CONCOMITANT TREATMENT OF COLORECTAL CANCER WITH PLATINUM-BASED CHEMOTHERAPY AND RADIATION: STUDIES ON CYTOTOXICITY, PHARMACOKINETICS AND CONCOMITANT IN VITRO AND IN VIVO EFFECTS

By

Thititip TIPPAYAMONTRI

Département de Médecine Nucléaire et Radiobiologie

Thesis submitted to the Faculté de médecine et des sciences de la santé

for the Degree of

Doctor of Philosophy (Ph.D.) in Radiation Sciences and Biomedical Imaging

Sherbrooke, Québec, Canada

August 2013

Doctoral Committee:

Thesis Examiner Prof. Thierry M. Muanza, McGill University
Thesis Examiner Prof. Johannes van Lier, Université de Sherbrooke
Thesis Examiner Prof. David Mathieu, Université de Sherbrooke
Thesis Supervisor Prof. Rami Kotb, Université de Sherbrooke
Thesis Supervisor Prof. Leon Sanche, Université de Sherbrooke
Thesis Supervisor Prof. Benoit Paquette, Université de Sherbrooke
NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.
ABSTRACT

CONCOMITANT TREATMENT OF COLORECTAL CANCER WITH PLATINUM-BASED CHEMOTHERAPY AND RADIATION: STUDIES ON CYTOTOXICITY, PHARMACOKINETICS AND CONCOMITANT IN VITRO AND IN VIVO EFFECTS

By
Thititip Tippayamontri
Department of Nuclear Medicine and Radiobiology

Thesis submitted to the Faculté de médecine et des sciences de la santé for the graduation of philosophiae doctor (Ph.D.) in sciences des radiations et imagerie biomédicale, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4

Advances in curing rectal cancer came from successful chemoradiotherapy. Platinum-based drugs such as oxaliplatin have also been studied and integrated in treatment strategies against rectal cancer. Although platinum-based drugs can act as radiosensitizers, their radiosensitizing activity is limited by their narrow therapeutic index which avoids the dose escalation. In addition, it is important also to optimize the schedule of drug administration with radiation treatment to gain advantage of drug-radiation interactions and maximize tumor response. We evaluated the new liposomal formulation of cisplatin and oxaliplatin (Lipoplatin™ and Lipoxal™, respectively) that should increase the anticancer effectiveness while minimizing the side effects.

We investigated different chemoradiation schedules to assess the best antitumor efficacy with regard to our hypothesis of the "true" concomitant chemoradiotherapy which consist in the addition of radiation at the time of maximum accumulation of platinum in the DNA of cancer cells. We performed in vitro studies using human colorectal carcinoma HCT116, and in vivo using nude mice HCT116 xenograft. Pharmacokinetic studies on platinum accumulation were measured by inductively coupled plasma mass spectrometry. Regarding the results of DNA-platinum concentrations, the synergy with radiation was assessed for in vitro and in vivo studies. Cytotoxicity was determined by a colony formation assay, while the resulting tumor growth delay in animal model was correlated to induction of apoptosis and histophatology analyses. The synergism of combined treatments was evaluated using the combination index method.

In this study, a radiosensitizing enhancement was observed with combining radiation treatment with cisplatin, oxaliplatin and their liposomal formulations in both in vitro and in vivo studies. Variations of platinum accumulation with incubation time in normal and tumor tissues and in different cell compartments, as well as platinum-DNA were measured. A higher level of synergism was observed when radiotherapy was performed in vitro at 8 h of exposure and in vivo at 4 and 48 h after drug administration, which corresponded to the times of maximal platinum binding to tumor DNA. These results were correlated to a highest induction of apoptosis and a low mitotic activity. In conclusion, the optimal treatment schedule of chemoradiotherapy is dependent on the time interval between drug administration and radiation, which was closely associated to the kinetics of platinum accumulation to DNA and the intracellular concentration of the platinum drugs. Regarding our hypothesis, administered radiotherapy to the time intervals of maximum synergism could improve efficacy of chemoradiation treatment. This should be confirmed in clinical trials.

Keywords: Cisplatin, oxaliplatin, Lipoplatin™, Lipoxal™, radiotherapy, pharmacokinetic, colorectal cancer, concomittance.
RÉSUMÉ

Traitement concomitant du cancer rectal avec la chimiothérapie basée sur des dérivés de platin et la radiothérapie: Études sur la cytotoxicité, la pharmacocinétique et l'effet concomitant in vitro et in vivo

Par
Thititip Tippayamontri
Département de médecine nucléaire et radiobiologie

Thèse présentée à la Faculté de médecine et des sciences de la santé pour l'obtention du grade de philosophe doctor (Ph.D.) en sciences des radiations et imagerie biomédicale, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4


Nous avons étudié la séquence optimale de radio-chimiothérapie en lien avec à notre hypothèse de "vraie" concomitance qui favorise l’ajout de rayonnement au moment de l’accumulation maximale de platine dans l’ADN des cellules cancéreuses. Nous avons effectué des études in vitro utilisant le carcinome colorectal humain HCT116, et in vivo sur des souris nue HCT116 xénogreffe. Les études pharmacocinétiques sur l’accumulation de platine ont été mesurées par spectrométrie de masse couplée au plasma induit. La cytotoxicité a été déterminée par un essai de formation de colonie, tandis que le retard de croissance tumorale obtenue en modèle animal est corrélu à l’apoptose et analyses histopathologiques. La synergie des traitements combinés a été évaluée en utilisant l’indice de combinaison.

Dans cette étude, nous avons observé une amélioration de la radiothérapie combinée avec le cisplatine, l’oxaliplatine et leurs formulations de liposomes à la fois in vitro et in vivo. Des variations entre l’accumulation du platine dans les cellules cancéreuses, de tissus normaux et tumoraux, ainsi que des adduits du platine à ADN en fonction de la cédule d’administration du médicament ont été observées. Un fort effet concomitant in vitro a été observé lorsque la radiothérapie a été délivrée à 8 h et in vivo à 4 et 48 h après l’administration du médicament, ce qui correspondait au temps de liaison maximale du platine à l’ADN tumoral. L’augmentation de la radiosensibilisation a été corrélée à une élévation de l’apoptose et une réduction de l’activité mitotique. La cédule de traitement optimal de la chimio-radiothérapie dépend de l’intervalle de temps entre l’administration de la radiation et de la drogue, ce qui a été étroitement associé à la cinétique d’accumulation de platine à l’ADN et de leurs concentrations intracellulaires. En conclusion, les meilleurs résultats in vitro et in vivo pourraient être ultérieurement confirmés en essai clinique pour valider ces concepts.

Mots-clés: Cisplatine, oxaliplatine, Lipoplatin™, Lipoxal™, radiothérapie, pharmacocinétique, cancer colorectal, concomittance
ACKNOWLEDGEMENTS

I would like to thank to the Department of Nuclear Medicine and Radiobiology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, to give me the opportunity to perform my Ph.D.

I would like to express my thankful to Prof. Léon Sanche, Prof. Benoit Paquette and Dr. Rami Kotb, for their supervision, encouragement, and kindness throughout the course of this work. I am thankful to Prof. Sherif Abou Elela, co-supervisor of my comité d'encadrement.

I would like to thank Dr. Theirry Muanza, Dr. David Mathieu, Prof. Johan E. Van Lier, for having accepted to be on my thesis committee.

I wish to thank the professors of the Department of Nuclear Medicine and Radiobiology for their great courses and suggestions. I am thankful to Dr. Ana-Maria Crous-Tsanaclis for her kindly advices and interesting discussions.

I am grateful to Gabriel Charest, Hélène Therriault and Rosalie Lemay for their advices and help to begin my experimental work in the laboratory. I also appreciated the help, friendship and cheerful support of all my colleagues in our laboratory and in the department.

Most importantly, my warmest thanks go to my always-supporting family for their generosity and understanding, their encouragement, and their great love.

This research was supported by the Canadian Institute of Health Research (grant # 81356). Prof. Léon Sanche, Prof. Benoit Paquette and Dr. Rami Kotb are members of the Centre de recherche Clinique-Étienne Lebel supported by the Fonds de la Recherche en Santé du Québec. Sanofi-Aventis Canada has offered a partial unrestricted grant to support this project. Regulon has provided liposomal formulation drugs (Lipoplatin™ and Lipoxal™) for this project.
TABLE OF CONTENTS

ABSTRACT .................................................................................................................. i
RÉSUMÉ ..................................................................................................................... ii
ACKNOWLEDGEMENTS .............................................................................................. iii
LIST OF ILLUSTRATIONS (TABLES AND FIGURES) .............................................. vi
LIST OF ABBREVIATIONS/SYMBOLS ......................................................................... x

CHAPTER I -- INTRODUCTION ................................................................................. 1
Colorectal cancer .......................................................................................................... 1
Platinum (Pt) compounds -Cisplatin and Oxaliplatin .................................................... 6
  History ...................................................................................................................... 6
  Chemical structure ................................................................................................. 7
  Chemistry .............................................................................................................. 8
Mechanism of action .................................................................................................... 9
  Interaction with the cell ......................................................................................... 9
  Interaction with the DNA ..................................................................................... 11
Liposomal formulation of platinum compounds .......................................................... 15
  Lipoplatin™ and Lipoxal™ .................................................................................. 16
  Mechanism of action ........................................................................................... 17
  Preclinical and clinical studies of Lipoplatin™ and Lipoxal™ ............................... 18
Chemoradiation treatment ............................................................................................ 19
Objectives of this study ............................................................................................... 28

