

Influence of Irinotecan and SN-38 on the Irradiation Response of WHO3 Human Oesophageal Tumour Cells under Hypoxic Conditions

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Abstract. Irinotecan and its metabolite SN38 were evaluated for their cytotoxicity and influence on radiosensitivity in WHO3 human oesophageal cells under hypoxic conditions. The IC_{50} 's of Irinotecan and SN-38 were found to be 0.8 and 0.04 μ M, respectively, with SN-38 emerging as the more potent drug. The toxicities were similar under anoxic conditions. Given in conjunction with irradiation under hypoxic conditions, the two drugs restored the radiosensitivity of WHO3 cells in a dose-dependent manner by factors of 1.5 – 2.1 as compared to a control oxygen enhancement ratio (OER) of 2.1 in this cell system. In the subtoxic concentration range of 10^{-2} μ M SN-38 still generated a marked sensitisation of hypoxic tumour cells by factors of 1.2-1.6. It is concluded that the topoisomerase inhibitor Irinotecan and in particular the metabolite SN-38 may be clinically useful for radiotherapy of notoriously hypoxic tumour pathologies.

Irinotecan (CPT-11), a derivative of camptothecin, is converted by the enzyme carboxylesterase to a more active antitumour compound, SN-38, which has been reported to be 200 to 1000 times more potent than Irinotecan (1-3). Irinotecan has broad antitumour properties and clinical trials have shown that this drug is effective against lung, colorectal, cervical and ovarian carcinomas (4-7). It has also been reported that Irinotecan and SN-38 potentiate the cytotoxic effect of irradiation on various carcinoma cell lines under aerobic conditions (8-11).

Hypoxia severely limits the therapeutic efficacy of the irradiation of tumours, including carcinomas of the larynx

and pharynx (12). The identification of drugs, which preferentially act on hypoxic cells and sensitise them to irradiation, is therefore a great challenge. The potential of Irinotecan and its SN-38 metabolite to enhance the radiosensitivity of human oesophageal carcinoma cells under both aerobic and hypoxic conditions was investigated.

Materials and Methods

Drugs. Irinotecan and SN-38, supplied by Rhône Poulenc Rorer (Lyon, France), were solubilised in dimethylsulfoxide (DMSO) at a concentration of 2 mg/ml and were diluted in medium to the required concentration immediately before use.

Culture. The WHO3 human oesophageal carcinoma cell line was provided by Dr A Thornley (Department of Zoology, University of the Witwatersrand, South Africa) and was maintained in a 50:50 v/v mixture of MEM and Hams F12 supplemented with 10% heat inactivated foetal bovine serum (FBS) and 0.1 mg/ml of penicillin and streptomycin in complete medium.

Cytotoxicity assay. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was obtained from Sigma Diagnostics Inc. Chicago, IL, USA. The conditions for the MTT assay (13) were optimised to measure the response of cells to ionising irradiation as described by Price and McMillan (14). The cells were seeded at 3×10^3 /well in 96-well microtiter plates in a final volume of 200 μ l complete medium in the presence or absence of the drugs. The appropriate solvent control systems were included. After incubation at 37°C for 7 days, 20 μ l of a 5 mg/ml solution of MTT were added to each well and the plates were incubated for 4 h at 37°C. The cells were then washed with phosphate-buffered saline and the intracellular formasan crystals were solubilised with DMSO and the MTT reduction product was measured at 540 nm in a plate reader (13).

