Evaluation of the efficiency of chemoradiotherapy *in vitro* and *in vivo*

Ph.D. Thesis

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PUBLICATIONS OF THE AUTHOR RELETED TO THE THESIS

I. **Nagy B**, Mucsi I , Molnár J, Thurzó L: Combined effect of Cisplatin and 5-Fluorouracil with irradiation on tumor cells *in vitro. Anticancer Research* 22:135-138, 2002

II. **Nagy B**, Tiszlavicz L, Eller J, Molnár J, Thurzó L: Ki-67, Cyclin D1, p53 and Bcl-2 expression in advanced head and neck cancer. *In vivo* 17:93-96, 2003

III. **Nagy B**, Mucsi I, Molnár J, Varga A, Thurzó L: Chemosensitizing effect of vitamin C in combination with 5-Fluorouracil in vitro. *In vivo* 17: 289-292, 2003

IV. Molnár J, Molnár A, Mucsi I, Pintér O, **Nagy B**, Varga A, Motohashi N: Reversal od multidrug resistance in mouse lymphoma cells by phenothiazines. *In vivo* 17:145-150, 2003

V. **Nagy B**, Molnár J, Rovó L, Paczona R, Thurzó L: Effective chemoradiotherapy without additive toxicity in locoregionally advanced head and neck cancer. *Anticancer Research* 23: xxx-xxx, 2003

CONTENTS

INTRODUCTION

”Nil nocere!” – this is the basic principle in medical science. However, in oncology or radiotherapy this aim is very difficult to achieve because of the harmful adverse effects of the medicines and methods applied. It is essential therefore to seek an optimum balance between the effectiveness and tolerability of these treatment modes.

1. 1. General introduction

A combination of chemotherapy and radiotherapy was applied first in the 1960s by Heidelberg, who examined the effects of 5-fluorouracil (5-FU) in combination with radiotherapy in several tumor localizations (1). There have recently been an increasing number of studies that have proved the benefit of combination therapy (2). However, the pathomechanism of such combined treatment is not completely clear.

Radiation induces many changes in tumors, and their surroundings, but just why treatment is efficient in one and inefficient in other tumor has not been explained by researchers (3).
On the other hand, the response to chemotherapy is also a complex question. It is necessary to take into account the route of administration, the drug resistance and the drug metabolism in chemotherapy. In the event of the combination of chemotherapy and radiotherapy, their effects may be additive, and interactions may also occur between.
A knowledge of the pathomechanism of such interactions is very important, since this can be utilized to maximize the tumor cell kill and to select cases where combination therapy is expected to be more efficient.

In our experimental study we set out to model the clinical situation (concomitant chemoradiotherapy) in order to demonstrate its efficiency. To potentiate the action of this treatment, we investigated tumor resistance sensitizers in combination with 5-FU chemotherapy in cell cultures. The special feature of this study is that an attempt was made to create a bridge between the clinical and the experimental scientific work, which in most cases run separately.

1. 2.Tumor heterogeneity

Like normal tissues, tumor cell populations are not homogeneous. This phenotypic heterogeneity is a result of at least two major factors. First, the fact that neovascularization often fails to keep abreast of tumor cell population growth means that hypoxic and anoxic regions are created: hypoxia results in little or no cell proliferation, while anoxia leads to necrosis (4).

The second factor contributing to heterogeneity is the ability of tumors to display patterns of differentiation, which resemble the maturation sequences seen in the parent tissue, thereby partially recapitulating the phenotypic diversity of the tissue of origin (5).
Many malignant tumor cells contain an irregular number of chromosomes, and are referred to as aneuploid, aneuploidy being a state where a cell has a DNA content which is an inexact multiple of the normal (diploid) content. Aneuploidy is associated with increased aggressiveness in tumors, and is responsible for the variations in nuclear size and staining seen in many tumors; this is known as nuclear pleomorphism (4).

1. 3. Radioresistance

The radiosensitivity of cells varies considerably as they pass through the cell cycle. Although this has not been studied in a large number of cell lines, there seems to be a general tendency for cells in the S-phase (in particular the latter part of the S-phase) to be the most resistant, and for cells in G2 and mitosis to be the most sensitive. The reason for the resistance in S may be related to the conformation of DNA at that time, while the sensitivity in G2 probably results from the fact that the cells have little time to repair radiation damage before they are called upon to divide.

The biological factors that influence the responses of normal and neoplastic tissues to fractionated radiotherapy are as follows:
Repair: as evidenced by cellular recovery during the few hours after exposure.
Reassortment: a cell-cycle progression effect, otherwise known as redistribution. Cells that survive a first dose of radiation will tend to be in a resistant phase of the cell cycle, and within a few hours they may progress into a more sensitive phase.

Repopulation: during say a 5-7- week course of radiotherapy, the cells that survive irradiation may proliferate and hence cause an increase in the number of cells that must be killed.
Reoxygenation: in tumors, cells that survive a first dose of radiation will tend to be hypoxic, but thereafter their oxygen supply may improve, leading to an increase in radiosensitivity.
Repair and repopulation will tend to make the tissue more resistant to a second dose of radiation, while reassortment and reoxygenation tend to make it more sensitive.

The overall radiosensitivity of the tissue depends on the intrinsic radiosensitivity, which means that certain organs or certain tumors are more radioresponsive than others, this largely being due to differences in radiosensitivity. Thus, for a given fractionation course (or single-dose irradiation) the hemopoetic system exhibits a greater response than the kidney, even allowing for the different timing of the response (6).

1. 4. Drug resistance

Drug resistance is a major cause of chemotherapy failure. Two types of drug resistance can be distinguished: inherent and induced. Induced drug resistance is most apparent when patients who originally exhibited a good response to a first course of chemotherapy display a much reduced or no response to the same therapy when given for recurrent disease. Drug resistance can be induced in cultured tumor cells by repeated treatment for most drug types (7).

Many causes of induced drug resistance have been elucidated, including a reduced drug uptake, decreased activation and increased activation. Some drugs elicit multidrug resistance, in which resistance induced to one drug results in cross-resistance to a group of others. The mechanism usually involves production by the resistant cells of higher levels of a membrane p-glycoprotein which participates in the active efflux of drugs from the cell (8).

1. 5. Multidrug resistance

The effectiveness of chemotherapy is limited by the emergence of multidrug resistance (MDR). MDR is conferred by an eregy-dependent drug efflux pump, P-glycoprotein (170) encoded by the MDR1 gene (5). MDR is associated with P-glycoprotein overexpression and

with reduced drug accumulation secondary to enhanced drug efflux, a process sensitive to metabolic poison. In normal tissues P-glycoprotein is localized on the luminal surfaces of renal tubeles, colon, small intestine, bile canliculi, and vascular epithelia of the brain and spinal cord and is associated with phisiological functions. Pgp is responsible for the transport of toxic compounds into the celebrospinal fluid, bile, urine, etc.

In vitro, the MDR1 gene is frequently amplified or transcriptionally activated, or both, in cell lines selected for high levels of MDR. Additionally, it has been reported that increased P-glycoprotein mRNA may be induced heat shock, heavy metals, cytotoxic drugs, toxic and ablative liver damage, ionizing radiation, and altered expression of the tumor supressor gene, p53, or its mutant forms (9,10).

1. 6. Oxygen effect

Oxygen plays an important role in the radiation response of tumors. The growth of solid tumors requires the induction of a blood supply, a process referred to as angiogenesis. Tumor regions are surrounded by vascular stroma, from which the tumor cells obtain their nutrient and oxygen requirements. As these regions expand, areas of necrosis appear at the center.
Since hypoxic cells are resistant to radiation, their presence in tumors is critical in determining the response of tumors to treatment. At the end of the treatment, the tumor response will be dominated by the hypoxic cell population. However, if reoxygenation occurs between fractions, the radiation killing of initially hypoxic cells will be greater and the hypoxic cells will then have less impact on the response (11).

Hypoxic cells are resistant to chemotherapy as well. Hypoxic cells are often situated far from blood vessels, and may therefore be difficult to kill for two separate reasons: they may be non-cycling and therefore resistant to many drugs, and they may be exposed to lower drug levels than are attained close to blood vessels. Compounds have therefore been developed which are specifically toxic to hypoxic cells. These compounds undergo reduction to toxic products only under hypoxic conditions. There are three major classes of bioreductive drugs: quinones. (e.g. mitomycin C), nitroimidazoles (e.g.misonidazole) (12) and benzotriazine-di-N-oxides, of which tirapazamine (13) is the lead compound. Clinical trials with various of

these compounds are under way. They could be combined with radiation and with drugs which specifically kill the well-oxygenated, cycling cells, from which a therapeutic benefit may be expected (14, 15).

1. 7. Genes associated with tumor response

The most frequently examined genes which are involved in tumor proliferation and cell death are Ki-67, cyclin D1, p53 and bcl-2.

The Ki-67 index and thecyclin D1 rate are related to the tumor proliferation rate. The Ki-67 labeling index assesses the growth fraction of a tissue composed of proliferative and non-proliferative cells. Cyclin D1 is a member of the family of regulatory molecules which act at specific points of the cell cycle and govern the progression of cell replication (16).
P53 is a nuclear-acting growth-suppressing protein product of the p53 gene, a transcription factor for the *Cipl* gene encoding p51, which causes interruption of the cell cycle to facilitate the repair of damaged DNA. Under certain circumstances, p53 induces apoptosis.

Literature data on the p53 status and radiation treatment of head and neck carcinomas have been reported frequently, but the results of the findings are very inconclusive (17). A majority of the studies were not able to identify any correlation between the p53-labeling index and clinical endpoints. Some investigators have suggested that the defects in apoptosis caused by inactivation of the normal p53 function may explain an increased resistance to chemotherapeutic drugs and ionizing radiation (16).

Bcl-2 is the prototype of a family of genes that regulate apoptosis. Apoptosis is considered to be an important component of the tumor response to radiation treatment, although its significance has yet to be established for the major solid cancers. Since the Bcl-2 protein can rescue cells normally destined to die by widely dissimilar stimuli, it must mean that it acts close to the final irreversible step where the various afferent pathways converge and at which the effector processes are activated. The bcl-2 protein probably takes part in an antioxidant pathway, acting as a free radical scavenger (18).

1. 8. Action of radiotherapy

Irradiation of any biological system generates a succession of processes that differ enormously in time-scale. These processes are divided into three phases.

The physical phase consist of the interactions between charged particles and atoms of which the tissue is composed. A high speed electron takes about 10^{-18} seconds to traverse the DNA molecule and about 10^{-14} seconds to pass across a mammalian cell. As it does so, it interacts mainly with orbital electrons, ejecting some of them from their atoms (ionization) and raising others to higher energy levels within an atom or molecule (excitation). If sufficiently energetic, these secondary electrons may excite or ionize other atoms near which they pass, giving rise to a cascade of ionization events.

The chemical phase describes the period in which these damaged atoms and molecules react with other cellular components in rapid chemical reactions. Ionization and excitation lead to the breakage of chemical bonds and the formation of free radicals. These are highly reactive and they engage in a succession of reactions that lead eventually to the restoration of electronic charge equilibrium. Free radical reactions are complete within approximately 1 millisecond of radiation exposure. An important characteristic of the chemical phase is the competition between scavenging reactions, for instance with sulfhydryl compounds that inactivate the free radicals, and fixation reactions that lead to stable chemical changes in biologically-important molecules.