CHAPTER II -- ARTICLE NO. 1 ................................................................................ 29

CHAPTER III -- ARTICLE NO. 2 ............................................................................... 51

CHAPTER IV -- ARTICLE NO. 3 ............................................................................... 80

CHAPTER V -- ARTICLE NO.4 ................................................................................. 115

CHAPTER VI -- DISCUSSION ..................................................................................... 147
The scope of this thesis ............................................................................................... 147
Cytotoxicity potential of cisplatin and oxaliplatin ....................................................... 147
Pharmacokinetics of platinum drugs.................................................................148
Platinum-based radiosensitizer and cell cycle progression to chemoradiotherapy in human colorectal cancer HCT116 cell..........................................................151
Radiosensitizing activity of Lipoplatin™ and Lipoxal™ ........................................156
Histopathological analysis ..............................................................................156
Emerging strategies for improvement in chemoradiotherapy: Clinical aspects ......166

CHAPTER VII -- CONCLUSIONS........................................................................171

BLIBIOGRAPHY .................................................................................................173
LIST OF ILLUSTRATIONS (TABLES AND FIGURES)

CHAPTER I -- INTRODUCTION

Table 1. Staging for colorectal cancer, percentage of occurrence and percentage of survival .2

Table 2. Example of preclinical and clinical studies on oxaliplatin-based chemoradiotherapy of colorectal cancer .................................................................5-6

Figure 1. The chemical structures of cisplatin and oxaliplatin ..........................................................7

Figure 2. The hydrolysis reaction scheme of cisplatin ........................................................................8

Figure 3. The hydrolysis reaction scheme of oxaliplatin ....................................................................9

Figure 4. Mechanism of platinum uptake and efflux ........................................................................10

Figure 5. Formation of cisplatin- and oxaliplatin–DNA adducts ........................................................12

Figure 6. Interaction of cisplatin and oxaliplatin with the DNA .........................................................13

Figure 7. Mechanism of cell death after platinum chemotherapy .......................................................15

Figure 8. Depiction of a liposomal formulation ..................................................................................17

Figure 9. Liposome-cell interactions .................................................................................................18

Figure 10. The theoretical framework for the prediction of the outcome of combined treatments with cytotoxic chemotherapy and radiaotheraphy ..............................................................................................21

Table 3. The mechanisms of chemotherapy and radiation interaction ................................................22

Figure 11. Comparison of the yield of SSB and DSB as a function of different cisplatin to plasmid DNA ratios .................................................................................................................24

Table 4. Example of previous studies of combined treatment of cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™ plus radiation ............................................................26-27
CHAPTER II -- ARTICLE No.1

Figure 1. Chemical structures of platinum-based drugs studied and schematic of a liposome................................................................. 34

Table 1. IC\textsubscript{50} values of the platinum compounds for the HCT116 cells.................................................. 37

Figure 2. Time course of platinum derivatives accumulation in HCT116 cells.......................... 38

Figure 3. Cell proliferation in presence of the platinum derivatives............................................. 39

Figure 4. Amount of platinum bound to DNA after exposing the HCT116 cells to platinum drugs for 4 and 24 h......................................................... 40

Figure 5. Time course of platinum binding to DNA after exposing the HCT116 cells ....... 41

Figure 6. Distribution of platinum compounds in cytoplasm or bound to DNA...................... 42

CHAPTER III -- ARTICLE No.2

Figure 1. Dose response curve for radiotherapy alone on HCT116 cells......................... 61

Figure 2. Response curves for chemoradiotherapy on HCT116 cells at 8 h incubation time.. 62

Figure 3. Response curves for chemoradiotherapy on HCT116 cells at 48 h incubation time............................................................................................................. 63

Figure 4. The IC\textsubscript{50} values of cisplatin, oxaliplatin and their liposomal formulation (Lipoplatin\textsuperscript{TM} and Lipoxal\textsuperscript{TM}) in combination with radiation.......................................................... 64

Figure 5. The combination index (CI) of chemoradiotherapy on HCT116 cells .................. 66

Figure 6. Flow cytometric measurement of cell-cycle distribution of the HCT116 cell ...... 69

Figure 7. Induction of apoptosis by cisplatin, oxaliplatin, Lipoplatin\textsuperscript{TM} and Lipoxal\textsuperscript{TM} combination with radiation................................................................. 70
CHAPTER IV -- ARTICLE No.3

Table 1. Change in body weight of male nu/nu nude mice treated with i.v. oxaliplatin or LipoxalTM in combination with GK irradiation ................................................................. 91

Figure 1. The experiment setup for chemoradiotherapy in nu/nu nude mice model .......... 92

Figure 2. Pharmacokinetic profiles of platinum concentration in blood, tumor tissues and normal tissues after a single i.v. injection of oxaliplatin or LipoxalTM ................................ 95-96

Figure 3. Time dependence of platinum concentration in cytoplasm, nucleus and platinum-DNA adducts obtained after a single i.v. injection of oxaliplatin or LipoxalTM .... 99

Figure 4. The tumor response after nu/nu nude mice were treated initially with chemotherapy and followed by radiotherapy ................................................................................. 100

Table 2. In vivo efficacy of platinum-based chemotherapy alone and in combination with radiation treatment .............................................................................................................. 101

Figure 5. The combination index of chemoradiotherapy in nude mice model ............... 103

Figure 6. TUNEL assay after chemoradiotherapy of oxaliplatin and LipoxalTM ............ 104

CHAPTER V -- ARTICLE No.4

Table 1. Change in body weight of male nu/nu nude mice treated with i.v. cisplatin or LipoplatinTM in combination with Gamma Knife irradiation ............................................. 124

Figure 1. Pharmacokinetic profiles of platinum concentration in whole blood, serum, kidney, liver, muscle, and tumor after a single i.v. injection of cisplatin or LipoplatinTM ................................................................. 126-127

Figure 2. Time dependence of platinum concentration in cytoplasm, nucleus and platinum-DNA adducts obtained after a single i.v. injection of cisplatin or LipoplatinTM .... 129

Figure 3. The tumor growth delay after initial treatment of nude mice bearing HCT116 colorectal tumor with cisplatin and LipoplatinTM followed by radiotherapy ............ 131
Table 2. Efficacy of platinum-based chemotherapy alone and in combination with radiation treatment .......................................................................................................................... 133

Figure 4. Assessment of apoptosis after chemoradiotherapy of cisplatin and Lipoplatin™ ........................................................................................................................................ 134

CHAPTER VI -- DISCUSSION

Table 1. The platinum-DNA binding after exposure of HCT116 to cisplatin, oxaliplatin, and their liposomal formulation for 4 h and 24 h of drug incubation ................................................. 151

Figure 1. Diagram illustrated phases of cell cycle and their relative radiosensitivity or radioresistance .............................................................................................................................. 156

Figure 2. Induction of the mitochondrial apoptotic pathway by cisplatin.................................................. 161

Figure 3. Histopathologic changes of HCT116 human colorectal cancer tumors treated with platinum drugs and radiation ......................................................................................... 163-166

Figure 4. Possible improvement for clinical chemoradiation treatment apply from our preclinical studies......................................................................................................................... 168

Table 2. Lists of other chemotherapeutic agents that are commonly combined with platinum drugs .......................................................................................................................... 170
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt</td>
<td>Platinum</td>
</tr>
<tr>
<td>DACH</td>
<td>Diaminocyclohexane</td>
</tr>
<tr>
<td>CDDP</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>FA</td>
<td>Folinic acid</td>
</tr>
<tr>
<td>DPPG</td>
<td>Dipalmitoyl phosphatidyl glycol</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>MMR</td>
<td>Mitmatch repair</td>
</tr>
<tr>
<td>HMG</td>
<td>High-mobility group</td>
</tr>
<tr>
<td>OCT</td>
<td>Organic cation transporter</td>
</tr>
<tr>
<td>CTR</td>
<td>Copper treansporter</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>DNA</td>
<td>Dioxynucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>SSB</td>
<td>Single strand break</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometer</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>$^{60}$CO</td>
<td>Cobalt-60</td>
</tr>
<tr>
<td>GK</td>
<td>GammaKnife</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
</tbody>
</table>
eV  Electronic volt
\( t_{1/2} \)  Half-life
h  Hour
\( \mu M \)  Micromolar
\( \mu g \)  Microgram
ng  Nanogram
mg/kg  Milligram/kilogram
Td  Tumor growth delay
CHAPTER I
INTRODUCTION

Colorectal cancer

Colorectal cancer occurs when abnormal cells grow in the lining of both the colon and rectum. Colorectal cancer is one of the most common types of cancer worldwide, accounting for a significant percentage of cancer mortality (International Agency for Research on Cancer-World Health Organization, www.iarc.fr). In Canada, mortality rates continue to decline in both sexes by 2.6% per year since 2003, however, colorectal cancer is still the second leading cause of death from cancer, behind lung cancer in men and breast cancer in women. The Canadian Cancer Society has estimated that there will be about 23,300 new cases of colorectal cancer in 2012. On average, 64 Canadians will be diagnosed with colorectal cancer and 25 Canadians will die of this disease each day (Canadian Cancer Society, www.cancer.ca).
Table 1. Staging for colorectal cancer, percentage of occurrence and percentage of survival
(Greene and Page, 2002)

<table>
<thead>
<tr>
<th>Stage</th>
<th>% Occurrence</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>14 T1-2, N0, M0</td>
<td>&gt;90</td>
</tr>
<tr>
<td>IIA</td>
<td>28 T3, N0, M0</td>
<td>60-85</td>
</tr>
<tr>
<td>IIB</td>
<td>T4, N0, M0</td>
<td></td>
</tr>
<tr>
<td>IIIA</td>
<td>37 T1-2, N1, M0</td>
<td>25-65</td>
</tr>
<tr>
<td>IIIB</td>
<td>T3-4, N1, M0</td>
<td></td>
</tr>
<tr>
<td>IIIC</td>
<td>T (any), N2, M0</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>21 T (any), N (any), M1</td>
<td>5-7</td>
</tr>
</tbody>
</table>