Irradiation and drug treatment. Irradiation was performed at room temperature using an 8 MV photon beam. The cell cultures were plated in 5 ml glass tubes at densities of 600 cells per tube and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO₂. Solutions of either Irinotecan (3.1 μ M) or SN-38 (0.046 nM and 0.092 nM) were added to the tubes before radiation. At these drug

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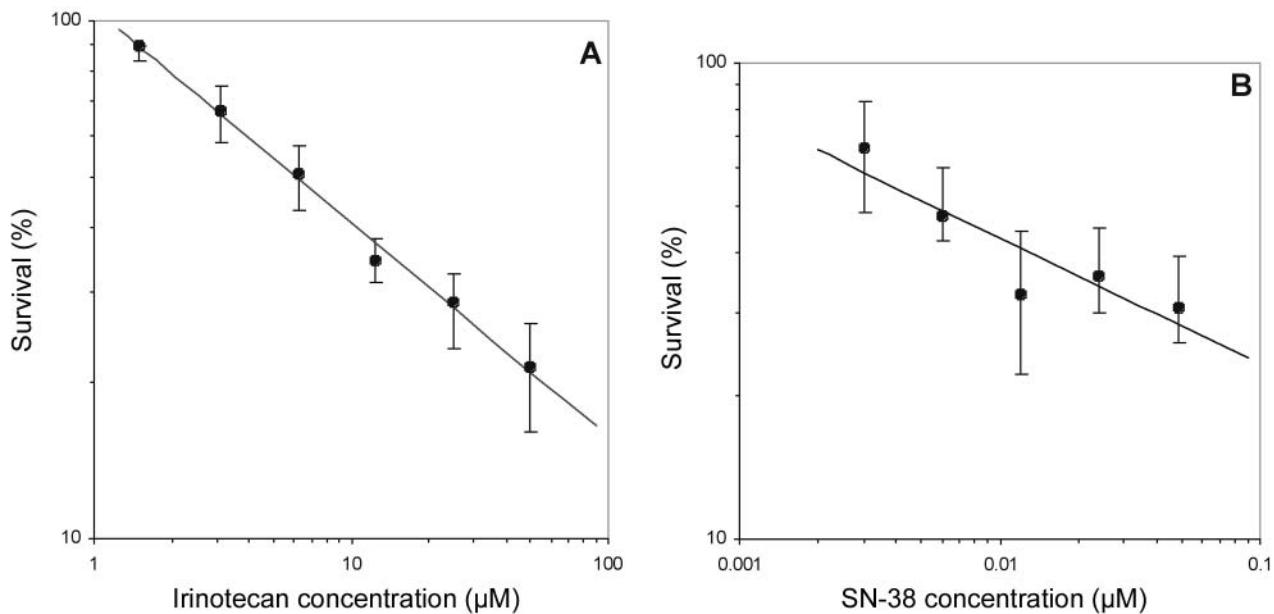


Figure 1. Survival curves of WHO3 cells after a 6-day exposure to various concentrations of Irinotecan (A) and SN-38 (B). Each point represents the mean of 3 experiments with the standard error. From these plots, the IC_{50} for Irinotecan and SN-38 were 0.8 and 0.004 μ M, respectively.

concentrations and in the absence of irradiation, there was 75% cell survival. The tubes were then placed into a modular incubator chamber (Billups-Rothenburg Inc., Del Mar, CA, USA) with a wax build-up equivalent to 2 cm tissue (8 MV d_{max}) cast into the bottom of the chamber. The chamber was placed on a shaker incubator for 30 min, continuously passing either a mixture of 5% CO₂/95% air or 5% CO₂/95% N₂ through before sealing. The cells were then incubated for an additional 1 h at 37°C to deplete residual O₂ by cellular respiration, before irradiation with doses of 1-10 Gy at a dose rate of 1 Gy/min. Immediately after irradiation, the cell cultures were placed in an incubator at 37°C and given air/ 5% CO₂ for an additional 4 h after which the medium containing the drugs was replaced with complete medium and the tubes incubated for 6 days. The cell survival was assessed using the MTT assay as described above (14). The appropriate solvent control systems were included.