The biological phase includes all subsequent processes. These begin with enzymatic reactions that act on the residual chemical damage. The vast majority of lesions, for instance in DNA, are successfully repaired. Some rare lesions fail to undergo repair and it is these that lead to eventual cell death. Cells take time to die; indeed, after small doses of irradiation they may undergo a number of mitotic divisions before dying. It is the killing of the stem cells and the subsequent loss of the cells that they would have given rise to that causes the early manifestations of normal-tissue damage during the first weeks and months after radiation exposure. A secondary effect of cell killing is compensatory cell proliferation, which occurs both in normal tissues and in tumors. At later times after the irradiation of normal tissues, late

reactions appear. An even later manifestation of radiation damage is the appearance of second tumors (19).

1. 9. Action of chemotherapy

Despite the complexity, the basic principles of chemotherapy are in many respects similar to those of radiotherapy. Important factors such as the limited effectiveness due to cellular resistance, and the limited value of tumor regression as compared with disease-free survival as an indication of tumor response, are common to both therapies.

Cytotoxic drugs can be classified according to their mode of action and their source, and also on the basis of dose-response curves for cell killing. The last classification arose from the concept that tumor cells usually proliferate faster than stem cells in normal tissues. Three classes of cytotoxic agent have been distinguished:

Proliferation non-specific agents (class I.)

The rapidly and slowly proliferating cells are similarly sensitive.

Cell-cycle phase-specific agents (class II.)

Agents that kills cells only in a particular phase of the cell cycle.

Proliferation dependent agents (class III.)

Rapidly proliferating cells are much more sensitive to these agents (e.g. 5-FU)

Table I. Predominant phase of the cell cycle blocking or cell killing

Drug	**Cell killing**	**Progression delay**
Adriamycin	Late S, M	S, G2
Cisplatin	G1, G2	S, G2, G1/S
5-FU	All phases	G1/S1
Hydroxyurea	S	G1/S1
Methotrexate	Early G1, G1/S, S	G1/S1
Mitomycin C	G1, G2, M	S/G2
Etoposide	S, G2	S, G2
Vincristine	S	M
Radiation	G1/S, G2, M	G1, S, G2

This classification is more useful than that of drug type, for it leads to concepts of drug scheduling. For instance, phase-specific agents must be infused or given repeatedly, since single doses will be ineffective because the mitosis of cells is asynchronic. The level of cell killing with these agents depends critically on the treatment duration. The classification also throws light on drug resistance in kinetically heterogeneous tumor cell populations, where slowly-proliferating or resting cells may fail to be killed, since proliferating cells tend to be more sensitive to drug treatment than resting cells (20).

1. 10. Rationale of chemoradiotherapy

The exact mechanism by which chemotherapeutic agents can potentiate the radiation effect is poorly in most cases poorly understood. A wide variety of biological mechanisms have been proposed to explain the interactive processes between radiation and cytotoxic drugs. The biological factors that influence the responses of normal and neoplastic tissues to fractionated radiotherapy are: the repair mechanism, the cell-cycle, the cell-proliferation rate, the oxygen supply and the intrinsic radiosensitivity of the tumors (3).

Many drugs have the property of inhibiting the repair of radiation damage (21). Consequently, there is an attractive possibility of complementary action between drugs and radiation. Many cytotoxic drugs exhibit some degree of selectivity in killing cells in a certain phase of the cell cycle. Proliferating cells have generally been shown to be more sensitive to chemotherapy than non-proliferating cells (2). Radiation is also cycle-dependent, and the effects of irradiation may therefore be modified by applying chemotherapeutic agents (23). Positive effects have been found in cell cultures of fast-growing experimental tumors, but with slowly-growing or resting cells, synchronization therapy has been disappointing.

There is a strong rationale for the concurrent administration of radiation and chemotherapy. Potential interactions between the two modalities include the targeting of different cell subpopulations within the tumor, and the chemotherapy-associated inhibition of the repair of DNA damage induced by radiation. Moreover, a cell cycle redistribution may take place, resulting in an increase in the fraction of cells in radiosensitive phases of the cycle (23).

1. 11. Chemoradiotherapy in head and neck cancer

The accurence of head and neck cancer increasing with an annual death of approximately 1400 cases in Hungary (24). In general, a majority of these patients present with locoregionally advanced disease. For these patients, surgery and radiation have been traditionally used in sequence. Despite this agressive bimodality treatment approach, cure is achieved in only a minority of the patients. Most patients die of locoregional persistence or recurrence of the disease. The addition of chemotherapy to the overall treatment plan has been studied intensively during the past 30 years. Research strategies have mainly included the use of induction or adjuvant chemotherapy, as well as concomitant chemoradiotherapy. Given the anatomic location of the tumor, the surgery and the preservation of the organ function is an important second treatment goal.

Recently reported randomized trials conducted to study the role of chemotherapy have yielded the consistent finding that the concurrent administration of chemotherapy (5-FU with or without cisplatin) and radiotherapy results in improved locoregional control and overall survival as compared with radiotherapy alone (25–30). Conversely, sequential (usually neoadjuvant) chemotherapy has yielded only a marginal, non-significant benefit (29).

In these studies, different chemotherapy regimens were applied, either as single agents or in combination, and the results virtually uniformly supported the superiority of chemoradiotherapy over radiotherapy alone in terms of both locoregional control and overall survival. Given the toxicity of concurrent chemoradiotherapy, the careful selection of patients is critical.

In clinical practice, high-dose chemotherapy or combination regimens may be difficult to tolerate. Only two-thirds of patients receive all planned cycles of chemotherapy (2). Lower doses of chemotherapy in combination with radiotherapy may therefore be appropriate, especially for patients with a compromised performance status or with comorbidities.

AIMS OF THE THESIS

Concomitant chemoradiotherapy is a new approach in cancer therapy. Randomized studies prove its superiority, but it has higher toxicity. Careful selection of the patients is essential to avoid fatal side-effect. We achieved our first experience with advanced head and neck patients, who suffered not only the local toxicity of the radiotherapy, but also the systemic side-effects of the chemotherapy. Additionally we observed that the condition of most of these patients was too poor for them to tolerate this treatment.
Accordingly, we designed a study with a view to understanding the pathomechnism of the combined treatment in order to establish the optimum rationale schedule of chemoradiotherapy.

We planned our examinations as follows:

1. To avoid unnecessary toxicity caused by chemoradiotherapy, it is important to identify patients who have no chance of benefiting from this approach. Many genes are involved in chemotherapy and radiotherapy sensitivity. In our study, we aimed to examine Ki-67, cyclin D1, p53 and bcl-2 oncoprotein overexpression retrospectively in advanced head and neck cancer patients in order to determine relationship of its value and the tumor response to radiotherapy.

2. When cytotoxic drugs are administered with radiation therapy, it may be supposed that the two modalities interact in aa asynergistic or supra-additive way. In reality, the combined therapy can worsen the results if the chemoradiotherapy has severe additive toxicity. To analyze this interaction, we decided to model the effects of two chemotherapeutic modalities commonly used in clinical practice, i.e.5-FU, and cisplatin in combination with radiation on HEp-2 and, mouse lymphoma cell line.

3. The effectiveness of chemotherapy is limited by the emergence of multidrug tesistance. With regard to possible targets on MDR and Pgp a study was initiated to see the action of anticancer agent (5-FU and cisplatin) on MDR efflux pump.

4. There is abundant evidence that hypoxic areas within solid tumors present a barrier to effective therapy. An approach to an improvement of the effectiveness of standard therapy and circumvention of the tumor resistance brought on by hypoxia is represented by the bioreductive hypoxic-specific cytostatics. One representative such agent is tirapazamine, which has been extensively investigated in the literature and in clinical studies. We examined its antiproliferative effect in combination with 5-FU in order to compare its activity that of with vitamin C, which exhibited a chemosensitizing effect *in vitro* studies.

5. To test our theory stemming from the results of our preclinical study, we designed an investigation in advanced head and neck cancer patients, in which we focused on the locoregional control and on an assessment of acute toxicity of this combined treatment.

MATERIALS AND METHODS

3. 1. Chemicals

5-FU (Fluorouracil inj., Sigma, Ebewe, Unterach, Austria), cisplatin (Platidiam inj. La Chema, A.S., Brno, Czech Republic), vitamin C (Vitamin C inj. 10%, EGIS Pharma Rt, Budapest, Hungary), Ftorafur (Tegafur caps, Grindex, Riga, Latvia), 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MD, USA) and sodium dodecylsulfate (SDS) (Sigma Chemical Co., St. Louis, MO, USA) were used in our experiments. Rhodamine 123 (R123) and colchicine were obtained from Sigma Chemical Co., (St. Louis, MO, USA)

3. 2. Cell cultures

The L5178Y mouse T lymphoma (parent) cell line was grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum. The HEp-2 (human epidermoid carcinoma) cell line was cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum. MRC-5 (normal lung fibroblast from human fetus) cells were cultured in RPMI 1640 medium supplemented with 10% heat-activated fetal bovine serum.

3. 3. Cell proliferation and cytotoxic assay

The antiproliferative effect and cytotoxic activity of the chemotherapeutic agents alone and in combination were tested by the MTT method. For cell proliferation assay, the drugs were diluted with culture medium in 96-well flat-bottomed culture plates in a 100 µl volume per well; 100 µl (or 50 µl) of cell suspension was added to the wells, with the exception of the medium controls. 1x104 cells /well for the mouse lymphoma (parent) cells and for the HEp-2 cells, and 1.5x104 cells /well for the MRC-5 cells were used in the cell proliferation

assays. The culture plates were further incubated for 72 hours. The drug interactions were studied in combination with checkerboard methods.

In this case, the different dilutions of drug A were made in 3-12 rows in a volume of 50 μl per well and each dilution was combined with 50 μl of drug B in decreasing concentrations in the columns from A to H. The concentrations of drug A decreased from left to right and for drug B from up to down from A to H. The cell suspension was then added to the wells a volume of 50 (or100) μl containing the above-mentioned cell number. In the cell proliferation experiments, the cultures were further incubated for 48-72 hours, and at the end of the circulation period 10 μl of MTT solution (from a 5 mg/ml stock solution was added to each well (100 μl of medium). After incubation at 37 ^{o}C for 4 hours, 100 μl of SDS solution (10%) was added to each well and the plates were further incubated overnight at 37 ^{o}C.

The inhibitory effect on the cell proliferation was determined by measuring the optical density (OD). The absorbance was recorded at 540 nm; the reference wavelength was 630 nm; a Multiscan EX reader was used for evaluation. The average OD values of parallel wells of each sample and the controls were calculated. The percentage inhibitory effect or cytotoxic activity was determined according to the formula:

$$100 - \left[\frac{\text{OD sample - OD medium control}}{\text{OD cell control - OD medium control}} \right] \times 100$$

3. 4. Assay of cytotoxicity:

Wells containing 4x10^{4} mouse lymphoma cells and the monolayer cultures of adherent cell-lines (HEp-2 and MRC-5) were treated with 100 ul of medium containing the compounds at different concentrations for 24 hours. The cell viability was tested by the MTT assay and the cytotoxicity was evaluated as described above.

