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Experimental report

The influence of hypoxia on the cytotoxicity of concomitant KW-2149 and ionizing irradiation in Chinese hamster fibroblasts

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7-*N*-((2-((2-(γ-L-glutamylamino)ethyl)dithio)ethyl))-mitomycin C (KW-2149) is a newly synthesized water-soluble mitomycin C (MMC) analog. Preclinical testing showed an interesting activity profile and a superior hematological tolerance in murine models. The aim of this study was to investigate the interaction of this compound with ionizing radiation, both under normoxic and hypoxic conditions, in Chinese hamster fibroblasts (V79). V79 cells were irradiated both under normoxic conditions and after a 1 h period of hypoxia. Paired irradiation dose–response curves confirmed the significance of radioresistance under hypoxia with an oxygen enhancement ratio of approximately 3. In contrast to MMC, KW-2149 showed no increased cytotoxic effect on hypoxic V79 cells. The cytotoxic effect of KW-2149 increased with increasing concentration, irrespective of the ambient oxygen pressure. When KW-2149 was combined with irradiation under hypoxic conditions, cytotoxicity was significantly enhanced under these conditions. The difference in survival between normoxic and hypoxic conditions was statistically significant ($p < 0.004$). These data suggest a radiosensitizing effect of KW-2149, more pronounced under hypoxic conditions. This effect increases with radiation dose. It also corroborates earlier suggestions of a different mode of action of KW-2149 as compared to MMC.

Key words: Hypoxia, irradiation, KW-2149.

Introduction

The effectiveness of radiation therapy is considered to be limited by the existence within tumors of hypoxic cells.[1] Recent studies measuring the degree of hypoxia in human tumors have underscored the importance of this phenomenon.[2,3]

Neoangiogenesis is mainly situated in the outer rim of the tumor, leaving central parts relatively underperfused. This lack of central tumor perfusion increases with increasing tumor volume and increasing interstitial hydrostatic pressure.[4] An acute and more chronic form of hypoxia should be distinguished when discussing tumor hypoxia. Data from recent studies indicate acute hypoxia as an important mechanism of treatment resistance.[5] Hypoxic cells are highly resistant to radiation damage and the diffusional limitations for oxygen also apply to the reactive cytotoxic agents used in chemotherapy.

Different attempts have been made to eliminate hypoxia-related resistance either by increasing the delivery and/or availability of oxygen to the tumor prior to radiotherapy, e.g. hyperbaric conditions or hemoglobin–oxygen affinity modifiers, or sensitizing hypoxia cells to radiation.[6] One group of such agents is the bioreductive drugs.[6–8] These are preferentially activated under conditions of hypoxia. The effectiveness of these agents seems to be independent of the duration of hypoxia.

There are three main types of bioreductive agents. The first group includes the nitroimidazole compounds, such as misonidazole, pimonidazole, etanidazole and many others. A second group of bioreductive cytotoxic drugs includes the benzotriazine di-*N*-oxides of which SR4233 is the prototype compound. The third group is the quinone group with agents such as mitomycin C (MMC), porfiromycin and EO9.[9]

MMC is a cytotoxic agent that requires biotransformation, preferentially under anaerobic conditions, before drug activation results in DNA cross-linking. Activation is supposed to be either a one-electron reduction generating a semiquinone free radical intermediate or a two-electron reduction generating a hydroquinone intermediate.[10] For MMC, despite the variability in general hypoxic cytotoxicity against different cell lines, values obtained by different investigators are in the range from 1 to 5.[11–15]

This has led to the combination of MMC with radiotherapy in the treatment of different solid tumors, e.g. in head and neck cancer, and anal cell cancer. Although some randomized trials in these diseases have corroborated the increased efficacy of the MMC-containing treatment arm, these data cannot be seen as proof for an *in vivo* selective hypoxic cytotoxic effect of MMC.[16] For porfiromycin, a structure analog of MMC, the selectivity of hypoxic cytotoxicity is much more pronounced.[17,18] The same holds true for EO9, a new MMC analog, with an even higher degree of selective cytotoxicity for hypoxic cells.