Data analysis. The cell growth fraction (S), relative to untreated control samples was plotted against the irradiation dose (D) and data were fitted to a linear-quadratic equation as $\log_e = -AxD - BxD^2 - C$, where A and B are inactivation constants (15). When one of these parameters was found to be negative, it was set at zero and the other parameter re-estimated. The overall cellular responses were expressed in terms of the mean inactivation dose calculated from the respective inactivation parameters (15). This parameter quantifies radiosensitivity as a 2-dimensional parameter which is proportional to the area under the dose-response curve. As the mean inactivation dose is determined predominantly by the response of cells within the first decade of cell survival, it is particularly suitable for use with the current set of data where cell growth in most measurements was not less than 10% of control samples. Furthermore, the ratio of mean inactivation doses obtained with the MTT assay yields dose modifying factors similar to those obtained using conventional clonogenic assays (19).

Results

Cytotoxicity. The cell growth curves of the WHO3 monolayer cultures served to analyse the effect of Irinotecan or SN-38, when present for 6 days under hypoxic conditions as compared to untreated controls. It can be seen that 1-10 μ M Irinotecan produces a marked decline of cell growth (Figure 1A) and that SN-38 is much more toxic and requires only 0.001 to 0.01 μ M to inflict a marked decline of growth in these cells (Figure 1B).

Irradiation and drug effect. Hypoxic conditions effectively reduce the sensitivity of WHO3 cells to irradiation (Figure 2). The mean oxygen enhancement ratio for this cell system was found to be 2.1 (Table I), confirming the marked resistance of the cells to irradiation in the absence of oxygen (16, 21).

Under hypoxic conditions, SN-38 emerged as a typical radiosensitiser, as shown by the increase in the radiosensitivity of WHO3 cells (Figure 3). In the case of Irinotecan, an increase of radiosensitivity was also noted but the effect was small and additive (Figure 4). Additive cytotoxicity was also observed when the cells were exposed to the combination of radiation and either Irinotecan or SN-38 under aerobic conditions (results not shown). Interestingly, the mean inactivation dose obtained under hypoxic conditions in the presence of 0.092 nM SN-38 was lower than the mean inactivation dose under aerobic conditions (Table I). The dose-modifying factor(s) for

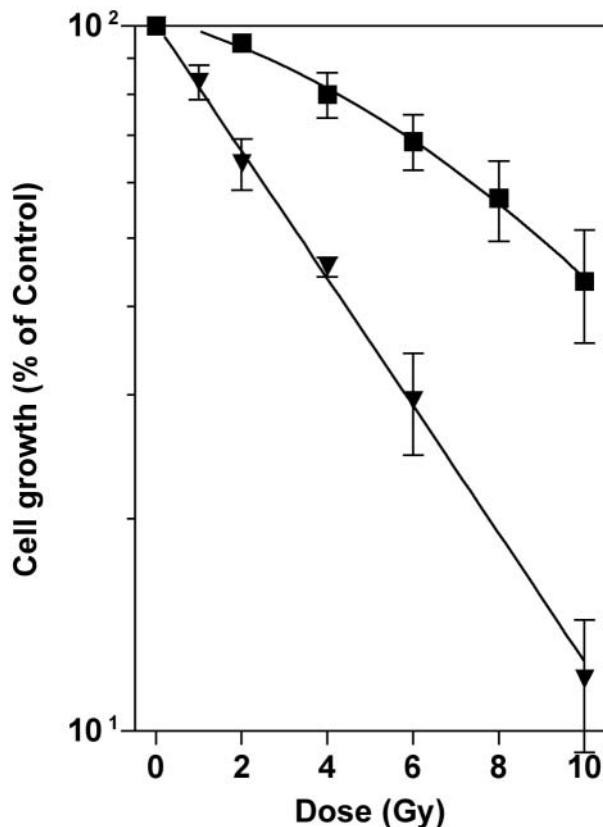


Figure 2. Growth fractions of WHO3 cells following irradiation under aerobic (▼) and hypoxic (■) conditions.

Irinotecan at 3.1 μ M was 1.5 and for SN-38 at 0.046 and 0.092 nM were 1.6 and 2.1, respectively (Table I).