3. 5. Fluorescence uptake assay.

The L5178Y mouse T cell lymphoma cell line was infected with the pHa MDR1/A retrovirus. For MDR1 expression, the cells were selected by culturing the infected cells in 60 ng/ml colchicine-containing media. The L5178Y MDR cells and L5178Y parental cells were grown in McCoy's 5A medium supplemented with 10 % heat-inactivated horse serum, glutamine and antibiotics. The cells were adjusted to a density of 2 $x10^6$ /ml, resuspended in serum-free McCoy's 5A medium and then distributed as 0.5 ml aliquots in Eppendorf centrifuge tubes. The test compounds were added to the cells in quantities from 1.0 to 10 µl of the 1.0 mg/ml stock solutions and the samples were incubated for 10 minutes at room temperature. Then, 10 µl of the indicator R123 (5.2 µM final concentration) was added to the samples and the cells were incubated for a further 20 minutes at 37 ^oC. The cells were washed twice and resuspended in 0.5 ml phosphate-fuffered saline for analysis. The fluorenscence of the cell population was measured by flow cytometry with the Beckton Dickinson FACScan instrument.

3. 6. Histological analysis

The tumor samples were fixed in neutral 10% formalin, dehydrated and embedded in paraffin. 4 µ tissue sections stained with hematoxylin-eosin were prepared for routine histological examination.

Automated immunohistochemistry (Dako Techmate ^{TM} 500 Plus, Dako, Glostrup, Denmark) was carried out according to the streptavidin-biotin-peroxidase technique. The sections were incubated with commercial primary monoclonal antibody against p53 oncoprotein (Clone DO7, 1:100), bcl-2 oncoprotein (Clone 127, 1:200), cyclin D1 (Clone DCS 6,1:80), Ki-67 (1:1000) from Dako (Glostrup, Denmark) and MDR (Clone JSB1, 1:50) from Biogenex. Appropriate positive and negative controls were included in each run.

3. 7. Irradiation

Experimental study

We irradiated the cell cultures in microplates by means cobalt 1.25 MV machine.

Clinical study

Study I (Radiotherapy alone)

We administered a standard dose of 70 Gy of conventional fractionated radiotherapy. Radiotherapy was delivered with a cobalt 1.25 MV machine, using a three-field technique with two lateral coaxial fields, including the primary and upper neck nodes and an anterior lower neck and supraclavicular field . All patients received 50 Gy to this lower volume following a 2 Gy daily dose schedule. The spinal cord was protected after 40 Gy. The total dose to the primary site and metastatic lymph nodes was 66-70 Gy.

Study II (Chemoradiotherapy)

Chemotherapy (Ftorafur) was administered every day throughout the radiotherapy in an oral dose of 30 mg/m^2 2 times per day.

3. 8. Patient selection

To be considered eligible for this study, patients had to meet the following criteria: stage III or IV (International Union Against Cancer [UICC] criteria) squamous cell unresectable carcinoma of the oral cavity, oropharnx, hypopharynx or larynx or carcinoma of unknown origin with cervical metastatic nodes.

Patient group I: Between 1998 and 2001, 33 patients (2 females and 31 males) were observed. The primary aim of the study was to evaluate the radiosensitivity of the tumors in accordance with the genetic alterations.

Patient group II: Between November 2000 and January 2003, 50 patients (13 females and 37 males) were enrolled into the study. The main goal of the study was to evaluate the remission rate and toxicity of chemoradiotherapy. The patients were stratified according their performance state (Eastern Cooperative Oncology Group [ECOG]): groups 0-2, and 3-4.

3. 9. Statistical analysis

For the comparision of mean values, the *t*-test (31, 32, 33) and one-way analysis of variance were used, together with the Mann-Whitney and Kruskal-Wallis tests (31, 32, 33) in cases of non-normality. The normal distribution of the samples was tested by using Kolmogorov-Smirnov test (31). The Spearman correlation coefficient (31, 32, 33) was applied to assess correlations between continuous variables. Survival curves were constructed by the Kaplan-Meier method (32, 33) and compared by using the long-rank test. The dependence of the survival curves on the predicting factors was analyzed by means of Cox regression (proportional hazard model) (32, 33). The cut-off values for the categorization of the predicting factors were chosen so as to ensure the appropriate case numbers for the statistical procedures. To evaluate the possible correlation between the condition of the patients and the tumor response, we calculated Tschuprow coefficient (34).

RESULTS

4. 1. Evaluation of predictive factors

In order to evaluate the value of the most commonly examined genetic alterations (Ki-67, cyclin D1, p53 and bcl-2 overexpression) in predicting the radioresponsiveness, we designed a study to determine the most important pathways responsible for the complex event of tumor response to irradiation and survival.

33 patients with advanced head and neck cancer were analyzed as concerns the remission rate and survival The results indicate that there is no correlation between the tumor response and the examined predicting factors (Table II).

Table II. Comparison of predicting factor levels for survival and remission

Factor	Survival time (months)†				Remission (%)			
	Mean	S.E.	n (cens.)	p	Mean	S.E.	n	p
Ki-67				0.636				0.477
0-30%	10.5	2.4	11 (1)		53.2	9.5	11	
31-60%	14.5	3.0	9 (0)		65.0	8.2	9	
61-90%	13.1	2.9	13 (2)		67.6	9.0	11	
Cyclin-D1				0.279				0.121
–	13.8	1.9	20 (3)		68.2	6.4	19	
+	11.1	2.9	13 (0)		51.6	8.2	12	
p53				0.765				0.821
–	12.0	2.4	10 (1)		60.0	8.1	10	
+	13.5	2.3	23 (2)		62.6	6.7	21	
bcl-2				0.024				0.054
–	10.8	1.5	29 (2)		57.9	5.5	27	
+	27.5	5.2	4 (1)		87.5	7.5	4	
Total	13.2	1.8	33(3)		61.7	5.2	31	

Survival curves were constructed by the Kaplan-Meier method and ccompared by using long-rank test. Analyzing the effects of all four predicting factors, only the bcl-2 levels resulted in a significant difference (Table II).The estimated survival curves for the bcl-2-positive and negative groups clearly indicates a better survival for the bcl-2-positive group (Fig. 1), but the survival curve for this group is less reliable because of the small number of bcl-2-positive cases. Although the correlation is significant, more data acquisition is required for a more precise comparison.

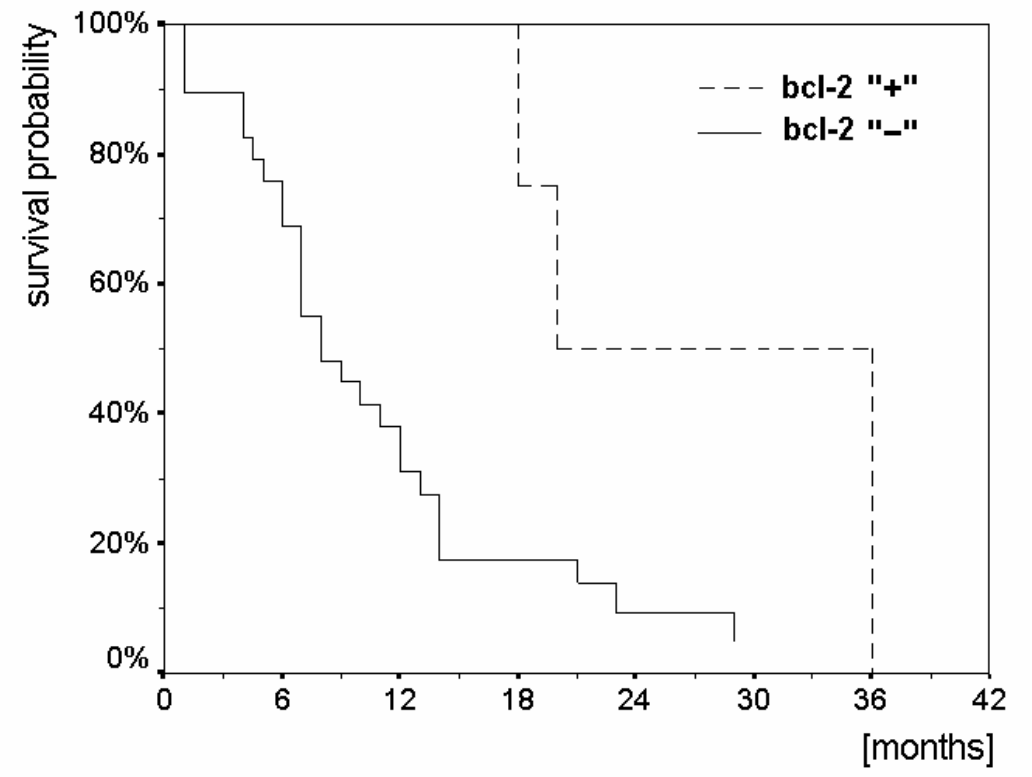

Figure 1. Overall survival of advanced head and neck patients according to bcl-2 overexpression.

4. 2. Preclinical results with a combination of irradiation and chemotherapy

To analyze the interaction of chemotherapy and radiotherapy, we decided to model the effects of two chemotherapeutic modalities commonly used in clinical practice, i.e. 5-FU, and cisplatin, in combination with radiation on HEp-2 and mouse lymphoma parent (PAR) cell lines.. Before the combination of irradiation with cisplatin and 5-FU, the optimum dose of irradiation was determined separately on mouse lymphoma and HEp-2 cells, and was found to be 6 Gy and 30 Gy, respectively.

We found that in the case of HEp-2 cells even extremely high doses of irradiation hardly affected the growth rate of the HEp2 cells.However in the case of mouse lymphoma cells relatively low dose of radiation achieved high tumor growth inhibition(Fig. 2.3). Based our results we considered the mouse lymphoma cells as radiosensitive, while HEp-2 cells as radioresistant cell line.

