oxygen enhancement ratios calculated for the different drugs, concentrations, incubation periods and photon energies are summarized in Figure 4. Dose modification factors (DMF) are given in Table I.

## Discussion

We synthesized four different nitroimidazole derivatives, three of them containing one or two iodine atoms. The purpose of introducing the iodine atoms was two-fold; first, we hypothesized that adding atoms with a high absorption

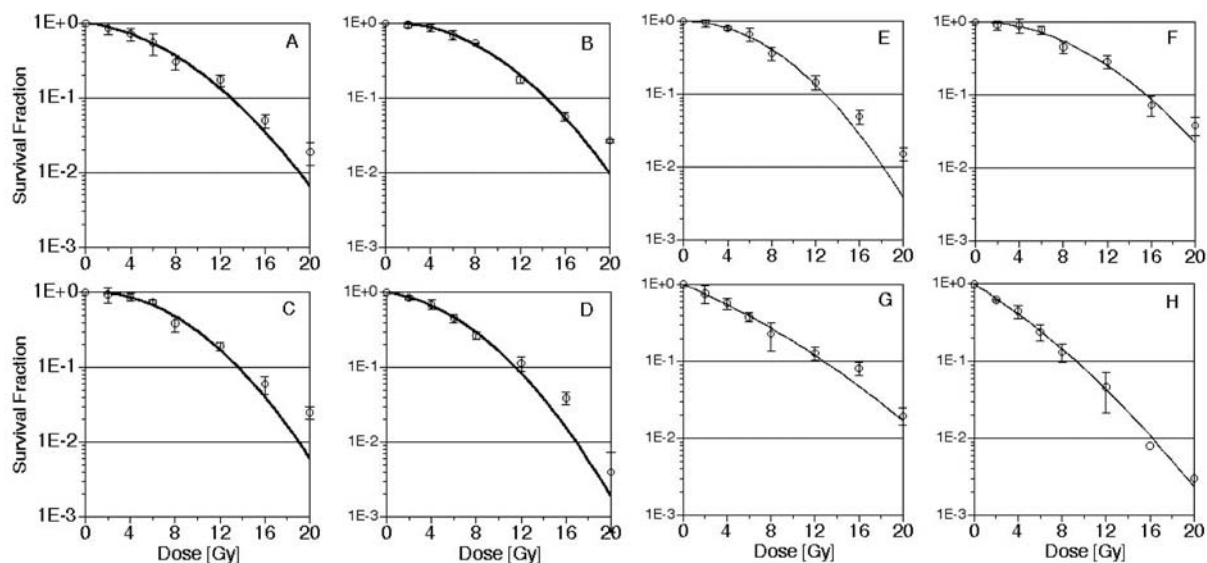


Figure 3. Radiosensitizing efficacy of ZK 236039 (C, G) and ZK 852714 (D, H) in comparison to metronidazole (A, E) and hypoxic control (B, F). Incubation time was 4 h. Photon energy was 50 kV (A-D) or 20 MV (E-H), total dose was 20 Gy.

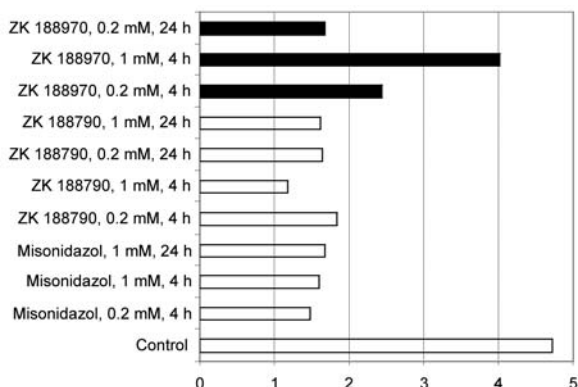


Figure 4. Oxygen enhancement ratios (OER) for different nitroimidazoles and testing conditions.

potential for X-rays might increase the efficacy of the nitroimidazoles as radiosensitizers, and, second, the iodine atoms could potentially be used for imaging purposes, particularly in computed tomography (CT). Iodinated contrast agents are benzene derivatives with three iodine atoms and three hydrophilic groups for achieving sufficiently high water solubility. Approx. 1 mg of iodine per ml is required to achieve a signal increase of 30 Hounsfield Units in CT (15). If an iodine atom is added to a radiosensitizer that accumulates in tumor tissue due to its hypoxia-seeking molecular properties, then imaging of the tumor might become possible. Testing of the latter hypothesis was, however, not part of this investigation.