**T (Primary tumor)**
- TX: Primary tumor cannot be assessed
- Tis: Carcinoma in situ
- T1: Tumor invades submucosa
- T2: Tumor invades muscularis propria
- T3: Tumor penetrates muscularis propria and invades subserosa
- T4: Tumor directly invades other organs or structures or perforates visceral peritoneum

**N (Nodal status)**
- NX: Regional lymph nodes cannot be assessed
- N0: N metastases in regional lymph nodes
- N1: Metastases in one to three regional lymph nodes
- N2: Metastases in four or more regional lymph nodes

**M (Distance metastases)**
- MX: Presence or absence cannot be determined
- M0: No distant metastases detected
- M1: Distant metastases detected
Table 1 shows the colorectal cancer staging, the percentage of occurrence and percentage of survival (Greene and Page, 2002). Surgical resection at an early stage is still the only chance for a cure in patients with colorectal cancer (Canadian Cancer Society, www.cancer.ca). The role of adjuvant chemotherapy (5-fluorouracil) in stage II (T3/4, N0) is debatable, there is a minimal benefit. For patients with stage III (TX, N1) rectal cancer, adjuvant chemoradiotherapy was considered the standard treatment because it improved local control and overall survival compared with surgery alone or surgery plus radiation (Krook et al., 1991). Neoadjuvant chemoradiotherapy protocols were mainly used for locally advanced rectal cancers, aiming to achieve subsequent curative resection by decreasing tumor size and decrease the risk of local recurrence (Kapiteijn et al., 2001). The radiotherapy is the use of carefully measured dose of radiation (e.g. adjuvant radiotherapy is typically given 1.8 Gy per day for 5 days per week for a number of weeks, total dose of 50.4 Gy). When the dose per day is increased, the total number of days is correspondingly decreased to limit the risks for injury to normal tissue from the increasing dose per fraction (Robertson, 2008). Fractionation radiotherapy allows normal cells to recover and also allows tumor cells that were in a relatively radio-resistant phase of the cell cycle during one treatment to cycle into a sensitive phase of the cycle before the next fraction is given (Hall and Giaccia, 2005). Palliative therapy is recommended for patients with stage IV (Andre and Schmiegel, 2005).

Three recent completed clinical phase III trials (ACCORD-0405, STAR-01 and NSABPR-04) that added oxaliplatin to 5-FU or capecitabine treatment have failed to improve results of chemoradiotherapy (Aschele et al., 2011; Gerard et al., 2010 and Roh et al., 2009), with only the results from the German CAO/ARO/AIO-04 study (Rodel et al., 2012) that showed an increase in the treatment efficacy when added oxaliplatin to 5-FU plus radiation.
(Table 2). From the various treatment studies, non-optimal schedule for rectal cancer treatment may be one of the important factors in clinical treatment failure. Despite the fact that approximately 70-80% of patients are eligible for curative surgical resection at the time of diagnosis (Faivre-Finn et al., 2002), 5 years overall survival rate is only 50-60% et al., 2003). Some 66% of patients who undergo curative resection will experience local recurrence or distant metastases. In 85% of people, relapse was diagnosed within the first 2.5 years after surgery (Gill et al., 2004). Upon diagnosis of metastatic disease, patients have a median survival rate of only 6 months and the 5-year overall survival rate was less than 10% (Greene and Page, 2002).

Importantly, severe side effects of either chemotherapy or radiotherapy can limit the potential for dose escalations. Chemotherapy is known to cause severe adverse reactions including renal toxicity, gastrointestinal toxicity, peripheral neuropathy, asthenia and ototoxicity (Howell, 1991; Kweekel et al., 2005 and O'Dwyer et al., 2000). Radiotherapy potentially can cause skin irritation, nausea, diarrhea, trouble controlling of bowels, rectal or bladder irritation, and tiredness (Hall and Giaccia, 2005). This will limit the anticancer efficiency of both modalities. Moreover, the optimal schedule for adding platinum-based drugs to the standard chemoradiotherapy for treating rectal cancer is not yet determined. Investigations to improve the chemoradiation schedule for rectal adenocarcinoma to attain the maximum concomitant effect, while minimizing the systemic toxicities is thus very important.
Table 2. Example of preclinical and clinical studies on oxaliplatin-based chemoradiotherapy of colorectal cancer. (OXA = Oxaliplatin; 5FU = 5-Fluorouracil; Cape = Capecitabine; pCR = Pathological complete response; RT = Radiation)

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blackstock et al, 1999</td>
<td>HT29 colorectal cancer; OXA 0.48 μM; 1 or 24 h incubation; 2 or 4 Gy before or after drug treatment</td>
<td>Oxaliplatin induced synergistic effect with independent of the sequence of the combination</td>
</tr>
<tr>
<td>Magné et al, 2003</td>
<td>SW403 colorectal cancer; OXA 6.5-100 μM; 1 or 4 Gy ((^{60})CO gamma-rays, 1 Gy/min); RT 2 h before or 12 h after or 24 h after the start of OXA</td>
<td>OXA induced the supra-additive effect with no influenced of p53</td>
</tr>
<tr>
<td>Kjellström et al, 2005</td>
<td>WiDr colorectal cancer; OXA 1-4 μM; 2 h incubation; 0.5 – 4 Gy</td>
<td>Oxaliplatin induced radiosensitization</td>
</tr>
<tr>
<td>Folkwords et al, 2008</td>
<td>HT29 colorectal cancer; OXA 10 mg/kg; d1 weekly; 2 Gy x10 ((^{60})CO gamma-rays, 0.6 Gy/min) RT 30 min after i.v.</td>
<td>Weak endpoints o tumor growth delay after the combined treatment</td>
</tr>
<tr>
<td>Tippayamontri et al, 2012</td>
<td>HCT116 colorectal cancer; OXA 2.5-10 μM; 8 h incubation; 0.025-0.5 μM: 48 h incubation; 2.3 Gy after OXA ((^{60})CO gamma-rays, 1.64 Gy/min)</td>
<td>Oxaliplatin induced radiosensitization with influence of the level of Pt-DNA adducts</td>
</tr>
<tr>
<td>Gerard et al, 2010</td>
<td>ACCORD-0405; n = 598; Cape 1600 mg/m²/day d1-5 weekly x5; OXA 50 mg/m² d1 weekly x5; 50 Gy for 5 weeks</td>
<td>Compared Cape- 45 Gy versus Cape-OXA-50 Gy: pCR = 14% Vs 19%; Grade 3 toxicity = 1% Vs 25%</td>
</tr>
<tr>
<td>Aschele et al, 2009</td>
<td>STAR-01, n = 747; 5FU 225 mg/m²/day d1-35; OXA 60 mg/m² d1 weekly x6; 50.4 Gy in 28 daily fractions</td>
<td>Toxicity significantly increased without affecting local tumor response</td>
</tr>
<tr>
<td>Roh et al, 2011</td>
<td>NSABP R-04; n = 1608; 5FU 225 mg/m²/day or Cape 1650 mg/m²/day d1-5 weekly x5; OXA 50 mg/m² d1 weekly x5; 45 Gy in 25 fractions over 5 weeks</td>
<td>Compared group with OXA versus without OXA: pCR = 19% Vs 20.9%; Grade 3 toxicity 6.6% Vs 15.4%</td>
</tr>
</tbody>
</table>
Platinum (Pt) compounds –Cisplatin and Oxaliplatin

History

In 1965, Rosenberg and his group discovered the potential of platinum-based drugs to inhibit cell division (Rosenberg, 1980). They investigated the effect of electronic current on bacterial mobility and, noticed that the cells under study formed a filamentous structure, which is a characteristic of un-dividing cells. The chemical compound causing this structure was cis-diamminedichloroplatinum (II), or cisplatin, a compound known since the 1860’s as “Peryone’s Chloride” (Rosenberg et al., 1969). After this finding, several studies of cisplatin for cancer treatment were performed, and cisplatin was approved for clinical use in 1979 (Rosenberg et al., 1969).

The treatment with cisplatin consists of a course of intravenous injections administered every 3-4 weeks at a dose of 50-120 mg/m² (Adelstein et al., 2003). Such treatment has a reported high efficacy for testicular, ovarian and bladder cancers, and other solid tumors (Begg, 1990). Over the years, various platinum complexes have been studied in an attempt to overcome the severe side effects of cisplatin et al., 1998). Oxaliplatin or (trans-R,R)1,2-diaminocyclohexaneoxalotoplatinum (II), is a third generation platinum-based compound in the alkylating family of anticancer agents and the first clinically available diaminocyclohexane (DACH) platinum compound. Oxaliplatin is distinctive from cisplatin and is non-cross-resistant with some platinum-resistant tumors. Although oxaliplatin was discovered 34 years ago, it only
gained Food and Drug Administration (FDA) approval 8 years ago, specifically for the management of advanced human colorectal cancer, for use in combination with 5-fluorouracil (5FU) and folinic acid (FA) (National Cancer Institute at the national institute of health, www.nih.gov). Unlike cisplatin, oxaliplatin is non-nephrotoxic with the main toxicity being neuropathy. Neurotoxicity is dose-limiting. Oxaliplatin is commonly given at the dose of 85 mg/m² i.v. injection every second week (Alcindor and Beauger, 2011).