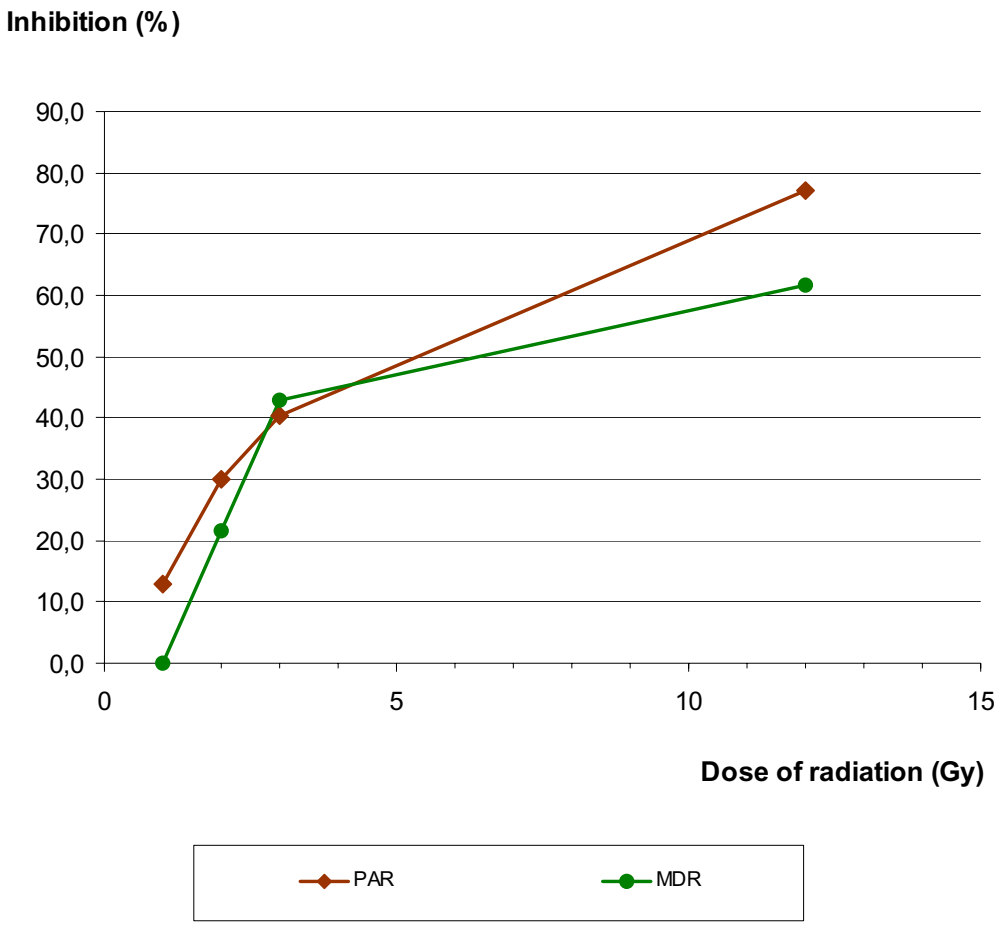

Figure 2. Dose-dependent cytotoxic effect of irradiation on mouse lymphoma cells .

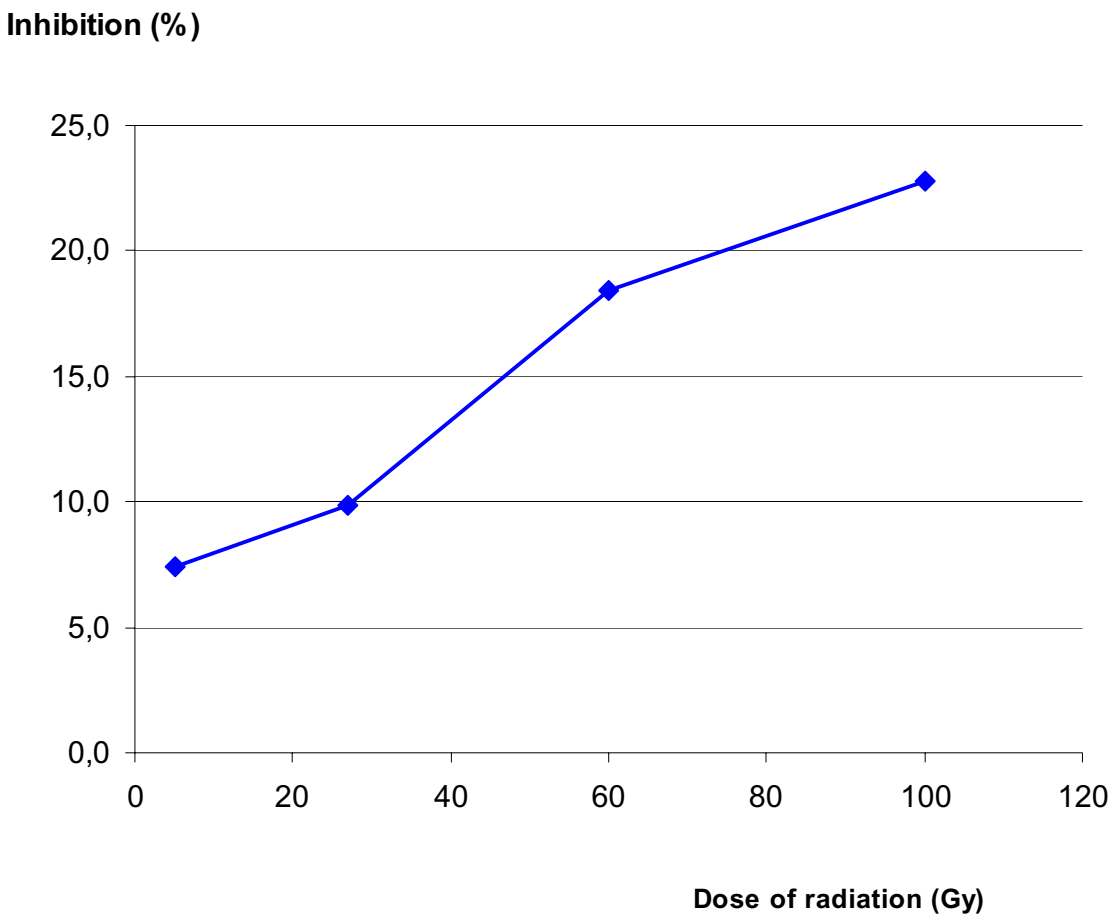

Figure 3. Dose dependent cytotoxic effect of irradiation on HEp-2 cells.

Combined effect of chemotherapy (Cisplatin and 5-FU) and radiation was examined on both cell lines (mouse lympoma PAR and HEp-2 cells).

Cisplatin alone exerted a dose-dependent inhibitory effect on the cells. When the optimum dose of irradiation was combined with various concentrations of cisplatin, a noteworthy, synergistic increase in growth inhibition was found in the case of the drug-sensitive PAR cells at a low level of cisplatin (fig.4.)

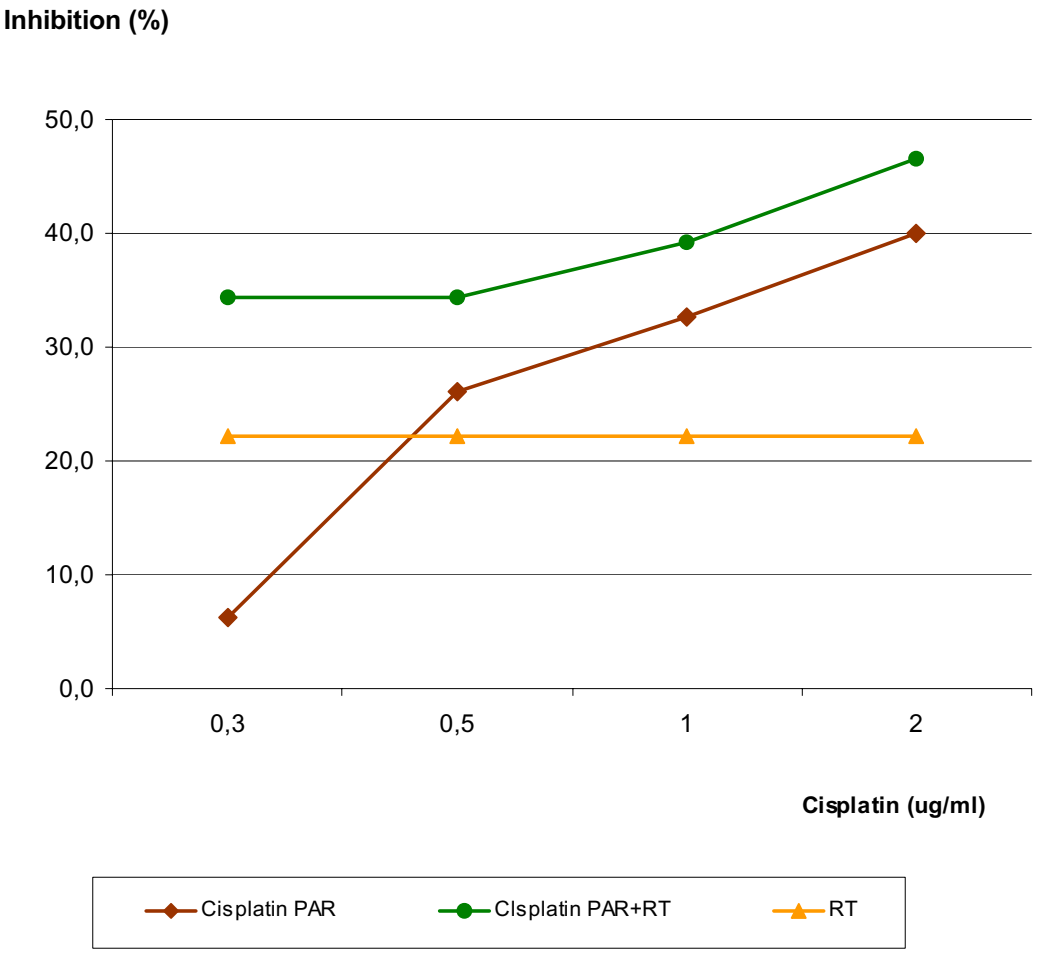

Figure 4. The effect of irradiation on the dose-dependent growth inhibition of Cisplatin in mouse lymphoma (PAR) cells.
RT= radiotherapy

We also examined the combined effect of 5-FU and irradiation.

The effect of the combination of 5-FU and irradiation on the mouse lymphoma PAR cells was dose-dependent, and the additive effect of radiation and 5-FU was similarly more relevant in the lower 5-FU concentration range than at higher concentrations (Fig.5).

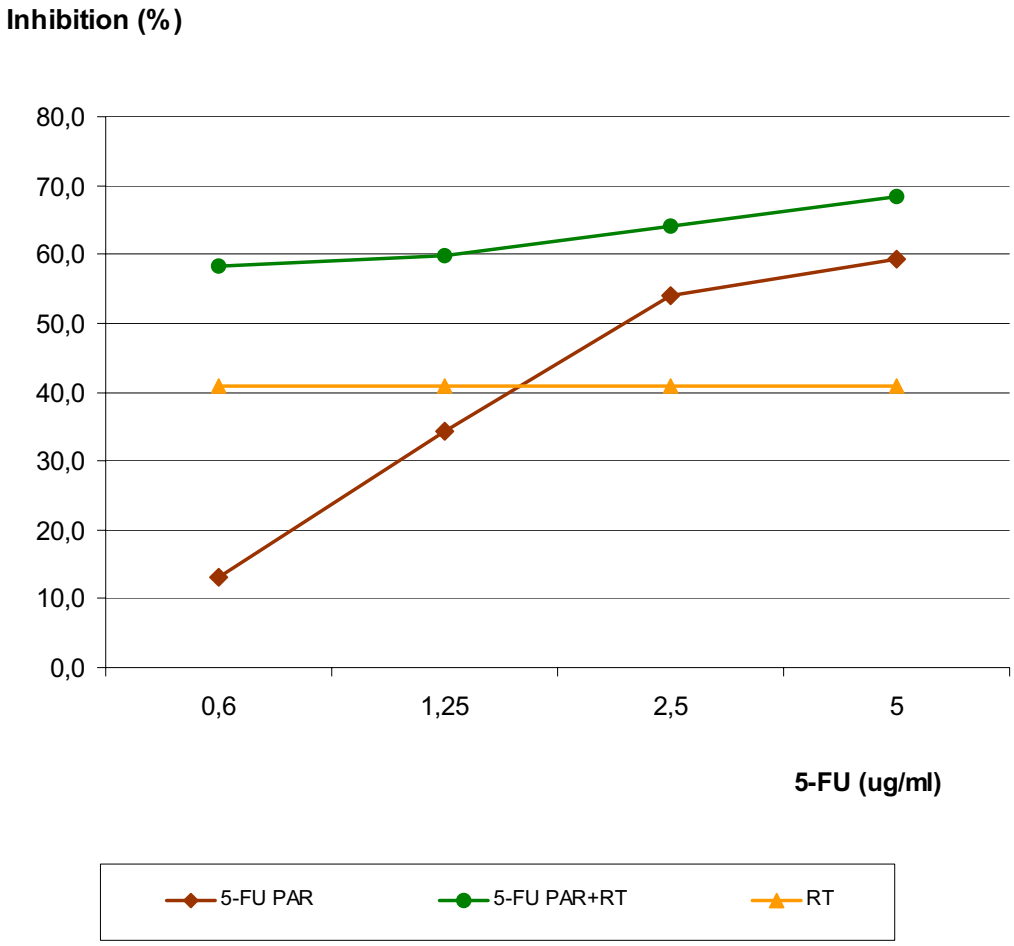

Figure 5. The effect of 5-FU combined with 6 Gy irradiation on the growth inhibition of sensitive (PAR) mouse lymphoma cells.