Another objective was to study the impact of increasing hydrophilicity on the cytotoxicity of nitroimidazoles. We used misonidazole and metronidazole for comparison. Additionally, we studied both high- and low-energy photons to measure the radiosensitizing efficacy of the drugs. We observed clear differences in the cytotoxicity ranging from ZK 188970 with the highest potential to ZK 188790, which was similar to misonidazole and metronidazole. The ranking according to decreasing cytotoxicity was: ZK 188970 >> ZK 832714 > ZK 236039 ≥ ZK 188790 ≈ misonidazole ≈ metronidazole. The addition of one or two iodine atoms to the nitroimidazole ring system and the reduction of hydrophilicity, e.g. by eliminating carboxyl groups, significantly increased the toxicity of the compounds. The effect of one iodine atom could not totally be counteracted by the simultaneous introduction of a highly hydrophilic side chain such as a dihydroxyethyl carboxamide group.

Due to the high intrinsic cytotoxicity of the new imidazole derivatives, results from radiosensitizing experiments at high concentrations (>1.0 mM) have to be considered cautiously. At this concentration, cytotoxicity alone could potentially contribute to the observed effects. However, at lower concentrations, the contribution of cytotoxicity *per se* might be negligible. This difference in potency can be evaluated from Figure 2, in which the radiosensitizing potential of ZK 188790 at 1.0 and 0.2 mM was compared. At the higher concentration of 1.0 mM, ZK 188790 exhibited a greater radiosensitizing efficacy than at 0.2 mM. For misonidazole, there was no difference between a concentration of 1.0 or 0.2 mM.

Table I. Dose modification factors for different imidazole derivatives.

Drug	Conc. (mM)	Incubation (h)	Energy	$\alpha$ -term value	SE	$\beta$ -term value	SE	$\alpha_{\text{sens}}/\alpha_{\text{M}}$	$\beta_{\text{sens}}/\beta_{\text{M}}$	$\alpha_{\text{sens}}/\alpha_{\text{C}}$	$\beta_{\text{sens}}/\beta_{\text{C}}$
Metronidazole	0.2	4	50 kV	0.06840	0.02400	0.00710	0.00180	1.00000	1.00000	18.48640	0.71710
ZK 236039	0.2	4	50 kV	0.00760	0.01620	0.01020	0.00130	0.11110	1.43660	2.05400	1.03030
ZK 852714	0.2	4	50 kV	0.06260	0.02070	0.01020	0.00200	0.91520	1.43660	16.91890	1.03030
Control	-	4	50 kV	0.00370	0.01630	0.00990	0.00130	0.05410	1.39440	1.00000	1.00000
Metronidazole	0.2	4	20 MV	0.01180	0.01540	0.01170	0.00120	1.00000	1.00000	0.80820	1.48100
ZK 236039	0.2	4	20 MV	0.14360	0.01990	0.00210	0.00110	12.16950	0.17950	9.83560	0.26580
ZK 852714	0.2	4	20 MV	0.19700	0.01810	0.00540	0.00160	16.69490	0.46150	13.49320	0.68350
Control	-	4	20 MV	0.01460	0.01560	0.00790	0.00110	1.12730	0.67520	1.00000	1.00000
Misonidazole	1.0	4	20 MV	0.01720	0.01220	0.00840	0.00150	1.00000	1.00000	0.22340	1.44830
ZK 188790	1.0	4	20 MV	0.03180	0.02000	0.00610	0.00280	1.84900	0.72600	0.41290	1.05170
ZK 188970	1.0	4	20 MV	0.00000	0.01500	0.01240	0.00240	0.00000	1.47600	0.00000	2.13800
Control	-	4	20 MV	0.07700	0.03760	0.00580	0.00460	4.47700	0.69000	1.00000	1.00000
Misonidazole	0.2	4	20 MV	0.02630	0.01306	0.00760	0.00162	1.00000	1.00000	0.34160	1.31030
ZK 188790	0.2	4	20 MV	0.04430	0.01851	0.00800	0.00207	1.68440	1.05260	0.57530	1.37930
ZK 188970	0.2	4	20 MV	0.00000	0.00942	0.01070	0.00134	0.00000	1.40780	0.00000	1.84480
Control	-	4	20 MV	0.07700	0.03760	0.00580	0.00461	2.92770	0.76310	1.00000	1.00000
ZK 188790	1.0	4	60 kV	0.04100	0.00770	0.01740	0.00170	ND	ND	0.56710	3.34610
ZK 188970	1.0	4	60 kV	0.18050	0.09630	0.02660	0.02550	ND	ND	2.49650	5.11540
Control	-	4	60 kV	0.07230	0.03760	0.00520	0.00460	ND	ND	1.00000	1.00000
Misonidazole	1.0	24	20 MV	0.02640	0.02100	0.00780	0.00270	1.00000	1.00000	0.34290	1.34480
ZK 188790	1.0	24	20 MV	0.00000	0.01120	0.00760	0.00130	0.00000	0.97440	0.00000	1.31030
Control	-	24	20 MV	0.07700	0.03760	0.00580	0.00461	2.91660	0.74360	1.00000	1.00000
ZK 188970	0.2	24	20 MV	0.00000	0.01450	0.00500	0.00160	ND	ND	0.00000	0.86200
ZK 188790	0.2	24	20 MV	0.00000	0.01250	0.00580	0.00130	ND	ND	0.00000	1.00000
Control	-	24	20 MV	0.07700	0.03760	0.00580	0.00461	ND	ND	1.00000	1.00000