**Chemical structure**

The chemical structure of cisplatin and oxaliplatin in 2D and 3D are shown in Figure 1. Cisplatin has a square-planar structure with the central platinum (Pt) coordinated by two chlorides (Cl) ion ligands and other two ammonia (NH₃) ligands in cis to each other. Oxaliplatin is a much bigger organic compound, with the two amine NH₃ ligands in cisplatin substituted by the (trans-R,R)1,2-diaminocyclohexan (DACH) ligand to improve antitumor activity; and the two chloride ion ligands replaced by the oxalatobidentate ligand derived from oxalic acid to improve water solubility.

![Chemical structures of cisplatin and oxaliplatin](image)

**Figure 1.** The chemical structures of cisplatin and oxaliplatin
**Chemistry**

Cisplatin of molecular formula H$_6$Cl$_2$N$_2$Pt has molecular weight 301.1 g/mol. There is evidence suggesting that due to a high concentration (about 100 mM) of chloride ions outside of cell membranes, cisplatin remains in the dichloro form until it passes through the cell membrane. Inside the cell membrane, the chloride ion concentration is about 4 mM which allows the hydrolysis process to take place (Hall et al., 2008). In aqueous solution, the Cl$^-$ ion is slowly lost and replaced by a water molecule (H$_2$O) to form the mono-aqua product, cis-[Pt(NH$_3$)$_2$(OH$_2$)Cl]$^+$. Further substitution of the remaining chloroligand leads to the formation of the diaqua-platinum complex, cis-[Pt(NH$_3$)$_2$(OH$_2$)$_2$]$^{2+}$ (Figure 2). At physiological pH (pH=7.87), the equation shifts and the majority of molecules present are in the diaqua form, the active form of cisplatin (Dionisi et al., 2011; Esteban-Fernandez et al., 2010).

![Figure 2. The hydrolysis reaction scheme of cisplatin (Reprinted with permission from Lau and Deubel) (Lau and Deubel, 2006).](image-url)
The oxaliplatin molecule has the formula $C_8H_{14}N_2O_4Pt$ and a molecular weight of 397.3 g/mol. Oxaliplatin slowly hydrolyzes in water according to following reaction:

$$[(DACH)Pt(C_2O_4)] + 2H_2O \rightarrow [(DACH)Pt(H_2O)]^{2+} + C_2O_4^{2-}$$

(Esteban-Fernandez et al., 2010). The oxalate group can be shifted by nucleophile groups such as $Cl^-$ or $HCO_3^-$ and $H_2O$ (Figure 3).

Upon entering the cell, cisplatin and oxaliplatin are biotransformed to their reactive species. Through non-enzymatic reactions, the chloride atom of cisplatin and oxalate group of oxaliplatin are displaced by $H_2O$ forming biaquated biotransformation products that readily react with nucleophiles in the cells, including DNA, RNA and protein (Hall et al., 2008).

![Figure 3. The hydrolysis reaction scheme of oxaliplatin. Permission license # 3034930431133 (Kweekel et al., 2005).](image)

**Mechanism of action**

**Interaction with the cell**

Figure 4 illustrates the mechanisms affecting and controlling the cellular accumulation of platinum drugs. In the extracellular environment, platinum drugs can be associated with water molecules as explained previously. These may enter the cell or cross-react with extracellular proteins such as serum albumin, reducing its bioavailability. Platinum drugs can enter the cell by passive diffusion across the lipid bilayer as well as by fluid phase endocytosis.
The main organic cation transporters such as OCT1-3, CTR1, and sodium-dependent process have been identified.

Inside the cell, platinum drugs can be deactivated by binding to the thiol-rich metallothionein (MT) or chelated by glutathione (GSH) and effluxed from the cell via the GS-X pumps. Platinum drugs can also be entrapped in subcellular organelles such as vesicles or melanosomes in melanoma cells and then subsequently exported out from the cells (Wang and Lippard, 2005). The accumulation of platinum is a steady-state phenomenon which depends on both the rates of influx and efflux of platinum drugs. When the shift of the balance between influx and efflux occurs, an increase of the extrusion process leads to a reduction in cellular accumulation of platinum. Drugs that evade the detoxification and efflux processes can enter the nucleus and bind to DNA, eliciting apoptosis if the DNA lesion is not repaired.

Figure 4. Mechanism of platinum uptake and efflux.
Interaction with the DNA

The clinical efficacy of platinum drugs against tumors is thought to be primarily due to the formation of platinum-DNA adducts which interfere with cellular repair and DNA replication and which therefore trigger a chain of cell regulatory events, ultimately leading to cell death (Saris et al., 1996).

On DNA, cisplatin and oxaliplatin preferentially react with the highly nucleophilic N7 position on guanine or adenine, and form coordinated covalent bonds (Figure 5). Platinum binds to DNA and causes a critical structure change in the DNA such as a bend of 45 degree, unwinding of DNA and causing destacking of the purine bases (Sharma et al., 2007).

Cisplatin and oxaliplatin are bifunctional agents. They can bind to two sites in DNA with the following order of sequence preference: -GG- > -AG- >> -GA. The resulting biadducts composed of approximately 60-65% 1,2-intrastrand GG, 25-30% 1,2-intrastrand AG, 5-10% 1,3-intrastrand GXG and 1-3% interstrand GG (Figure 6) (Eastman, 1987). Although cisplatin and oxaliplatin form the same types of adduct at the same sites on DNA, their structures are distinct (Sharma et al., 2007). The bulky DACH carrier ligand of oxaliplatin is thought to contribute to a much bulkier adduct than cisplatin.
**Figure 5.** Formation of cisplatin- and oxaliplatin–DNA adducts. Permission license # 3034940510767 (Sharma et al., 2007).
Figure 6. Interaction of cisplatin and oxaliplatin with the DNA.
The DNA repair system can detect many forms of damage. Repair of platinum-DNA adducts is through the nucleotide excision repair pathway (NER), a complex process which is essential in the repair of bulky covalent DNA lesions such as those attributed to platinum drugs (Wozniak and Blasiak, 2002). Once a platinum adduct is detected, excision repair removes the damaged DNA to form a gap. This gap is then filled by DNA polymerase. The order of DNA repair efficiency is 1,3-d(GTG) crosslink > AG > GG. This implies that 1,2-d(GG) cross-links, as formed by cisplatin or oxaliplatin are the most toxic. Cisplatin adducts, but not the bulky oxaliplatin adducts, are recognized by a functional mismatch repair (MMR) complex. Binding of the MMR complex to damage DNA is thought to mediate the apoptotic response (Sharma et al., 2007).

Alterations to DNA structure prevent both cell replication and activation of cellular repair mechanisms, and thus lead to cell death (Figure 7). The link between structure alteration and cell death is due to the prevention of DNA transcription which proceeds by three mechanisms of action; i) Participation of high-mobility group (HMG) proteins, which have a higher affinity to bind to platinum-modified DNA than to unmodified DNA. Possibly, the HMG–Pt–DNA lesion prevents the binding of DNA-repair proteins, thus preventing replication and causing cell death, ii) Irreversible platinum binding to transcription factors by replacing the zinc ion with platinum, and iii) Abortive correlation repair will upregulate the p53 due to DNA strand breaks, which leads to apoptosis (Zlatanova et al., 1998).
**Figure 7.** Mechanism of cell death after platinum chemotherapy. ( Adapted with permission from Zlatanova) (Zlatanova et al., 1998).

**Liposomal formulation of platinum compounds**

Liposomes are spherical, self-enclosed structures consisting of one or several concentrated lipid bilayers with an aqueous phase inside and between the lipid bilayers. Attractive biological properties of liposomes include; i) liposomes are biocompatible, ii) liposomes can entrap water-soluble (hydrophilic) pharmaceutical agents into the membrane, iii) liposome-incorporated pharmaceuticals are protected from the inactivating effect of external conditions, and iv) liposomes provide an unique opportunity to deliver drugs into cells or even inside individual cellular compartments (Torchilin, 2005).
Lipoplatin™ and Lipoxal™

Cisplatin and oxaliplatin have been frequently used in therapy against cancer, despite the strong side-effects often observed in patients. In this context, any improvements to platinum complexes, related to their efficacy or reduction in toxicity, would be a substantial boost for cancer therapy.

In recent years, liposomal encapsulations of cisplatin and oxaliplatin, Lipoplatin™ and Lipoxal™, respectively, were achieved using Regulon’s platform technology (Boulikas, 2007). They were developed to reduce the systemic toxicity of cisplatin and oxaliplatin while attempting to improve the anticancer efficiency. Figure 8 depicts a liposomal formulation. The lipid formulation is composed of soy phosphatidyl choline (SPC-3), cholesterol, dipalmitoyl phosphatidyl glycol (DPPG), and methoxy-polyethylene glycol-disteraryl phophatidylethanolamine (mPEG 2000-DSPE). The nanoparticles of liposomal formulation have a 110 nm diameter. Lipoplatin™ is composed of 8.9% cisplatin and 91.1% lipid (w/w). Concentrations of 3 mg/ml of cisplatin in Lipoplatin™, and 3 mg/ml of oxaliplatin in Lipoxal™ were used in this study.

Lipoplatin™ and Lipoxal™ formulations were developed to have several therapeutic advantages: i) the anionic lipid DPPG gives liposomal drugs their fusogenic properties presumably acting at the level of entry of the drug through the cell membrane after reaching the target tissue, ii) the total lipid to platinum compounds ratio is low in liposomal formulations which means that less lipid is injected into the patient, and iii) the PEG polymer coating used on liposomal formulations is meant to give the drug particles the ability to pass undetected by the macrophages and immune-system cells to remain in the circulation of body fluids and tissues for long periods ($t_{1/2} = \sim 72$ h for Lipoplatin™ and $\sim 6$ h for cisplatin), to extravasate
preferentially and to infiltrate solid tumors and metastatic tissue through the altered and often compromised tumor vasculature (Boulikas and Vougiouka, 2003).