As regards the HEp-2 cells, only high doses of chemotherapy influenced the inhibition of tumor cell growth. In addition, increasing doses of chemotherapy (cisplatin and 5-FU) in combination with irradiation did not change the effect of irradiation (Fig.6.7)

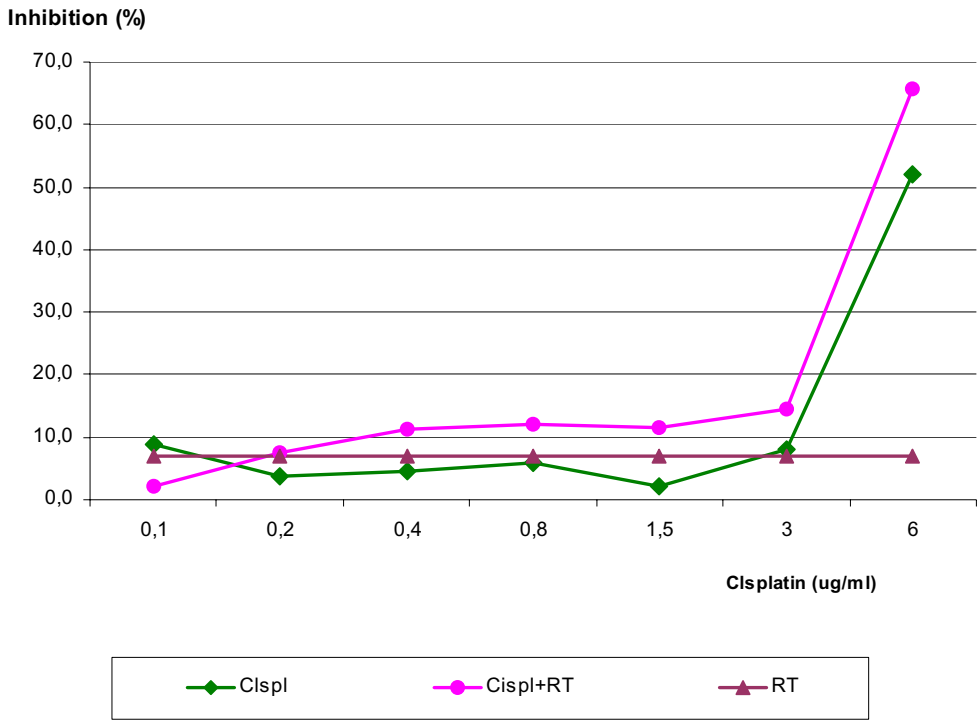

Figure 6. The effects of cisplatin in combination with radiation (30 Gy) on the growth rate of the HEp-2 cell line.

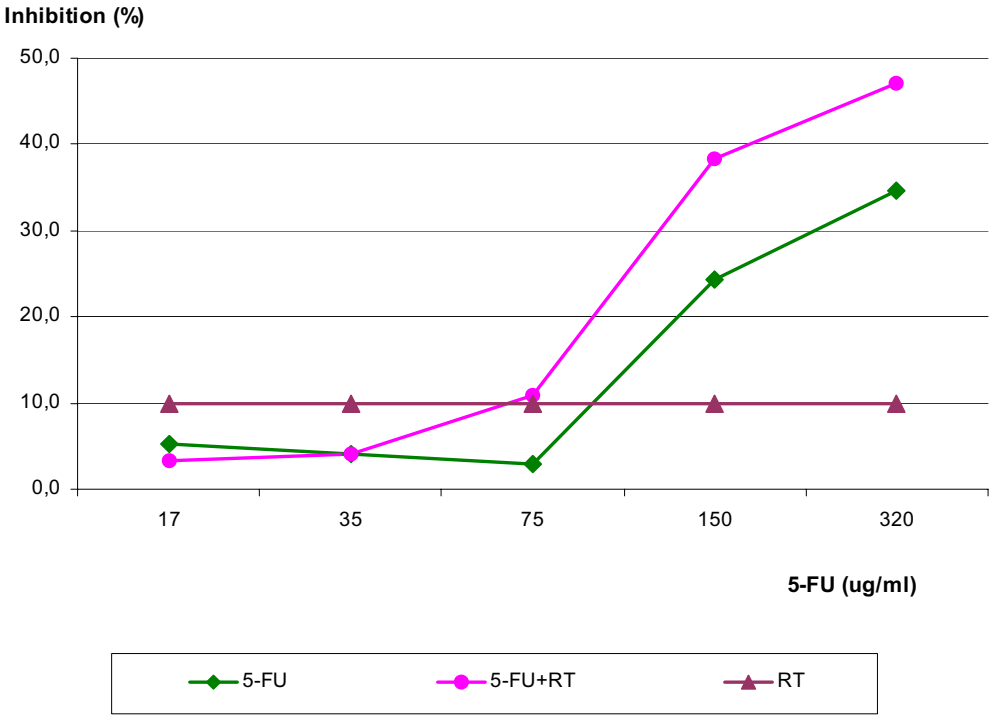

Figure 7. The effects of 5-FU in combination with radiation (30Gy) on the growth rate of the HEp-2 cell line.

4. 3. MDR reversal effect of Cisplatin and 5-FU

Seeking for the key of overcoming tumor resistance, we firstly analyzed the effect of chemotherapy (Cisplatin and 5-FU) on the MDR efflux pump. In a control experiment we found that the MDR reversal effect of the two tested drug did not change the accumulation of the human MDR1 infected mouse lymphoma cells (Table III).

Table III. The effect of cisplatin and 5-Fluorouracil on Rhodamine 123 accumulation by MDR infected mouse lymphoma cells.

Components	**Concentration** (μg/ml)	**Fluorescence Activity ratio**
Cisplatin	2.0	0.53
	20.0	0.60
Fluorouracil	2.0	0.47
	20.0	0.73
Control	-	1.0

In a control experi…t. MDR reversal of teste a cytosta…

4. 4. Preclinical results with tumor response modifier drugs in combination with 5-FU

Since our previous preclinical studies had demonstrated that neither chemotherapy alone nor combination of chemotherapy with radiation did not overcome the tumor cell resistance we decided to test new agents, easy to apply clinically in combination with the commonly used chemotherapy (5-FU). Accordingly, we examined the effectiveness of a supplementary drug modifying tumor hypoxia. One of the most promising and well-known such agents is tirapazamine. We examined this in combination in mouse lymphoma and HEp-2 cell cultures.

A synergistic interaction of tirapazamine in combination with 5-FU was observed for the antiproliferative effect both in the mouse lymphoma cells and in the HEp-2 cell line (Fig.8,9). However, the antiproliferative effect of 5-FU potentiated by tirapazamine was higher in the case of the mouse lymphoma cell line, than in the case of the radioresistant HEp-2 cells.

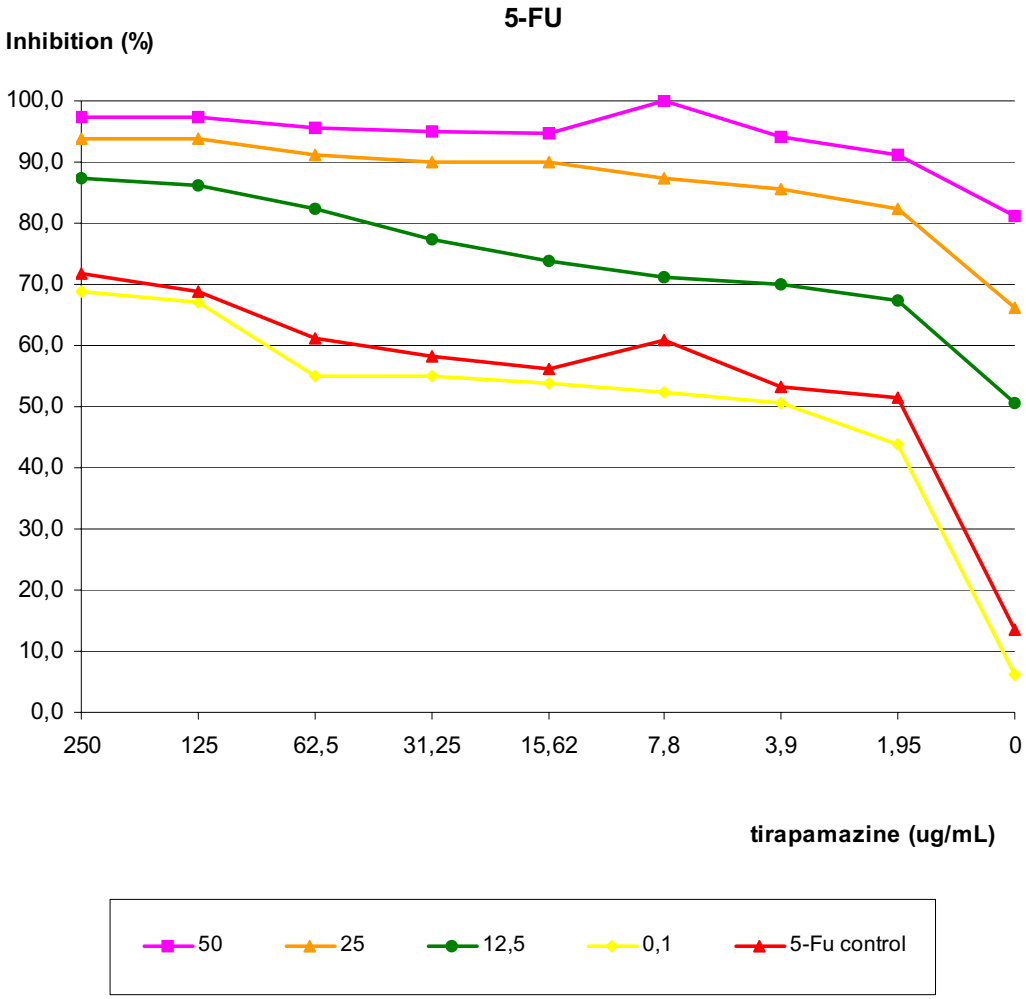

Figure 8. Antiproliferative effect of tirapazamine in combination with 5-FU on HEp-2 cells