ND = Not determined

At photon energy of 20 MV and a concentration of 0.2 mM, ZK 188790 and ZK 188970 had similar radiosensitizing efficacies, which were more or less identical to that of misonidazole (Figure 2). On the other hand, at the same energy (20 MV) and concentration (0.2 mM), ZK 852714 was more potent than metronidazole, which was superior to ZK 236039 (Figures 3H, 3E and 3G). At lower photon energies, *e.g.* 50 kV and a concentration of 0.2 mM, the same picture was obtained, showing that ZK 852714 was slightly more potent than ZK 236039 or metronidazole, which were similar in potency (Figures 3D, 3C and 3A).

From these results, it might be concluded that the introduction of additional iodine atoms does not increase the radiosensitizing potential of nitroimidazole derivatives. The OER's of the different drugs are relatively similar (Figure 4) with the exception of ZK 188970, which is much higher compared to the other drugs for a concentration of 1.0 mM and slightly higher for 0.2 mM. The high activity for

1.0 mM is probably due to the increased cytotoxicity of this compound. However, at 0.2 mM, ZK 188970 also was more potent than imidazoles with no iodine (misonidazole and ZK 188790).

The dose modification factor (DMF) is composed of an  $\alpha$ - and a  $\beta$ -term. Table I gives a comparison of DMF's for the different drugs and conditions. At photon energy of 20 MV and a radiosensitizer concentration of 1.0 mM, the efficacy judged from the  $\alpha$ -term relative to misonidazole or metronidazole ( $\alpha_{\text{sens}}/\alpha_{\text{M}}$ ) was greater for ZK 188790 without iodine and negligible for ZK 188970 with two iodines. On the other hand, this ratio was also greater for hypoxic conditions alone *vs.* hypoxic conditions plus misonidazole, indicating that, under these conditions, the nitroimidazoles do not exhibit radiosensitizing activity. However, upon lowering the concentration to 0.2 mM, ZK 236039 and ZK 852714 were superior to misonidazole and hypoxic conditions alone regarding the  $\alpha$ -term.