KW-2149 is a new MMC analog that proved superior to MMC in most *in vitro* models, and with both an increased activity against MMC resistant cell lines and an improved toxicity profile in animal models.[19,20] One of the more interesting characteristics of this compound is its apparent different mode of activation with a thiol-mediated non-enzymatic reduction mechanism.[21] This might explain the increased activity against MMC-resistant cell lines. Clinical development of this agent is ongoing.[22] Because of these differences in the activity profile and the superior preclinical toxicity profile we wanted to examine its activity in combination with ionizing radiation.

Materials and methods

Cell line

Chinese hamster lung fibroblasts, V79, were kindly provided by Professor GW Barendsen (Amsterdam, The Netherlands). These cells were grown as a monolayer in minimum essential medium (MEM) with Hanks' salts. The medium was supplemented with 10% fetal calf serum, 0.06 mM L-glutamine (Gibco), 0.01 mM L-asparagine (Gibco) and 0.03 M sodium bicarbonate. No antibiotics were added to exclude possible interaction. The mean plating efficiency (PE) of the untreated Chinese hamster fibroblasts was $70 \pm 5\%$. Cells were grown in 75 cm^2 culture flasks maintained in a humidified atmosphere at 37°C with 5% CO_2 and subcultured two to three times per week.

Drug

KW-2149 was supplied by Kyowa Hakko Kogyo (Tokyo, Japan). The drug was dissolved in physiological saline to a concentration of 25 μM. New dilutions were made prior to each set of experiments. The solution was dripped through antibacterial filters prior to each experiment.

Radiotherapy

Petri dishes with cells growing in a monolayer were irradiated with γ-rays from a Co (Theraton 780) clinical treatment unit at a dose rate of 98.4 cGy/min at 80 cm. Cells were exposed to acute single doses in the range of 2–12 Gy at room temperature.

Hypoxia environment

The use of glass material is recommended when performing experiments on hypoxia. A glass dessicator was used as an outer environment. The cells cultures in the Petri dishes were in the dessicator on a glass support. To produce hypoxia pure nitrogen was used with less than 0.5 p.p.m. oxygen. The dessicator was closed with both robinets open enabling a continuous flow of pure nitrogen with a flow of 3 l/min.

Clonogenic assay

Cell survival after irradiation and drug exposure was determined with a monolayer colony forming assay. Cells were plated at a density of 10^3 cells/ml in 9 cm glass Petri dishes (Schott) containing 8 ml medium and allowed to attach for 2 h. KW-2149 was added and cells were exposed for 1 h. Cells were incubated for 1 h in a gas exchange system to produce hypoxic and normoxic conditions. This was followed by exposure to acute single doses of irradiation with cells still in the dessicators. The control samples without irradiation were prepared in a similar manner.

After an incubation period of 7 days at 37°C in a 5% CO_2 atmosphere, medium was discarded and cells were washed with Hanks' Balanced Salt Solution (Gibco). Cells were fixed with 8% methanol and 30% formaldehyde, and stained with crystal violet. Colonies were then counted by the naked eye.

Survival is expressed as the ratio of the fraction of surviving colonies to the amount of colonies in the untreated control culture.

Oxygen enhancement ratio (OER)

The OER is defined as the ratio of radiation dose under hypoxic conditions to the dose under normoxic conditions needed to achieve the same biological effect. All these experiments were performed in triplicate.

Results

Chinese hamster fibroblasts (V79) and irradiation

These experiments confirmed the presence of a radioresistant effect of hypoxia on V79 cells. Figure 1 shows the survival fraction for these cells exposed to graded doses of ^{60}Co γ-rays under hypoxic and normoxic conditions. The acute single radiation dose ranged from 2 to 12 Gy per fraction. These curves illustrate resistance to radiation cytotoxicity for different irradiation doses ($p < 0.0007$). The OER was 2.8 at 50% cytotoxicity.