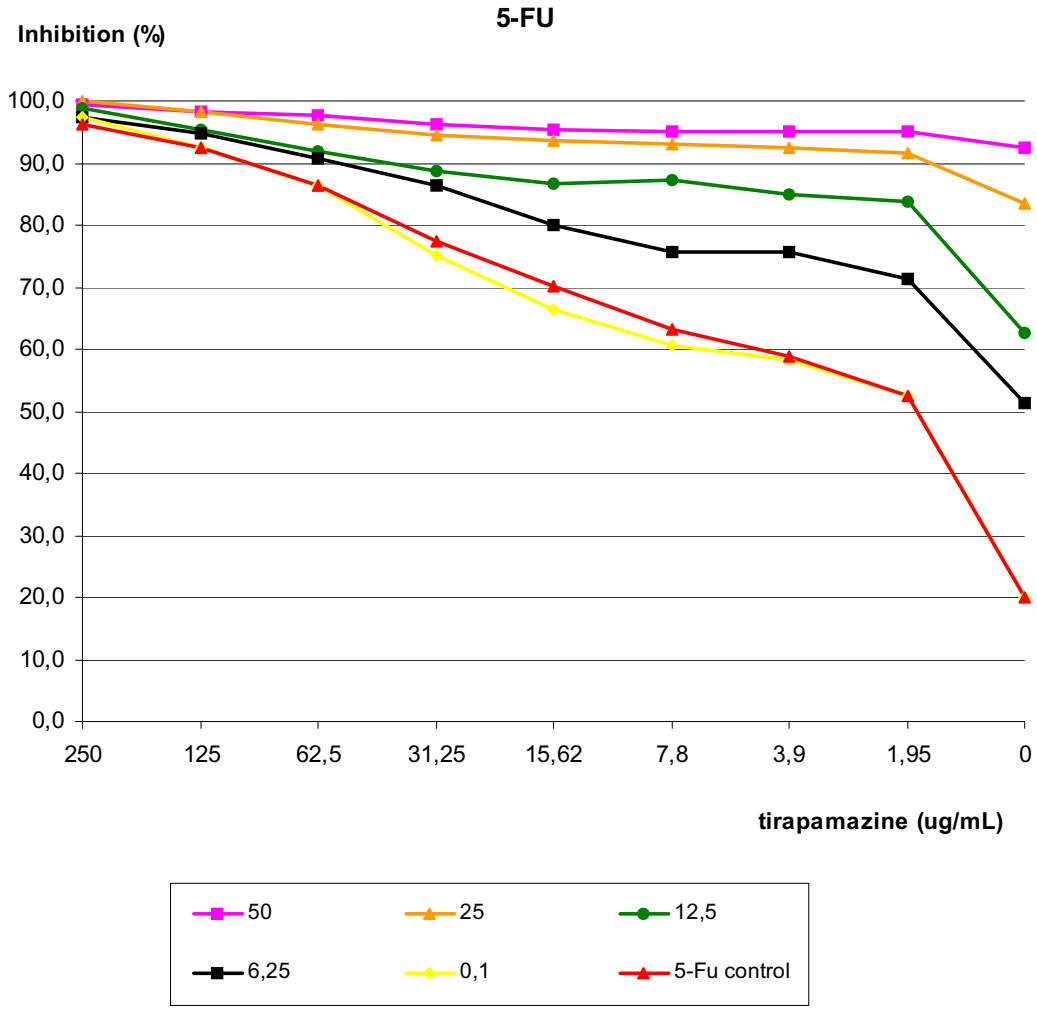

Figure 9. Antiproliferative effect of tirapazamine in combination with 5-FU on mouse lymphoma PAR cells

In a search for a clinically available agent that can be administered orally we found that vitamin C may act as a chemomodulator, potentiating the cytotoxic activities of various chemotherapeutic agents.

We therefore investigated the antiproliferative effects of vitamin C combined with 5-FU on the radiosensitive mouse lymphoma, the radioresistant HEp-2 and the normal fibroblast (MRC-5) cell line *in vitro*.

We found that different doses of vitamin C in combination with 5-FU chemotherapy had no effect on the normal fibroblast (MRC-5)cells (Fig.10.).

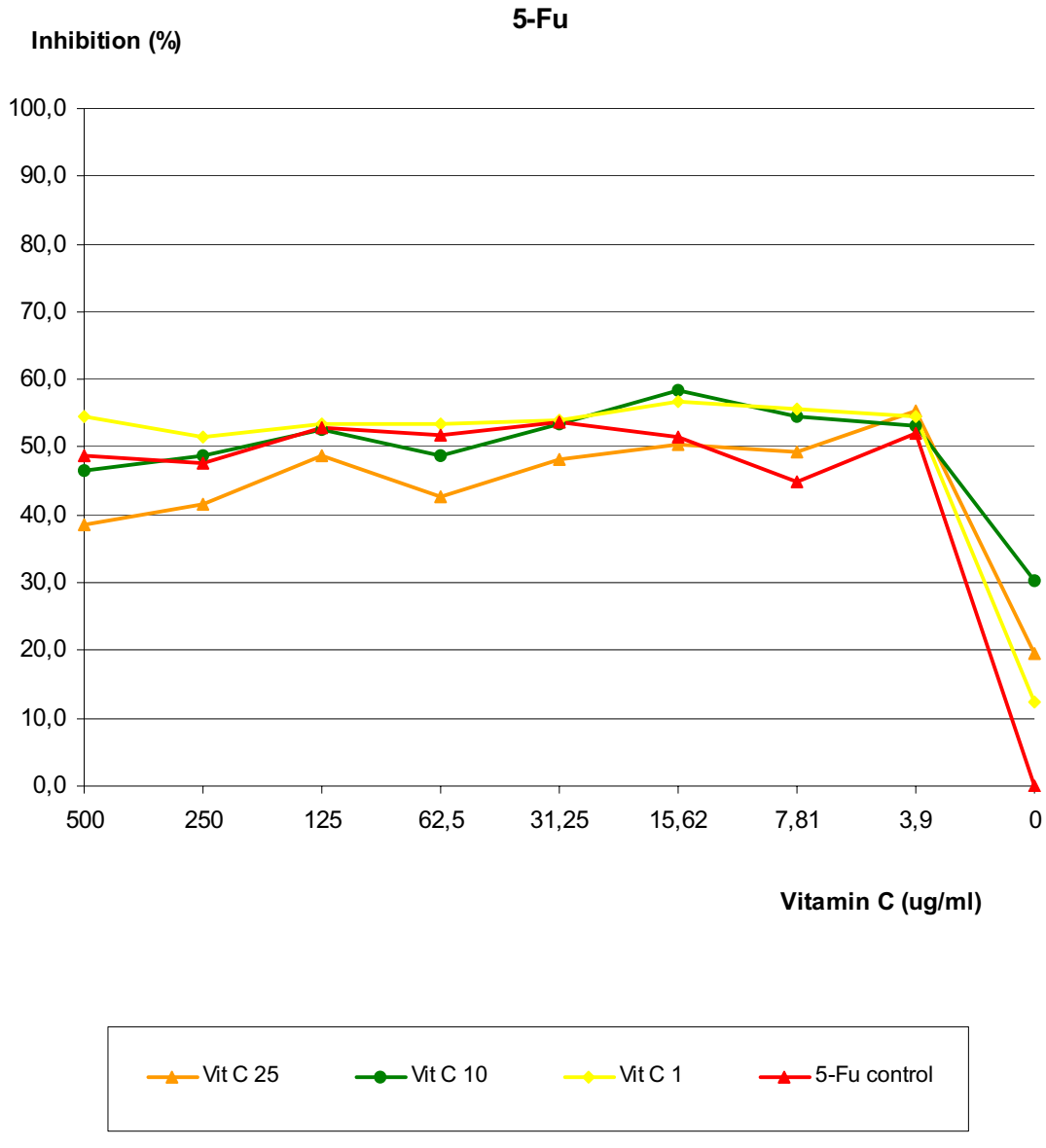

Figure 9. Antiproliferative effect of 5-FU in combination with vitamin C at different concentrations on normal fibroblast cells.

On the other hand we found, that vitamin C increased the anticancer effect of 5-FU in a dose-dependent manner. In the case of mouse lymphoma cell line 5-FU in combination with vitamin C proved to have an increased antiproliferative effect relative to that of 5-FU alone only when the vitamin C concentration was above 5 ug/ml. A lower dose did not modify the antiproliferative effect of 5-FU (Fig.11.).

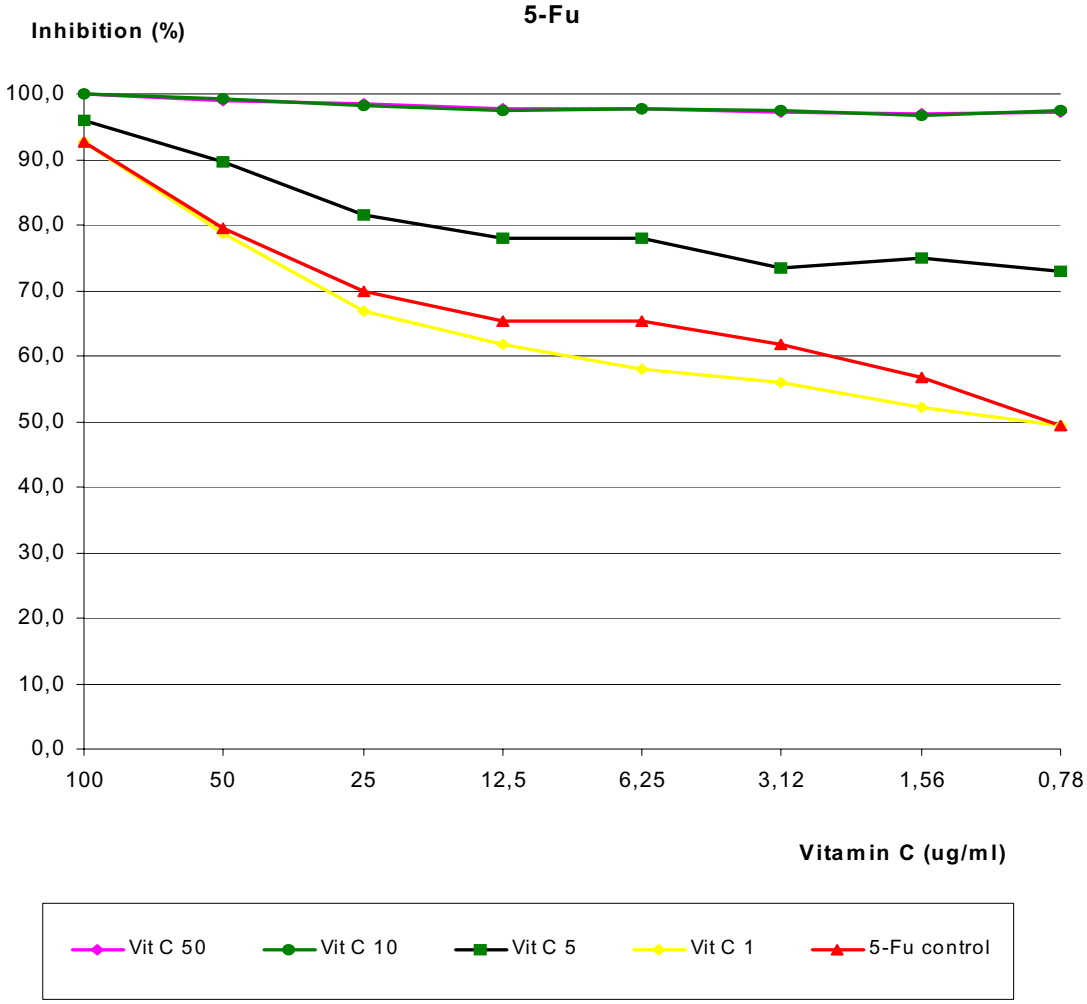

Figure 11. Antiproliferative effect of 5-FU in combination with vitamin C at different concentrations on mouse lymphoma cells.