Figure 8. Depiction of a liposomal formulation. Free platinum compounds are showed as blue spheres surrounded by the lipid bilayer with the PEGylated lipid sticking out like hair from the outer surface (Reprinted with permission from Stathopoulos and Boulikas) (Stathopoulos and Boulikas, 2012).

**Mechanism of action**

Drugs stably entrapped inside a liposome are not biologically active and must be released to gain access to their intracellular target. Figure 9 shows the liposome-cell interactions. Drug-loaded liposomes can specifically or non-specifically adsorb onto the cell surface. Liposomes can also fuse with the cell membrane, and release their contents into the cell cytoplasm, or can be destabilized by certain cell membrane components when adsorbed on the surface, so that the released drug can enter the cell via micro pinocytosis. Liposomes can undergo the direct or transfer-protein-mediated exchange of lipid components with the cell membrane or can be subjected to a specific or non-specific endocytosis. In the case of endocytosis, a liposome can be delivered by the endosome into the lysosome or, en route to the
lysosome, the liposome can provoke endosome destabilization, which results in drug liberation into the cell cytoplasm. It has been suggested that Lipoplatin™ is mainly taken up into the cell by phagocytosis, due to its 110 nm size, which is much greater than that of cisplatin (of molecular dimensions < 1 nm).

**Figure 9.** Liposome-cell interactions.

**Preclinical and clinical studies of Lipoplatin™ and Lipoxal™**

Devarajan and co-workers reported significantly less structural and functional evidence of nephrotoxicity in mice and rats after treatment with Lipoplatin™ compared to equal doses with cisplatin (Devarajan et al., 2004a). Phase I, II and III clinical trials have shown that Lipoplatin™ has a similar efficacy to that of cisplatin in pancreatic, head and neck cancer, breast cancer and non-small cell lung cancer, while reducing the severe side effects of cisplatin.
Lipoxal™ was a subject of a stability testing and a clinical study in 2003. As a single agent, Lipoxal™ has produced significant cytotoxicity in human colorectal cancer cells and showed adequate effectiveness in pre-treated patients with colorectal cancer in phase I study. Lipoxal™ is currently under study for phase II for gastric cancer and pancreatic cancer (Stathopoulos et al., 2006a).

**Chemoradiation treatment**

Sometimes, chemotherapy or radiotherapy alone is not the most effective treatment. The efficacy of cancer treatment using radiotherapy is often limited by the radiation tolerance of normal healthy tissues. Chemoradiation treatment is the combined modality treatment using chemotherapy and radiotherapy. Three clinical rationales support the use of combined treatment (Seiwert et al., 2007), these include:

i) That concomitant chemoradiotherapy can be used with organ-preserving intent, resulting in improved cosmesis and function compared with surgical resection with or without adjuvant treatment,

ii) The chemotherapeutic agent can act as radiosensitizer, by improving the locoregional tumor control,

iii) Chemotherapy given as part of concurrent chemoradiotherapy may act systemically and potentially eradicate distance metastases.

Figure 10 shows the classical framework for describing the possible interactions between chemotherapy and radiation treatment (Steel and Peckham, 1979) including:

i) Spatial cooperation: This concept is used to describe the scenario in which radiotherapy is designed to target locoregionally primitive tumor sites, and chemotherapy acts
against distant micrometastases, without interaction between the agents. This operative effect requires the agents to have non-overlapping toxicity profiles in order that both modalities can be used at effective doses without increasing normal tissue effects.

ii) Additivity: Where it is intended that both modalities interact in a purely additive mode with regards to sterilization of the target tumor.

iii) Supra-additivity: Synergism or radiosensitization, the use of combined modalities leads to increased killing of cells more than expected from the summation of the effects for each agent applied alone. The radiosensitizers should not have inherent cytotoxicity activity.

iv) Infra-additivity: Antagonism or radioprotective, where the chemotherapeutic agent inhibits tumor regression by radiation.

The mechanisms of chemotherapy and radiation interaction are summarized in the table 3. The interaction between chemotherapy agents and radiation may take place different level of organisation (Hennequin and Favaudon, 2002) specifically;

i) The molecular level, altering DNA repair or modifying of lesions induced by drugs and radiation,

ii) The cellular level through cytokinetic cooperation from differential sensitivity of the various compartments of the cell cycle to drugs or radiation,

iii) The tissue level, including reoxygenation, increased drug uptake and inhibition of repopulation or angiogenesis.
Figure 10. The theoretical framework for the prediction of the outcome of combined treatments with cytotoxic chemotherapy and radiotherapy.

Radiosensitizers are intended to modify tumor cells to become sensitive to radiation therapy while having minimal toxicity on normal tissue (Tannock, 1996). Heidlberger's preclinical studies in 1958 were the first to establish the concept of giving drugs concomitantly with radiation to enhance the effect of the radiation (Heidelberger et al., 1958).
Table 3. The mechanisms of chemotherapy and radiation interaction

<table>
<thead>
<tr>
<th>Process affected</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased radiation damage</td>
<td>Incorporation of chemotherapeutic agents into DNA/RNA generate inter/intra-strand cross-links with DNA/RNA</td>
</tr>
<tr>
<td>Inhibition of DNA repair process</td>
<td>Interfere with the DNA repair process after radiation</td>
</tr>
<tr>
<td>Cell cycle interference (cytokinetic corporation and synchronization)</td>
<td>Platinating agents are cell-cycle independent. Radiation is cell-cycle specific. Accumulation of cells in the G₂ and M phases are the most radiosensitive phases and in the S phase is the most radioresistant phase.</td>
</tr>
<tr>
<td>Reoxygenation and tumor shrinkage</td>
<td>A reduction in tumor volume resulted in improved blood supply to the tumor, leading to reoxygenation and increase radiosensitivity and drug sensitivity.</td>
</tr>
</tbody>
</table>

Platinum-based radiosensitizers induce additional damage or modification of radiation induced DNA damage

The biological impact of ionizing radiation results predominantly from the induction of a variety of lesions in cellular DNA via energy deposition into the DNA itself (direct effect) and its surrounding molecular environment, particularly water molecules (indirect effect) (Hall and Giaccia, 2005). The intermediate species (e.g. DNA subunit radical cations, free electrons and electronically excited DNA subunits) arising from the direct effect of radiation are non-thermal secondary electrons which carry most of the primary radiation energy. The majority of secondary electrons have energies below 30 eV with the most probable energy at about 10 eV (Pimblott and LaVerne, 2007). Even low energy electrons with energies less than that required
for ionization of DNA can induce DNA damage (M. Rezaee et al., 2013). In addition, low energy electrons can also induce DNA damage via indirect action, which due to dissociative electron transfer from water-interface electron traps to DNA bases, quenching of dissociative electron attachment to DNA, and quenching of dissociative electronically excited states of H₂O in contact with DNA (Alizadeh et al., 2013).

Ionizing radiation results in DNA damage and exerts its therapeutic activity primarily by inducing DNA strand breaks of two types: single strand break (SSB) and double strand break (DSB). The DSB is more difficult to repair than the SSB and thus apoptosis typically ensues following therapeutic application of chemoradiation treatment to a tumor. Radiation also can induce DNA base damage, DNA to protein cross-links and install replication forks. In addition, membrane damage leading to signal transduction may affect: i) gene expression of cell cycle regulations, ii) growth factor production, and iii) oxidative stress pathway activation (Hall and Giaccia, 2005).

Initial radiation induced cell death can be directly enhanced by the incorporation of a radiosensitizer into DNA. Cisplatin and oxaliplatin can exert their cytotoxicity through the formation of monofunctional adducts and bifunctional adducts, leading to the induction of intra- and inter-strand DNA cross-links. Sanche and co-workers have demonstrated increased yields of SSBs and DSBs in plasmid DNA after combining cisplatin with irradiation with 100 eV electrons (Figure 11). An enhancement in the electron cross section for damage to DNA at the cisplatin binding site is thought to be the cause of this increase in SSBs and DSBs. When cisplatin-DNA complexes are irradiated by high energy particles, secondary low energy electrons can be captured at the cisplatin site, increasing the probability of rupture of the DNA backbone, by an order of magnitude. Moreover the ionization cross section, which describes the
probability of generating secondary electrons, is increased near cisplatin due to the presence of high platinum atom ($Z = 78$), and this therefore also indirectly contributes to the huge increase in local damage. Thus, strand breaks in the DNA of cancer cells, leading to cells death, might be significantly increased if sufficient quantities of the drug were delivered or could accumulate preferentially in the DNA of cancer cells (Zheng et al., 2008).

![Figure 11](image.png)

**Figure 11.** Comparison of the yield of SSB (A) and DSB (B) as a function of different cisplatin to plasmid DNA ratios. The yields were obtained from exposure curves of 5 molecule cisplatin/plasmid mixture deposited on a tantalum substrate and bombarded with $100 \pm 0.5$ eV electrons (Copyright from American Physical Society by author) (Zheng et al., 2008).

*Platinum-based radiosensitizers inhibit or alter of radiation damage repair*

Radiation recovery is considered to be the main reason for the poor response of tissue to radiotherapy. Many studies have shown that sublethal DNA damage induced by radiation can be repaired. This repair could occur through two major pathways; non-homologous end-joining
(NHEJ) and homologous recombination (HR) (Haveman et al., 2004; Myint and Raaphorst, 2002).