Discussion

Over the past 20 years there has been considerable interest in developing effective hypoxic radiosensitisers, which are not toxic but which can sensitise hypoxic cells at the low irradiation dose of 2 Gy used in radiotherapy. The assessment of the effectiveness of an anoxic radiosensitiser by *in vitro* experimentation is not straightforward and various procedures have been utilised to generate a truly hypoxic atmosphere (17, 18). These methods are time-consuming and not suitable for high output. In the present study, we made use of a modular incubator chamber in which a wax build-up equivalent to 2 cm of soft tissue was cast. Ten test tubes containing monolayers of proliferating cells were grouped together for each radiation dose. By this procedure we were able to perform various drug treatments in one experiment (16). The control oxygen enhancement ratio (OER) value of 2.1 is in close agreement with that obtained by other investigators using therapeutic irradiation

Table I. Influence of Irinotecan and SN-38 on the radiosensitivity of WHO-3 human esophageal carcinoma cells to X-rays expressed as the mean inactivation dose \bar{D}

Conditions	\bar{D}	DMF
aerobic	5.81	-
hypoxic	9.18	1.60 - 2.10
hypoxic + IT ¹	6.70	1.37
hypoxic + SN-38 ²	7.60	1.20
hypoxic + SN-38 ³	6.50	1.40

DMF (Dose Modification Factor) = \bar{D} RT alone / \bar{D} RT + drug

¹0.31 μ M

²0.046 μ M

³0.092 μ M

doses (19). Very recent data have shown that the wide variation of OER values obtained in different cell systems may be due to the fidelity of repair pathways and that OER values considerably lower than 2.7 are obtained when homologous recombination (HR) repair is inhibited (26). Agents which reduce anoxic radioreistance thus may influence the fidelity of DNA repair. It is possible that the 2 topoisomerase inhibitors which are known to reduce religation of topological DNS single strand breaks after replication (28) display such additional specific inhibitory effect of HR repair. We have shown elsewhere that the well known radiosensitiser Pentoxifylline has emerged as an effective inhibitor of HR repair (27). The cytotoxicity and radiosensitising potentials of the topoisomerase I inhibitors Irinotecan and SN-38 described here for WHO3 human oesophageal carcinoma cells are clinically interesting in that the toxicity of SN-38 was found to be 600 times greater than that of Irinotecan. This is in agreement with Kawato *et al.*, (1) who reported SN-38 to be between 200 and 1000 times more potent than Irinotecan.

The new and important result of the present research is that sub-cytotoxic concentrations of SN-38 sensitise proliferating WHO3 cells to irradiation under hypoxic conditions. This is consistent with the findings of Omuru *et al.* (8) and of Falk and Smith (20) who reported that the radiosensitising effects of camptothecin and SN-38 are restricted to G₀ phase cells and are ineffective against rapidly proliferating tumour populations. Under aerobic conditions, we found the cytotoxicity to be additive for both drugs. When the effect of SN-38 alone and irradiation alone were normalised, we found that the resultant toxicity was considerably larger, suggesting a synergistic effect and hence displaying the properties of a true radiosensitiser. The capacity of Irinotecan or SN-38 to sensitise tumour cells under hypoxic conditions has, to our knowledge, not previously been described. As far as cell death mechanisms are concerned it has been shown that Irinotecan abolishes