For the radioresistant cell line HEp-2, low doses were ineffective, but high doses (higher than 50 µg/ml) of vitamin C markedly enhanced the antiproliferative effect of 5-FU (Fig. 12.)

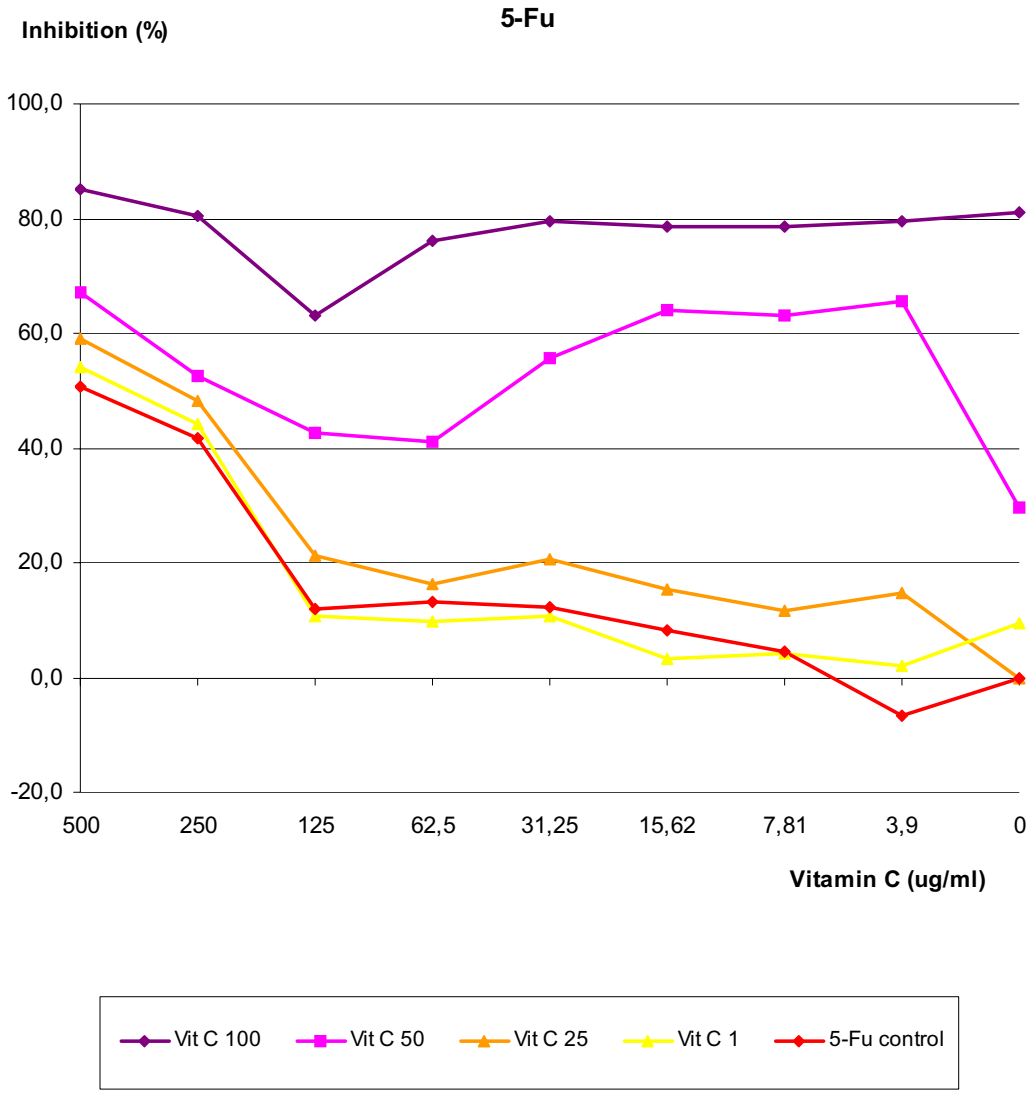

Figure 12. Antiproliferative effect of 5-FU in combination with vitamin C at different concentrations on HEp-2 cells.

4. 5. Clinical results in patients treated with chemoradiotherapy

On the supposition that concomitant chemoradiotherapy is superior to radiotherapy alone, we set out to attempt to find a simple form tolerable for all patients. Since our previous

preclinical work had revealed that low-dose, but not high-dose chemotherapy had a synergetic radiosenzitizing effect, we designed a study involving an orally administered well-tolerable drug (Ftorafur) in order to establish the treatment outcome in two patient groups: with good (ECOG 0-2) or poor (ECOG 3-4) performance states. Since the survival in these patient groups depended on many other factors, such as the nutrition, the patient's life style, the alcohol- drinking habits, the immune status etc., we focused our investigation only on the local control of the treatment which correlated best with symptom relief.

Response

The overall response ű rate for the 50 patients was 94% (95% confidence interval [CI] 88 to 99). A complete response was achieved in 60% (95%, [CI] 48 to 72) (Table IV).

Table IV. Tumor response according to performance state.

Performance state	**Response**			**Total**
	CR	**PR**	**SD**	
ECOG 0-2	20	11	0	31
ECOG 3-4	10	6	3	19
Total	30	17	3	50

The numbers indicate the numbers of patients

CR: complete response, PR: partial response, SD: stable disease

None of the patient characteristics proved to have an impact on the rate of response. According to Tschuprow coefficient [T] indicated that, the performance state (T=0.27), the age (T= 0.178) and the body weight (T=0.153) had hardly any effect on the tumor response. However, it can be seen that the performance state was the strongest, and the body weight was the weakest determining factor (Table V).

Table V. Correlation between the patient's condition and the tumor response.

Conditions	Tschuprow coefficient (T)
Performance state	0.270
Age	0.178
Body weight	0.153

T< 0.3 represents a weak correlation

Acute toxicity

The 50 patients were evaluated for acute toxicity in 2 groups (good and poor performance status) (Table VI). The hematologic toxicity was grade 3 in only 1 case in the good performance status group, and in 3 cases in the poor performance status group. The same patients exhibited gastrointestinal toxicity of grade 2-3. All the symptoms could be quickly eliminated by hydration and careful supportive care. Febrile neutropenia and sepsis were not experienced, but 1 patient died soon after the completion of the therapy, from tumor bleeding.

Mucositis was the dominant toxicity; it occurred in grade 2 in 44% of the patients, and in grade 3 in 12%. Skin toxicity was moderate: only 9 (18%) of the patients developed a moist desquamation. Loss of >10% of the initial body weight occurred in 6 patients (12%).

Table VI. Acute toxicity (WHO grade) according to performance state.

Performance state	Toxicity/grade			
	0 - 1	2	3	4
ECOG 0-2	12	14	4	0
ECOG 3-4	8	8	4	0

The numbers indicate the numbers of patients.

The data show that most of the patients, irrespective of their performance state, had grade 0-2 acute toxicity.

DISCUSSION

5. 1. Importance of predictive factors

A number of clinical parameters, i.e. the TNM (tumor, node, metastasis) staging system, the site of the primary tumor, the performance status and biological factors (markers of cell kinetics and apoptosis, etc.) were analyzed for clinical significance as predictive factors.

Our study, in contrast with an other study (35), did not indicate that the Ki-67 labeling index was a predictor of local control or survival. We observed that most of our patients had a high rate of tumor cell proliferation, but the levels did not display any prognostic impact on the locoregional control or overall survival. Accordingly, the proliferation parameters are only part of a spectrum of potential predictors of the tumor response, including hypoxia, intrinsic cellular radiosensitivity and other genetic mutations.

Our study did not reveal a correlation between cyclin D1 overexpression and the local control or survival after radiotherapy. This is in contrast with a report that cyclin D1-positive patients had a lower survival rate than cyclin D1-negative ones (36).

In patients with squamous cell carcinoma of the head and neck, the prognostic value of p53 is still controversial (37). Our data did not confirm that an abnormal p53 protein level is of predictive value in terms of locoregional control or overall survival.

The only examined factor which displayed significant corrrelation with the survival in our study, was bcl-2 in our study. It has recently been reported that bcl-2 appears to be a new class of prognostic indicator which, although a marker of advanced disease, defines a relatively small population (12.8 %) of such head and neck cases that have a favorable outcome independently of the treatment (38).

Our results demonstrated that the examined molecular biological markers did not allow the selection of patients who would respond worse or better to radiotherapy. We consider that tumor progression is a complex event with many forms of mutations, which cannot be

characterized by one or several genetic alterations. In conclusion, we believe that the bcl-2 protein is a fascinating molecule which may play a role in the therapeutic strategy for cancer in the near future.

5. 2. Preclinical study with combined chemoradiotherapy

Our experimental data suggested that radiochemotherapy, within the combination of the two different cytotoxic effects, is promising in radiosensitive cells, since extremely low concentrations of cytostatics are able to enhance the growth inhibition of irradiation, while in radioresistant cells, the combination of high-dose irradiation with high-dose cytostatics could result in a synergistic effect.

We assume that concomitant chemoradiotherapy can be more effective than either single-treatment modality in the case of radiosensitive tumors, if continuous low-dose chemotherapy (cisplatin and 5-FU) and radiation are applied simultaneously. The continuous use of chemotherapeutic agents might serve to rescue the optimum cell cycle synchronization and consequently cell populations more sensitive to radiation. In the case of chemoradioresistant tumors, only very high doses are effective where the induced damage is nonrepairable. The effectiveness of high doses of radiation and high doses of chemotherapy in the event of therapy resistance can be explained by the higher rate of lethal cell damage, which is unrepairable (33). In this case the benefit of combined therapy is less, since fewer repairable lesions and fewer surviving clones exist. On the other hand, at higher doses the repair system is depleted, since the pool of repair enzymes is used up during repair and is less able to repair all the induced damage (39).

Many drugs have the property of inhibiting the repair of radiation (1). If it is assumed that interaction between chemotherapy and radiation is based on the inhibition of radiation-induced damage by chemotherapy, our result seems to support the hypothesis that increasing doses of chemotherapy saturate the repair system, and that accordingly, beyond that saturating dose of chemotherapy, there is no additive effect of a higher level of chemoradiotherapy.

In conclusion, we consider that the protracted administration of low-dose chemotherapy in combination with radiotherapy is a useful method. By this approach, we can enhance the effect of the radiotherapy of sensitive tumors but not cause additive toxicity in resistant cases.