Platinum-based drugs can inhibit the sublethal damage repair that occurs after a cell population is exposed to a radiation dose insufficient to cause cell death (Seiwert et al., 2007). Repair involves enzymatic corrections of DNA breaks and aberrations caused by radiation and can result in tumor cell recovery to full mitotic potential. The mechanism by which platinum drugs inhibit DNA damage repair is related to the formation of platinum-DNA complexes that lead to the transformation of DNA structure into forms that could not be recognized by the DNA damage repair proteins (Hennequin and Favaudon, 2002). The integration of platinum drugs into DNA in close proximity to a radiation-induced SSB would act synergistically to make the DNA damages difficult to repair. If the cells are unable to successfully repair the DNA damage induced by ionizing radiation, the cells undergo programmed cell death or apoptosis.

A summary of previous studies of combined treatment of cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™ plus radiation is shown in table 4. The combination of chemotherapy with radiation treatment has shown positive outcomes in preclinical and clinical trials, both in terms of anticancer efficacy and toxicity, however, an optimal sequence for administration, is still required. The principle aim of improving combined treatments is to have the greatest impact on current, solid tumor treatment practice. Any postulated protocol for an optimal sequence for administration, should be first developed in the preclinical setting and be well-grounded on a comprehensive, rational understanding of experimental results, before any transition to clinical trials.
Table 4. Example of previous studies of combined treatment of cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™ plus radiation.

<table>
<thead>
<tr>
<th>Platinum drug plus radiation</th>
<th>Study</th>
<th>Treatment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>Zhang N, et al. (Zhang et al., 2009)</td>
<td>HNSCC cell lines, 0.038-0.330 μM + 2 Gy</td>
<td>Induce additive and supra-additive effect</td>
</tr>
<tr>
<td></td>
<td>Rave-Frank M, et al. (Rave-Frank et al., 2007)</td>
<td>CaSki cervical cancer, 2/24 h incubation + 0-6 Gy</td>
<td>Supra additive effect</td>
</tr>
<tr>
<td></td>
<td>HJ Groen, et al. (Groen et al., 1995)</td>
<td>GLC4 HSCLC, 30 min, 4/24 h incubation + 0-8 Gy</td>
<td>Synergism of cisplatin plus radiation</td>
</tr>
<tr>
<td></td>
<td>BC Boulmay, et al. (Boulmay et al., 2009)</td>
<td>Phase II,III,IV adjuvant H&amp;N cancer, 30 mg/m²/wk + 72 Gy</td>
<td>Improve clinical efficacy</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>Magné N, et al. (Magne et al., 2003a)</td>
<td>SW403 colorectal cancer; 6.5-1000 μM + 1 or 4 Gy</td>
<td>Oxaliplatin induced radiosensitization with no influence of p53</td>
</tr>
<tr>
<td></td>
<td>Folkword S, et al. (Folkvord et al., 2008)</td>
<td>HT29 colorectal cancer; 10 mg/kg repeated dose fractionated radiation</td>
<td>Weak endpoint of tumor growth delay</td>
</tr>
<tr>
<td></td>
<td>Kjellström J, et al. (Kjellstrom, Kjellen and Johnsson, 2005)</td>
<td>WiDr colorectal cancer; 2 h incubation 1-4 μM + 0.5-4 Gy</td>
<td>Oxaliplatin induce radiosensitization</td>
</tr>
<tr>
<td></td>
<td>Koivusalo R, et al. (Koivusalo et al., 2002)</td>
<td>SiHa cervical cancer; 0.1-300 μM + 5 Gy 6 h incubation</td>
<td>Increase apoptosis</td>
</tr>
<tr>
<td></td>
<td>Cividalli A, et al. (Cividalli et al., 2002)</td>
<td>Breast cancer; 6-14 mg/kg + fractionated IR</td>
<td>Synergism of oxaliplatin plus IR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase I study rectal</td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>Study Details</td>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Lipoplatin™</td>
<td>Charest G, et al. (Charest et al., 2010) F98 Glioblastoma; 18.22 μM of Lipoplatin + 2.21 Gy</td>
<td>Reduction of toxicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Koukourakis, et al. (Koukourakis et al., 2010) Phase I/II study advance gastric cancer; 120 mg/m²/week + 5FU + 3.5 Gy</td>
<td>High complete response rate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Charest, et al. (Charest et al., 2012) F98 Glioplastoma; 12 mg/kg + 1.5-6.6 Gy</td>
<td>Prolong survival rate and reduce toxicity</td>
<td></td>
</tr>
<tr>
<td>Lipoxal™</td>
<td>Charest G, et al. (Charest et al., 2010) F98 Glioblastoma; 4.58 μM of Lipoxal + 2.21 Gy</td>
<td>Improve the tumor uptake</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Charest G, et al. (Charest et al., 2012) F98 Glioplastoma; 12 mg/kg + 1.5-6.6 Gy</td>
<td>Prolong survival rate and reduce toxicity</td>
<td></td>
</tr>
</tbody>
</table>
**Objectives of this study**

**Overall aim**

To improve the chemoradiation treatment schedule for colorectal cancer based on “true” concomitant chemoradiation treatment to attain the best synergic effect, while minimizing the systemic toxicities. All studies were performed *in vitro* with the human colorectal cancer HCT116 cells model, and *in vivo* with nu/nu nude mice model.

**Specific aims**

1) Study the cytotoxic effects of platinum drugs, cisplatin and oxaliplatin and their liposomal formulations (Lipoplatin™ and Lipoxal™) and elucidate their pharmacokinetic characteristic such as difference tissues distribution, intracellular platinum accumulation, and DNA binding.

2) Study the effect of cell proliferation, cell cycle progression and cell death on the response to chemoradiotherapy at different treatment schedules to better understand of how chemotherapeutic agents act as radiosensitizers.

3) Determine the effect of “true” concomitant chemoradiation treatment i.e. response when radiation is delivered at such time that platinum-drugs have their highest concentration bound to DNA.
CHAPTER II

ARTICLE NO. 1

In this chapter, we report our preliminary study regarding the cytotoxicity and pharmacokinetics of cisplatin, oxaliplatin and their liposomal formulation in human colorectal cancer HCT116 cell.

This work is presented in the following article, entitled: “Cellular uptake and cytoplasm / DNA distribution of cisplatin and oxaliplatin and their liposomal formulation in human colorectal cancer cell HCT116”, by Thititip Tippayamontri, Rami Kotb, Benoit Paquette and Léon Sanche.

This article is published in Investigational New Drugs, 2011, 29(6):1321-1327.
Les formulations liposomales de cisplatine et d'oxaliplatine (Lipoplatin™ et Lipoxal™, respectivement) ont été récemment proposées pour leur capacité à réduire la toxicité systémique, tout en optimisant l'efficacité anti-cancéreuse de ces composés. Comme l'activité anti-tumorale ou l'action radio-sensibilisante de ces médicaments est attribué à leur liaison à l'ADN, nous avons évalué l'impact de ces formulations de liposomes en fonction du temps d'accumulation de ces composés de platine dans les lignées humaines de cellules de cancer colorectal HCT116 et leur distribution entre le cytoplasme et l'ADN a été mesuré. Leur cytotoxicité a été déterminée par un test de formation de colonies. La quantité de platine intracellulaire et de platine lié à l'ADN a été mesurée par plasma induit couplé à la spectrométrie de masse. Bien que, comme agent chimiothérapeutique, le cisplatine était aussi efficace que l'oxaliplatine après une exposition pendant une courte période, l'oxaliplatine et Lipoxal™ est devenu plus actif que le cisplatine contre les cellules HCT116 après 24 h d'incubation. Le Lipoxal™ a fait preuve d'une plus grande accumulation dans le cytoplasme des cellules HCT116 par rapport à l'oxaliplatine libre, en accord avec le mécanisme proposé de la fusion des liposomes avec la membrane cellulaire. La distribution cytoplasme / ADN du cisplatine libre et du Lipoplatin™ étaient similaires. À l'inverse, la distribution cytoplasme / ADN du Lipoxal™ et de l'oxaliplatin a été significativement différente : plus de 95% de l'oxaliplatine transportés par les liposomes a été pris au piège dans le cytoplasme, même après 48 heures d'incubation. Notre étude indique que le Lipoxal™ peut améliorer grandement l'absorption cellulaire de l'oxaliplatine, mais cela n'a pas mené à une augmentation similaire des bris à l'ADN.

**Mots clés:** liposomes, le cisplatine, l'oxaliplatine, Lipoplatin™ et Lipoxal™, cancer colorectal
Abstract

Liposomal formulations of cisplatin and oxaliplatin (Lipoplatin™ and Lipoxal™, respectively) were recently proposed to reduce systemic toxicity, while optimizing the anti-cancer effectiveness of these compounds. As the anti-neoplastic or radio-sensitizing activity of these drugs is attributed to their binding to DNA, we assessed the impact of the liposomal formulations on the time course of accumulation of these platinum compounds in the human colorectal cancer HCT116 cell lines and their distribution between cytoplasm and DNA. Their cytotoxicity was determined by colony formation assay. Intracellular platinum and platinum bound to DNA was measured by inductively coupled plasma mass spectrometry. Although, as a chemotherapeutic agent, cisplatin was as efficient as oxaliplatin after exposure for a short time, oxaliplatin and Lipoxal™ became more active than cisplatin against HCT116 cells after 24 h incubation. Lipoxal™ displayed a higher accumulation in the cytoplasm of HCT116 cells compared to free oxaliplatin, consistent with its proposed mechanism of fusion with the cell membrane. The distribution cytoplasm/DNA of free cisplatin and Lipoplatin™ were similar. Conversely, Lipoxal™ had a significantly different cytoplasm/DNA distribution from oxaliplatin: more than 95% of oxaliplatin transported by the liposome was trapped in the cytoplasm, even after 48 h incubation. Our study indicates that Lipoxal™ can largely improve the cellular uptake of oxaliplatin, but this was not followed by a similar increase in the DNA bound fraction.