Chinese hamster fibroblasts (V79) and KW-2149

A minor difference in cytotoxicity of KW-2149 was observed in this set of experiments. Figure 2 illustrates this cytotoxic effect at different concentrations. Cytotoxicity increases with concentration both under hypoxic and normoxic conditions. The cytotoxic activity of KW-2149 is superior under aerobic conditions. The difference between the curves is statistically significant ($p < 0.02$). This suggests a lack of hypoxic sensitization for the cytotoxic effect of KW-2149, as is known to exist for MMC.

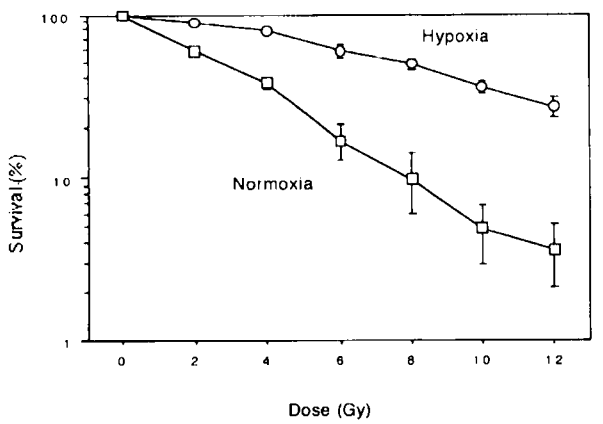

Figure 1. Survival fraction for cells exposed to graded doses of irradiation.

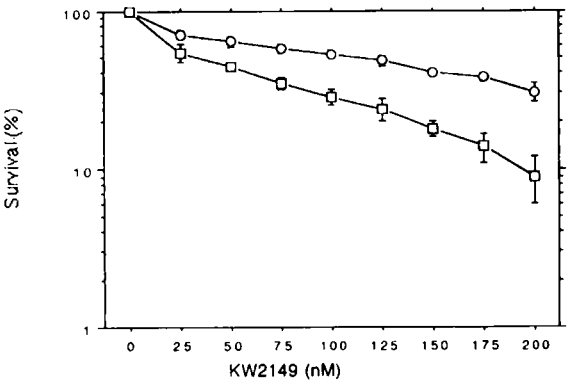

Figure 2. Cytotoxicity at different concentrations of KW-2149: , hypoxia/KW-2149; , normoxia/KW-2149.

Chinese hamster fibroblasts (V79) and KW-2149 and irradiation

The cells growth inhibitory efficacy of different concentrations of KW-2149 at 2 Gy, both under aerobic and hypoxic conditions, is shown in Figure 3. In these experiments an opposite effect was observed with hypoxic cells becoming more radiosensitive due to exposure to KW-2149. This difference in cell killing efficacy was statistically significant ($p < 0.004$). This effect of KW-2149 was observed at all concentrations tested and even increased with higher drug concentrations.

In Figure 4 the results of similar experiments are shown but performed after exposure to 50 nM KW-2149. These data again show an increased radiosensitization effect of KW-2149 under hypoxic conditions.

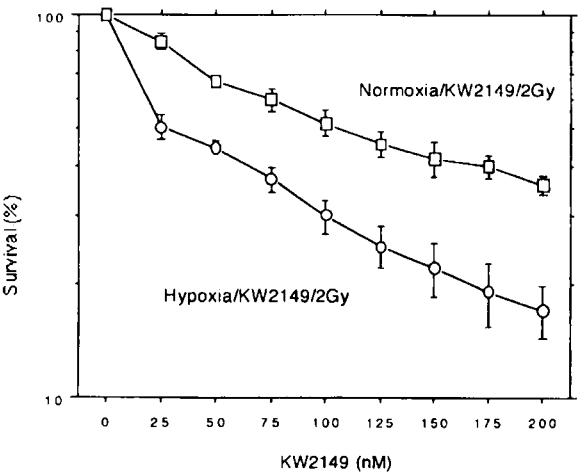

Figure 3. Cytotoxicity of irradiation at different concentrations of KW-2149.