If we could determine the radio- or chemosensitivity of the tumor prior to therapy, we could select the patient who would benefit from low-dose concomitant chemoradiotherapy and who might be treated alone by a higher dose of radiation without general toxicity.

5. 3. Effect of Cisplatin and 5-FU on the MDR efflux pump

The majority of anticancer drug (like Cisplatin and 5-FU) and intervention (like ionizing radiation) do not inhibit MDR but may induce the expression of the MDR1 gene ®. Taking into account these findings combination of chemotherapy and radiotherapy could not overcome MDR resistance, but may induce further expression of MDR1 gene. Therefore new targets and treatment strategies (combination anticancer treatments with P-glycoprotein decreasing agents) should be find to overcome tumorresistance.

5. 4. Chemosensitizing effect of tirapazamine and vitamin C

Our preclinical results proved that a combination of chemotherapy with irradiation could not overcome tumor resistance, although the precise mechanisms of radioresistance and chemoresistance are unknown (3). However it is assumed that one of the common causes of radio- and chemoresistance might be insufficient cell oxygenization. Nutritionally deprived hypoxic tumor cells are resistant to conventional chemotherapy or radiotherapy in consequence of their non-cycling status (G0 phase), limited drug diffusion, and limited oxygen perfusion (39, 40, 41). Treatment with these agents alone leaves the hypoxic regions of tumors unharmed. Viable hypoxic cells that survive cytotoxic therapy may result in cell proliferation and tumor regrowth, and also in mutation and the development of resistance.Agents which selectively target hypoxic cells may enhance the antitumor efficacy of chemotherapy and radiotherapy.

One such agent is tirapazamine. It causes DNA double-strand breaks in hypoxic cells by producing active tirapazamine radicals. Since tirapazamine acts primarily in hypoxic regions of tumors, additive tumor cell killing was expected from its combination with chemotherapeutic agents (cisplatin, melphalan, cyclophosphamide, etc).

The antitumor activity of tirapazamine has been attributed to its ability to damage DNA. Specifically, the damage is attributed to a free radical which is formed enzymatically via a one-electron reduction of tirapazamine. In the presence of oxygen, the free radical is spontaneously oxidized back to the parent drug. However, in poorly oxygenated (hypoxic) cells, back-oxidation is limited, allowing the free radical to damage cellular components, including DNA. The mechanism by which it produces DNA damage (single- and double-strand breaks) is not known with certainty, but it has been attributed to a direct interaction with DNA or to hydroxyl radicals which can be formed from the nitrogen-centered radical under hypoxic conditions. It has also been suggested that the nitrogen-centered radical can donate an electron to the DNA, forming a carbon-centered DNA radical which accepts oxygen from tirapazamine, resulting in cleavage of the DNA. The extent of DNA damage has been linked to the rate of strand break repair, which seems to be slower under hypoxic conditions, possibly explaining the greater sensitivity of hypoxic cells to the cytotoxic effects of tirapazamine (42, 43, 44, 45).

In our study, we found an enhancment of the antiproliferative effect of 5-FU chemotherapy in chemosensitive mouse lymphoma and chemoresistant HEp-2 cells; lower doses of tirapazamine were enough to achieve the same antiproliferative effect in mouse lymphoma cells. The results clearly indicate that in the case of the chemoresistant cell line (HEp-2) the administration of an agent able to reduce tumor hypoxia increases the effect of 5-FU chemotherapy.

Our experiment confirms the hypothesis that hypoxia is one of the major causes of tumor resistance, since administration of an agent that decreases tumor hypoxia, such as tirapazamine, effectively increased the antitumor effect of chemotherapy in the chemo- and radioresistant cell line (HEp-2) too. Further clinical studies should evaluate its efficacy and toxicity.

Similarly to the result of the experiments with tirapazamine, we found significant enhancements of the antiproliferative effect of vitamin C in combination with 5-FU chemotherapy in mouse lymphoma and HEp-2 cell cultures. As in the case of tirapazamine, lower doses of vitamin C could achieve the same antitumor effect in mouse lymphoma cells as high doses of vitamin C in combination with 5-FU chemotherapy in the chemo- and radioresistant HEp-2 cell line. Assuming that vitamin C can operate via electron transfer, we explain the results in terms of the oxidizing effect of vitamin C.

The results of *in vitro* studies demonstrate that vitamin C acts as a pro-oxidant and has antitumor activity (46, 47). The mechanism responsible for its antitumor activity appears to be related to the levels of L-ascorbate and its oxidative product dehydroascorbate, which generates intracellular hydrogen peroxid and other reactive oxygen species, which may deplete cellular thiol levels and initiate membrane lipid peroxidation (47). However, the study by De-Laurenzi et al. (49) suggests that the antitumor activity of vitamin C against neuroectodermal cancer cells may be related to the recycling of ascorbate by elevated levels of NADH-dependent semi-dehydroascorbate reductase, which leads to a pro-oxidant activation of the programmed cell death pathway (47, 49). In this process, ascorbate is transformed to dehydroascorbate, a potentially toxic product, which generates oxidative stress and triggers apoptosis (50). The pro-oxidant effect of vitamin C and the potentiation of cell death induced by free radicals seem to involve the production of hydrogen peroxide (51).

Nevertheless, cancerous cells appear to exhibit abnormal levels of several antioxidant molecules, and altered levels and activities of a variety of antioxidant enzymes (52). For example, vitamin C accumulates in some solid tumors at concentrations higher than those in the surrounding normal tissues; this appears to be selective for cancer cells, probably because of the deficiency of cancer cells in the enzymatic defense system against the toxic action of oxy radicals (53, 54). Whether the changes in antioxidant defense mechanisms observed in cancerous tissues play a role or not in carcinogenesis or are a result of the disease is not known.

Our study has demonstrated a noteworthy beneficial antiproliferative effect of vitamin C administration in combination with 5-FU chemotherapy on the chemoresistant cell line.

The effect was achievable only with high doses of vitamin C. For the chemosensitive cell line, even low doses of vitamin C increased the antiproliferative effect of 5-FU, while for the fibroblast cell lines, a harmful effect was not detected.

On the supposition that cancer cells have a defective antioxidant defense system, we consider that, in the event of chemoresistance, an excessive amount of antioxidant is needed to saturate the intracellular antioxidant defense system. As concerns the reason for the differential susceptibility of cell lines to vitamin C-induced cytotoxicity, we think that, since the cytotoxic activity of ascorbate is dependent on its oxidation (49), in the case of hypoxic tumors only a high concentration of vitamin C could inhibit tumor cell proliferation.

Our results suggest that consumption of a non-toxic pro-oxidant supplement such as vitamin C during long-term standard anticancer treatment, e.g. with oral 5-FU, could be counterproductive. A high dose of vitamin C may be useful as a chemosensitizer agent, since an enhanced antiproliferative effect of 5-FU in combination with vitamin C can be achieved in hypoxic, chemoresistant cases too. Incorporation of antioxidants in the treatment of cancer therapy could be meaningful and demands further examinations.

5. 5. Results on patients

Our study showed a high tumor response rate, which is comparable to that in other large studies in respect of locoregional control where radiotherapy was combined with high-dose intensive chemotherapy (55, 56) Apart from this, the treatment was tolerable even for old, or poor-risk patients (Table VI).

We assume that our good results could be explained in a part by the permanent interaction between the radiation and chemotherapy in consequence of the daily oral chemotherapy schedule, and the individually determined tolerable chemotherapy dose. Since the therapy does not involve extra toxicity, the full-dose radiation schedule could be administered without long breaks to almost every patient.

The fact that we did not find a correlation between the patient characteristics and the therapy outcome proves that the tumor response correlates best with the tumor behavior.

Different tumors consist of heterogeneous tumor cell populations in respect of chemosensitivity. Since the chemosensitive tumor population do better after chemotherapy, it would be reasonable to know initially which tumors are chemosensitive. Unfortunately to date have no reliable test to predict the tumor response before therapy. (57). As our preclinical study (3) indicated only low-dose chemotherapy has a synergistic effect on radiotherapy, and the additive effect of chemotherapy is pronounced only in chemosensitive tumors, the administration of a non-toxic dose chemotherapy to these poor-risk patients is the preferred rationale. Moreover, the adverse effect of this procedure is not a limiting factor.

We succeeded in demonstrating the possibility of achieving high antitumor activity with orally administered continuous low-dose chemotherapy in combination with concomitant radiotherapy. The major advantages of this approach are the easy administration, and the tolerability for all patients. Future studies should evaluate the effects of supplementary and maintenance therapy on the survival.

CONCLUSIONS

In order to select patients with radiosensitive tumors, we searched for molecular markers with which to predict the treatment outcome in patients with advanced squamous cell carcinoma of the head and neck. We investigated the effects of chemotherapy (5-fluorouracil and cisplatin) in combination with irradiation in cell cultures (mouse lymphoma and HEp-2 cell line). To improve the effect of chemoradiotherapy, we investigated the use of drugs (tirapazamine and vitamin C) to overcome tumor resistance. These were tested in patients with advanced head and neck cancer.

To summarize our results:

1. The examined predictive factors (Ki-67, cyclin D1, p53 and bcl-2 overexpression) in advanced head and neck cancer point to the high proliferating activity of these tumors, but the heterogeneity of our results do not allow us to conclude that these parameters are of prognostic value. However the high proliferating activity of these tumor should take into account in the treatment strategy (e.g.:starting anticancer treatment as soon as possible, applying hyperfractionated chemoradiotherapy, without long breaks may improve treatment outcome).

2. Our experimental study releated that 5-fluorouracil(5-FU) and cisplatin chemotherapy in combination with radiotherapy show synergistic effect in in case of (mouse lymphoma cell) line, but had no additive effect on HEp-2 (radioresistant) cells.In sensitive cases low dose of chemotherapy was sufficient for sensitisation the radiation effect.

3. Anticancer treatments (chemotherapy and radiation) do not inhibit MDR, but further increase the expression of MDR1 gene. Therefore combination these treatments with P-glycoprotein decreasing agents would be mandatory.

4. Searching for agents to overcome tumor resistance by other pathomechanism than MDR we found that vitamin C enhanced the antiproliferative effect of 5-FU chemotherapy- in vitro in chemomsensitive mouse lymphoma and resistant HEp-2 cell line as well, although only high doses of vitamin C can sensitize the effect of 5-FU chemotherapy in case of

hypoxic, chemoresistant tumor cells (HEp-2). The results were comparable with the antiproliferative effect of the hypoxia-selective agent tirapazamine, suggesting that vitamin C may influence the tumor oxidative status.

5. In our clinical study we found that continuous administration of the oral 5-FU derivate (tegafur) in combination with radiotherapy is efficiant treatment schedule in respect of locoregional control, and has no additive general toxicity.

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ABBREVIATIONS

5-FU	5-Fluorouracil
MDR	multidrug resistance
MTT	3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide
SDS	sodium dodecyl sulfate
OD	optical density
UICC	International Union Against Cancer
PAR	parent cell
RT	radiotherapy
CI	confidence interval
ECOG	Eastern Cooperative Oncology Group
CR	complete response
PR	partial response
SD	stable disease
T	Tschuprow coefficient
WHO	World Health Organization
TNM	tumor, node, metastasis system
R123	Rhodamine 123

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I dedicate my thesis to my patients.