Key words: Liposome, cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™, colorectal cancer
Introduction

Despite its nephrotoxicity and bone marrow toxicity, cisplatin (CDDP) has shown significant antineoplastic and radiosensitising activity for various solid tumours [1]. Oxaliplatin has shown a much better tolerance and similar or superior activity in different tumors compared to CDDP [2,3]. Combined with 5-fluorouracil and folinic acid, oxaliplatin improved the response rate, progression free and overall survival of patients with advanced colorectal cancer. Although no nephrotoxicity has been observed, the administration dose was still limited by the development of a neurotoxicity [4].

Lipoplatin™ and Lipoxal™, the liposomal formulation of CDDP and oxaliplatin, have been designed with the aim of reducing systemic toxicity, while simultaneously improving the targeting of drugs to primary tumor and metastases [5,6]. Preclinical studies in animal models supported an important reduction of nephrotoxicity when administrating Lipoplatin™ compared to CDDP [7]. In a phase I study, no nephrotoxicity was observed in the patients, even at a dose of 300 mg/m² [8]. Same trend was observed with Lipoxal™, which was better tolerated with fewer side effects than in patients receiving oxaliplatin [6]. Reduction of the systemic toxicity with Lipoplatin™ and Lipoxal™ did not seem to decrease their therapeutic potential, as suggested by preclinical and few clinical reports [9,10].

As radiosensitisers, only CDDP is routinely included in radio-chemotherapy protocols for different cancers [1,11]. Preliminary data on the added benefit of oxaliplatin to 5-fluorouracil and radiotherapy for rectal cancer are inconsistent, and requires further evaluation to determine the best combination and schedule [12].
For all these drugs, especially the liposomal formulations, important information regarding their ability to improve the accumulation of platinum in DNA is still missing, although it is of paramount importance to schedule radiotherapy in combination with any of these compounds.

In the present study, we assess the time course of intracellular platinum accumulation and DNA binding for each of these compounds in the human colorectal cancer HCT116 cells.

Materials and methods

Cell culture and drugs

The HCT116 human colorectal carcinoma cell line obtained from ATCC were routinely cultures in modified Eagle's medium (MEM) (Sigma-Aldrich, Oakville, Canada) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μM streptomycin in a fully humidified incubator at 37°C in an atmosphere containing 5% CO₂. Cisplatin was purchased from Sigma-Aldrich, while oxaliplatin was obtained from Sanofi-Aventis Canada through the pharmacy of the Centre Hospitalier Universitaire de Sherbrooke. Lipoplatin™ and Lipoxal™, the liposomal formulation of cisplatin and oxaliplatin respectively, were generously provided by Regulon Inc. (Athena, Greece). Their chemical structures are illustrated on figure 1. All platinum solutions were freshly prepared before usage in FBS-free MEM.
Figure 1. Chemical structures of platinum-based drugs studied and schematic of a liposome.

**Clonogenic assay**

The anticancer potential of platinum compounds was assessed by using a colony formation assay. Briefly, $10^3$ cells from a single-cell suspension were seeded into 100-mm cell culture dishes containing 10 ml of culture medium and incubated at 37°C in a humidified atmosphere containing 5% CO$_2$ for 24 h. After removal of the culture medium, the cells were washed with PBS before treatment with platinum compounds at appropriate concentrations and further incubated for the desired period. Thereafter, the drugs were removed and the cells were incubated for another 7 days to allow the formation of colonies. Cells were fixed and stained with 0.1% crystal violet, after washing with tap water. Colonies containing more than 50 cells were counted manually to calculate the clonogenic survival fractions. For each treatment, three parallel samples were scored and the assays were repeated for 3 independent experiments. The relative colony formation (% clonogenic survival) was plotted against drug concentrations, and
the concentration of platinum compound leading to a 50% reduction of colony formation (IC\textsubscript{50}) was calculated.

**Cellular uptake of platinum compounds**

HCT116 cells (6 x 10\textsuperscript{4}/plate) were incubated for 48 h in 100-mm tissue culture plates. Cells were then incubated at 37°C for 1, 4, 8, 24 or 48 h with a platinum compound at the IC\textsubscript{50} concentration previously measured after 4 h incubation with the drug. Cells were then washed twice with PBS, harvested by trypsinization, resuspended in PBS and counted. The quantity of platinum accumulated in HCT116 cells was determined with an inductively coupled plasma mass spectrometer (ICP-MS, ELAN DRC-II, PerkinElmer). Briefly, cell suspensions were treated with 23% of nitric acid, 8% hydrogen peroxide and wet autoclaved for 1 h. The solutions were then injected as is in the ICP-MS to quantify the intracellular platinum. Cell-uptake platinum was expressed as ng of platinum per 1 x 10\textsuperscript{6} cells. As internal controls, platinum and thallium (m/z 195 and 205, respectively) were quantified in untreated cells.

**Binding of platinum to DNA**

Cells (25 x 10\textsuperscript{4}/plate) were incubated with platinum compounds at their respective IC\textsubscript{50} for 1, 4, 8, 24 or 48 h. DNA was extracted according to a salting-out procedure [13]. Briefly, cells were washed twice with warm PBS, harvested after trypsinisation, and centrifuged at 1300 x g for 3 min. The cell pellets were cleaned one time with PBS and centrifuged another time, followed by resuspended in 3 ml of a lysis buffer containing 10 mM Tris-HCl, 400 mM sodium chloride and 2 mM EDTA. To this cell suspension, 0.1 ml sodium dodecylsulfate (SDS 20%) and 0.5 ml proteinase K (10 mg/ml, DNase free) were added and incubated overnight at 37°C. The DNA was precipitated by adding of 1.2 ml of 5 M sodium chloride. The tube was
agitated for 1 min, centrifuged at 2500 x g for 15 min and then the supernatant was transferred to another tube. The DNA was precipitated with 2.5 vol. of 95% ethanol, the tube was gently inverted for 30 s, and the DNA was spooled out and air-dried briefly. The DNA was dissolved in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and RNase A (0.05 ml of a 10 mg/ml solution) was added and incubated for 1 h at 37°C. The DNA was precipitated a second time with ethanol as described above and redissolved in TE buffer. The amount of DNA extracted was evaluated by measuring the absorbance of the DNA solution at 260 nm (A260) with a spectrophotometer (Synergy HT, BIO-TEX) and calculated using the following equation: A260 x 50 (pg/ml). Concentration of platinum was expressed as ng of platinum per µg DNA. No platinum was detected in the untreated control cells.

Statistical analysis

The mean ± SD were calculated. P < 0.05 was considered statistically significant (two-tailed paired Student’s t-test).

Results

Cytotoxicity potential

The IC₅₀ values for the platinum compounds tested are reported in Table 1. After 4 h incubation with the drugs, higher concentrations of Lipoplatin™ and Lipoxal™ were required to reduce the cell survival by 50% compared to their free analogs cisplatin and oxaliplatin. Since the rate of platinum accumulation in cancer cells in vitro could be reduced when the drugs were transported into liposomes, the incubation time with the drugs was extended to 24 h (Table 1). In this case, lower concentrations for all the platinum drugs were required to reach the IC₅₀. The free cisplatin and oxaliplatin still led to a better anticancer effect than their
respective liposomal formulation, the most effective platinum drug being oxaliplatin for treating the HCT116 cells.

**Table 1. IC₅₀ values of the platinum compounds for the HCT116 cells**

<table>
<thead>
<tr>
<th>Platinum compounds</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>7.5 ± 0.7</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>7 ± 2.2</td>
</tr>
<tr>
<td>Lipoplatin™</td>
<td>70 ± 10.5</td>
</tr>
<tr>
<td>Lipoxal™</td>
<td>21 ± 2.7</td>
</tr>
</tbody>
</table>

*IC₅₀ in μmol/l ± S.D.

**Time course of cellular accumulation**

The time course of platinum compounds accumulated in HCT116 cells is shown in Fig. 2. After 4 h incubation, cisplatin, oxaliplatin and Lipoxal™ accumulated at similar levels in the HCT116 cells (≈ 1 ng Pt / 10⁶ cells). Although incubation with Lipoplatin™ led to the same level of cytotoxicity (IC₅₀), the concentration of platinum measured in the HCT116 cells using this liposomal formulation was about 3-fold higher than the other platinum compounds.

Incorporation of oxaliplatin into liposomes considerably improved cellular uptake. While the cellular concentration of free oxaliplatin has reached a plateau after 24 h incubation, its liposomal formulation Lipoxal™ continued to accumulate in HCT116 cells even after 48 h incubation.
Regarding cisplatin and Lipoplatin\textsuperscript{TM}, the uptake was initially accelerated with the liposomal preparation. However, a similar accumulation in HCT116 cells was measured after 48 h incubation.

![Figure 2](image_url)

**Figure 2.** Time course of platinum derivatives accumulation in HCT116 cells. The cells were incubated at the IC\textsubscript{50} concentrations previously measured after 4 h incubation. The amount of platinum accumulated in the cells was measured by ICP-MS. Each point represents the mean ± SD (n=3).