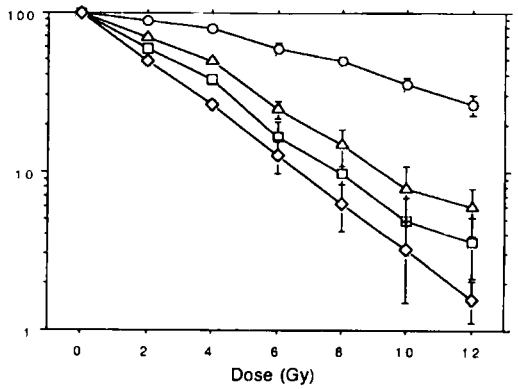

Figure 4. Cytotoxicity at different irradiation doses: , hypoxia/RT; , normoxia/RT; , hypoxia/RT/KW-2149; normoxia/RT/KW-2149.

This phenomenon is already observed at low radiation doses but increases and becomes more pronounced at higher radiation doses.

In combination with irradiation, cytotoxicity was strongly enhanced in hypoxic conditions and also but to a lesser extent in aerobic conditions. The dose enhancement ratio (DER) was 1.3 versus 1.7, respectively.

Discussion

KW-2149 is one of several MMC analogs with a *N*-7 substituent. These were developed because the *N*-7 position has a strong association with the reduction of the quinone ring. Studies on the interaction between MMC and DNA had previously shown that reduction of the quinone ring is a prerequisite in the activation process of MMC. Compared with MMC, KW-2149 was selected for further clinical development because of its similar anticellular spectrum, its cell growth inhibitory activity at 10 to 100 times lower concentration, its activity against MMC-resistant leukemias and its promising animal toxicity profile. Its clinical development is ongoing with already distinctive antitumor activity in phase I trials, but also with cumbersome pulmonary toxicity.[22]

Because KW-2149 is a MMC analog, we choose to investigate its potential role as a bioreductive agent to be used in combination with radiation treatment.

MMC was shown to be selectively activated by tumor cells under hypoxic conditions. The selective toxicity of MMC is more pronounced under conditions of very low levels of hypoxia (< 0.0% or 100 p.p.m.) and after conditions similar to chronic hypoxia after 4 h of pure nitrogen gassing prior to drug administration.

KW-2149 clearly behaves differently when exposed to V79 cells under conditions of acute hypoxia. In contrast to MMC, KW-2149 was not selectively toxic for hypoxic cells. Rather the reverse was true, with a small but significant increased killing efficacy being observed under aerobic conditions. These observations are in accordance with recent data from Ashizawa *et al.*[23] These investigators have used HeLa S_3 cells in cytotoxicity experiments with KW-2149 under aerobic and hypoxic conditions. These data confirm the superior activity of KW-2149 compared with MMC under aerobic conditions, but while MMC showed an increased cell killing under hypoxia, the opposite effect was observed with KW-2149.

These data and our own results support earlier studies suggestive of distinct differences in the drug activation process for both compounds. These results are also suggestive of some degree of oxygen dependence of KW-2149 for optimal cytotoxicity.

The impact of hypoxia on resistance towards radiation-induced damage was clearly confirmed in our experimental system. In contrast with our initial results was the observation of increased sensitivity of radiation-induced cytotoxicity in the presence of KW-2149. This enhancement factor under hypoxic conditions of KW-2149 for radiation-induced cytotoxicity is a phenomenon also observed with MMC. The reason why this selective hypoxic cytotoxicity might be evident only after combination with ionizing radiation might be due to the absence of absolute anoxia in our experimental design. The one-electron reduction to the hydroquinone intermediate of KW-2149 under conditions of moderate hypoxia might lead the production of the superoxide radical. Thus the superior cytotoxic effect under aerobic conditions might be compatible with more active radiation sensitivity under hypoxic conditions.

In conclusion the data presented on the sensitizing effect of hypoxia on the direct cytotoxicity of KW-2149 and the effect obtained after combination with radiotherapy add to the available evidence suggesting that KW-2149 is indeed substantially different from MMC. It also suggests that KW-2149 can sensitize hypoxic cells at modest radiation dose.

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