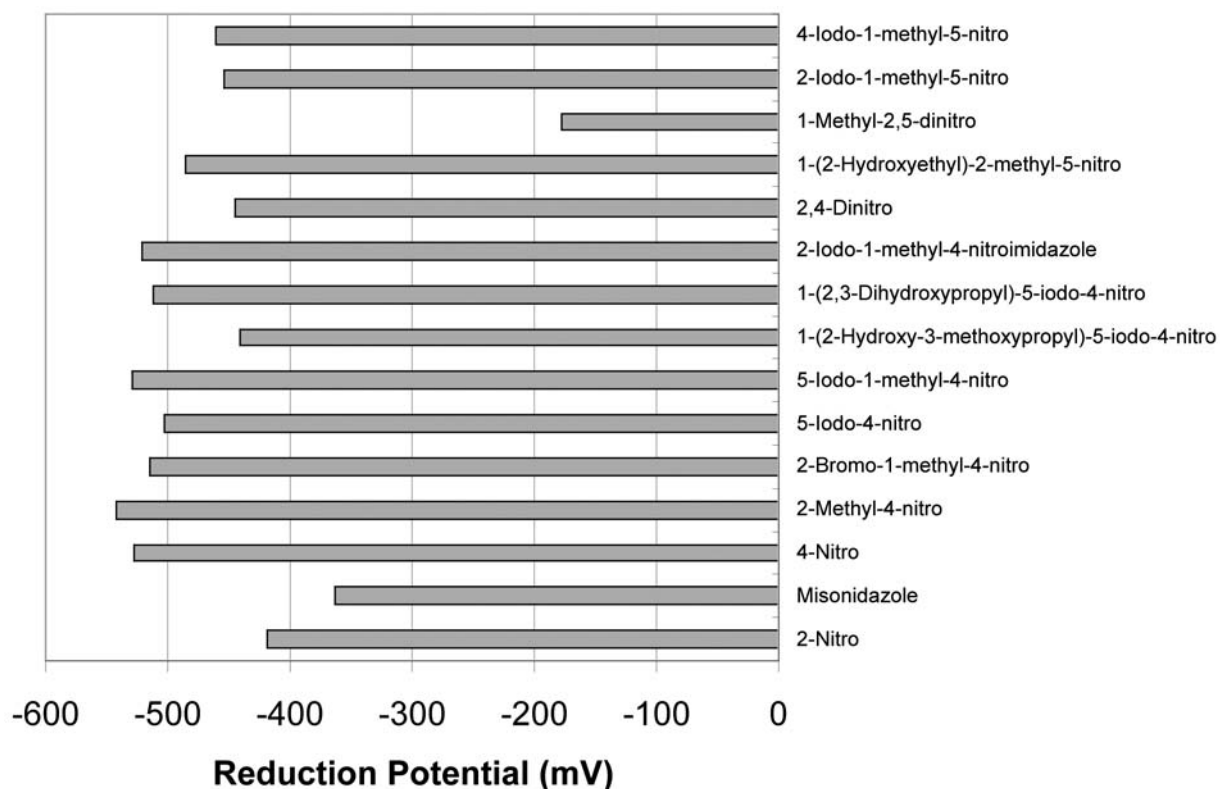


Figure 5. Reduction potentials of a number of substituted and unsubstituted nitroimidazoles. Data have been compiled from the literature (17-22).

More consistency was found for the  $\beta$ -term, particularly for lower photon energies. At 50 kV, the introduction of iodine atoms seemed to improve the radiosensitizing potency. At 20 MV, the results were not as clear. Potentially, this high energy is not as optimal as lower energies for radiosensitizing purposes.

What the biological consequence of this finding is remains unclear. There are, however, some indications in the literature that the  $\alpha$ -term plays a role in radiosensitization. The concept of intrinsic radiosensitivity has been reported to be strongly associated with the linear-quadratic (LQ) model, which is currently the most reliable method to fit the first three decades of a survival curve for tumor cell lines. This approach has led to the major conclusions that it is the initial part, and not the distal part of the survival curve which truly characterizes intrinsic cellular radiosensitivity. There is a correlation between the parameters describing mainly the initial part of the survival curve ( $\alpha$ , SF2, D) and the clinical radio-responsiveness (16).

Regarding a potential increase in efficacy, due to the introduction of an iodine atom, several approaches are possible to evaluate this hypothesis. One is to determine the reduction potential of the compounds that could give

an indication of their efficacy. We have compiled a number of reduction potentials from the literature (Figure 5). The general conclusions drawn from this comparison seem to be that the following steps lead to a further decrease in reduction potentials: moving the nitro group from position 2 or 5 to 4, introducing a second nitro group, substituting bromine by iodine, or iodine by hydrogen, increasing hydrophilicity. The reduction potential, however, will only give an indication of the radiosensitizing efficacy of the molecule *per se*. No conclusions can be drawn, however, regarding the radiation absorbing efficacy of the nitroimidazole moiety. Our hypothesis was that an increase in radio-absorption of the sensitizer could potentially result in an increase in efficacy without a concomitant effect on the reduction potential. Our *in vitro* measurements of the radiosensitizing potency of iodine-containing vs. iodine-free nitroimidazoles showed that there is no consistent increase in efficacy, if judged by their  $\alpha$ -term of the linear quadratic model compared to that of non-iodine containing nitroimidazoles. However, the  $\beta$ -term did indicate an increase compared to misonidazole or metronidazole. More investigations are necessary to evaluate the impact of this observation.

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