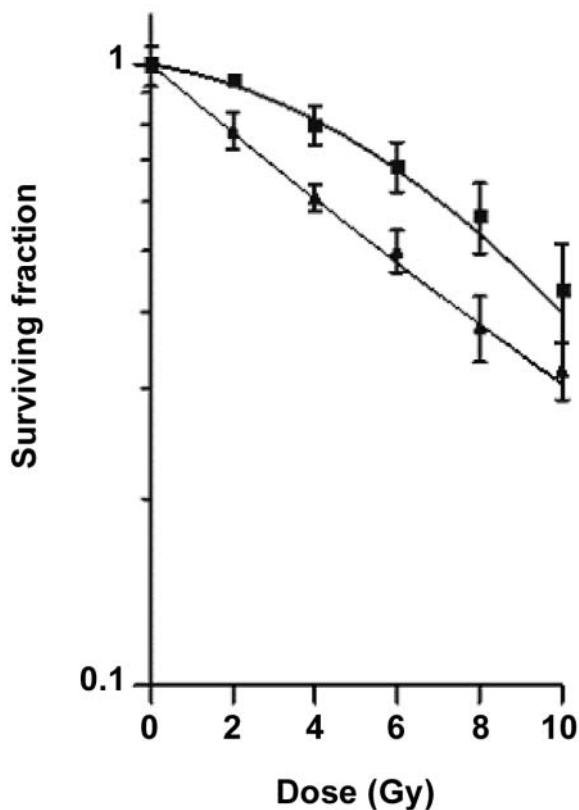


Figure 3. Survival of WHO3 cells following irradiation under hypoxic conditions in the presence of $3.1 \mu\text{M}$ Irinotecan (\blacktriangledown). The control irradiation response under hypoxia (\blacksquare) is shown for comparison. Each point represents the mean of 3 experiments with the standard error.

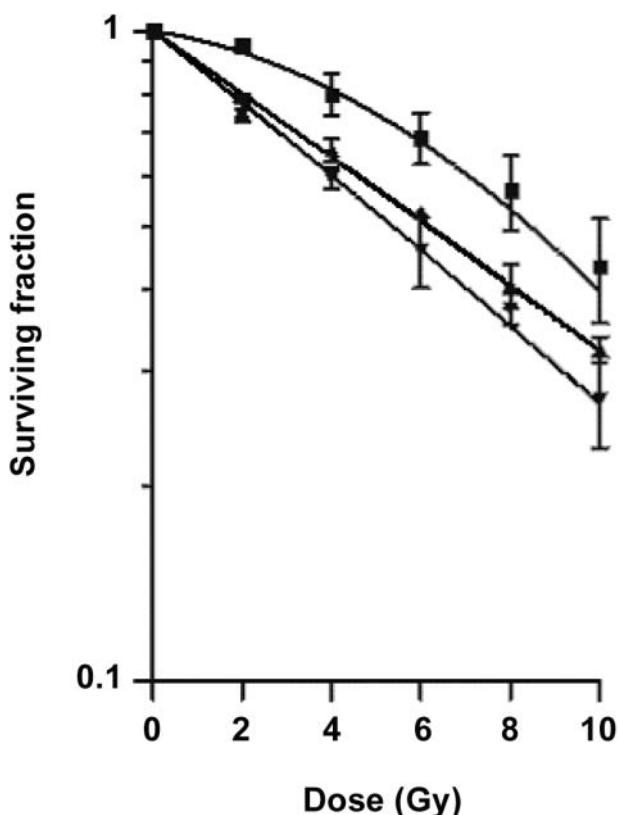


Figure 4. Survival of WHO3 cells following irradiation under hypoxic conditions in the presence of 0.045 nM SN-38 (\blacktriangledown) and 0.092 nM SN-38 (\bullet). The control irradiation response under hypoxia (\blacksquare) is shown for comparison. Each point represents the mean of 3 experiments with the standard error.

the resistance of LNCaP human prostate tumour cells to the apoptosis inducers Apo-L and TRAIL (22). But apoptotic propensity is not a reliable indicator of the effectiveness of an anticancer drug. We have demonstrated that some highly toxic drugs do not initiate apoptosis but rather activate the necrotic pathway directly (23). Indeed, there is now increasing evidence that factors which modulate apoptosis bear no relationship to reproductive cell death (23-25).

In conclusion our results suggest that Irinotecan and particularly the SN-38 metabolite may be clinically useful for the irradiation treatment of notoriously hypoxic tumours, such as cancer of the oesophagus.

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