Accumulation of the platinum compounds in HCT116 cells induces DNA damages that could result in cell death, and consequently a reduction of cell number. Cisplatin, oxaliplatin and Lipoxal\textsuperscript{TM} accumulated initially in the HCT116 cells at a similar level and they led to an equivalent reduction of cell number as shown in Fig. 3. Regarding Lipoplatin\textsuperscript{TM}, an initial reduction of cell number was followed 24 h later by a significant recovery of the cell proliferation capacity. This result contrasts with the higher ability of this liposomal formulation.
to accumulate platinum in the cells during the first 24 h incubation, compared to the other platinum compounds.

(◊) cisplatin
(☐) oxaliplatin
(♦) Lipoplatin™
(■) Lipoxal™

Figure 3. Cell proliferations in presence of the platinum derivatives. Cells were incubated at the IC50 concentration previously measured after 4 h incubation. Each point represents the mean ± SD (n=3).

DNA accumulation

Binding of the four platinum compounds to DNA are compared in Fig. 4. For the same level of toxicity (IC50) after 4 h incubation, cisplatin and oxaliplatin accumulated in DNA at a similar level. However, when included into liposomes, much lower quantities of cisplatin and oxaliplatin were linked to DNA for the same cytotoxicity level.
Figure 4. Amount of platinum bound to DNA after exposing the HCT116 cells to platinum drugs for 4 and 24 h. Cells were incubated at the IC\textsubscript{50} concentrations previously measured after 4 h (■) and 24h (■). The amount of platinum bound to DNA was measured by ICP-MS. Each point represents the mean ± SD (n=3).

After 24 h incubation, lower concentrations were required to reach the IC\textsubscript{50} for all platinum compounds (Table 1). A similar reduction (4-5 folds) of cisplatin and oxaliplatin accumulated in DNA was also measured (Fig. 4). Conversely, their respective liposomal formulation resulted in better accumulation in DNA than recorded with IC\textsubscript{50} after 4 h incubation. DNA binding measured for Lipoplatin™ was even higher than that measured with cisplatin.

The kinetic of platinum uptake in DNA was then followed up to 48 h, with concentrations of platinum compounds leading to the IC\textsubscript{50} measured after 4 h incubation. For the free cisplatin and oxaliplatin, the rapid initial accumulation in DNA was followed by a
progressive reduction which seem to slowly stabilized at about 0.14 ng Pt/μg DNA (Fig. 5). When included in their liposomal formulation Lipoplatin™ and Lipoxal™, their kinetics of accumulation in DNA were much slower starting at a level more than 10 times lower. After 48 h incubation, Lipoplatin™ led to a level of DNA accumulation approaching that of the free cisplatin. Lipoxal™ bound about 4 times less platinum to DNA compared to the other compounds (Fig. 5).

Figure 5. Time course of platinum binding to DNA after exposing the HCT116 cells for 4 h. Cells were incubated at the IC_{50} concentrations previously measured after 4 h. The amount of platinum accumulated in the cells was measured by ICP-MS. Each point represents the mean ± SD (n=3).
After entering the cancer cell, the distribution of platinum compounds between the cytoplasm and the nucleus could affect their anti-cancer potential. The kinetics of their distribution was therefore assessed to determine the impact of the liposomal formulation (Fig. 6).

A) Cisplatin

B) Oxaliplatin

C) Lipoplatin™

D) Lipoxal™

**Figure 6.** Distribution of platinum compounds in cytoplasm (■) or bound to DNA (■). Each point represents the mean ± SD (n=3).
After 4 h incubation with the HCT116 cells, the majority of the cisplatin was found in DNA. This equilibrium then moved to the cytoplasm where more than 90% of the cisplatin was measured after 48 h incubation with the cells (Fig. 6A). For oxaliplatin, the maximal accumulation and distribution in DNA was measured after 8 h incubation. As observed with cisplatin, most of the oxaliplatin accumulated after longer exposure to the cells was mainly found in the cytoplasm (Fig. 6B). Lipoplatin™ resulted in a generally lower distribution of platinum in DNA compared to the free cisplatin (Fig. 6C). Again, the platinum was mainly measured in the cytoplasm after 48 h incubation.

A completely different distribution compared to free oxaliplatin was observed (Fig. 6D) with Lipoxal™: despite the higher cell uptake reached (Fig. 2), most of the Lipoxal™ remained in the cytoplasm and the fraction bound to DNA never exceeded 5% (Fig. 6D).

**Discussion**

Liposomal formulations of cisplatin and oxaliplatin (Lipoplatin™ and Lipoxal™) have been proposed to reduce the systemic toxicity of these platinum compounds, while optimizing their anti-cancer effectiveness [5,6]. In the present study, effects of the liposomal formulations in the time course of the cellular accumulation of these platinum compounds and their distribution between cytoplasm and DNA were evaluated.

The relative cytotoxicity of cisplatin, oxaliplatin and their respective liposomal formulations Lipoplatin™ and Lipoxal™ varied according to the incubation time with the colorectal carcinoma cells HCT116. As expected, lower concentration of all these platinum compounds were required to reach the IC_{50} after 24 h incubation, than measured after 4 h incubation. Although cisplatin was as efficient as oxaliplatin after a short exposure time,
oxaliplatin either under its free form or transported in the liposome Liposomal™ became more efficient than cisplatin for treating the HCT116 cells after 24 h incubation. The highest effectiveness of oxaliplatin corresponds to clinical observations showing that DNA mismatch repair (MMR) deficient colorectal cancer cells, such as the HCT116 cells, are more sensitive to oxaliplatin than cisplatin. The oxaliplatin DNA-adducts are apparently not recognized or processed by the MMR system in the same way as cisplatin DNA-adducts. The structural basis for the lack of oxaliplatin recognition is unclear and could involve a distortion in DNA that is distinct from that produced by cisplatin [14].

In our study, the concentration of free cisplatin has to be about 8-fold higher than that of oxaliplatin to reach similar accumulations in DNA and lead to the same level of cancer cell toxicity. Similar results were reported by Kitada et al. [15]. These results further supported the hypothesis that oxaliplatin adducts are not efficiently processed by the MMR deficient HCT116 cells as it is the case with cisplatin adducts.

Mechanisms responsible for saturation of the cellular uptake of free oxaliplatin remain unclear. Platinum compounds accumulation was reported to occur through some copper transporters such as the uptake transporter hCtrl and the polyspecific organic cation transporter of hOCT1 [16]. Conversely, platinum compounds can be pumped out through the efflux transporters ATP7A and ATP7B, suggesting a contribution of these transporters to the sensitivity of cells [17-19]. A decrease in the accumulation of platinum is reported to be the most common mechanism of resistance [20]. Our study supports that incorporation of oxaliplatin in the liposome Liposomal™ largely improved its accumulation in the HCT116 cells. This result suggests that liposomes can bypass the uptake and efflux transporters, and potentially could increase the anti-cancer activity of oxaliplatin. These data further support the
proposal [10] that liposomal encapsulation promotes fusion of the liposome nanoparticle with the cell membrane resulting in higher cellular accumulation compared with the free drug. Thus, liposomal platinum drugs could find clinical application as second-line therapy treatments after front-line platinum drug-treatment against tumors in patients with resistance developed at the level of cell membrane rather than at the level of faster repair of DNA lesions or increased levels of glutathione.

Regarding cisplatin, it continued to accumulate in a time-dependent manner. Its incorporation into the liposome Lipoplatin™ accelerated its cellular uptake during the first 24 h of exposure; but no significant improvement was measured when the exposure time was extended to 48h. Therefore, the uptake and efflux transporters seemed to be less limiting for cisplatin in HCT116 cells.

The major target of platinum-based drug is DNA. Thus, when a platinum complex enters a tumor cells, cytotoxicity is not yet assured until drug enters the nucleus and reacts with DNA [21]. It was suggested that Lipoplatin™ and Lipoxal™ directly fusion with the membrane of the tumor cell to deliver their contents [22]. On the other hand, liposomes can also delivery their content by endocytosis. The resulting endosome ferry the platinum throughout the cytoplasm and fuse with a lysosome to form an endolysosome, followed by the release of the drugs. The rate of these events varies typically from half to several hours [23].

We have determined whether delivery of cisplatin and oxaliplatin through the liposome Lipoplatin™ and Lipoxal™ affected their cytoplasm/DNA distribution. Using isotoxic doses, Lipoplatin™ initially reduced the distribution of cisplatin to DNA, but a similar distribution cytoplasm/DNA was measured after 48 h incubation. On the other hand, Lipoxal™ has largely modified the distribution cytoplasm/DNA of oxaliplatin. More than 95% of oxaliplatin
accumulated in cancer cells was trapped in the cytoplasm, even after 48 h incubation. These results suggest that different pathways for delivering cisplatin and oxaliplatin occurred when in their liposomal formulation. The cell uptake study showed that Lipoxal™ and Lipoplatin™ led to similar platinum accumulations, although Lipoplatin™ initially accelerated the accumulation of cisplatin. However, the lower accumulation of oxaliplatin in DNA indicates that oxaliplatin seems to be entrapped in the cytoplasm when delivered by Lipoxal™. Conversely, Lipoplatin™ behaved quite differently, leading instead of an improvement of cisplatin accumulation in DNA. These results suggest that cisplatin transported by its liposomal formulation may be released at a faster rate from the endolysosome. Another hypothesis is that the delivery pathway through the direct fusion of the liposome with the membrane of the tumor cell could be more efficient for Lipoplatin™ than Lipoxal™.

In general, the degree of cytotoxicity correlated with the amount of platinum bound to DNA [18]. In our study, Lipoxal™ was a more efficient anti-cancer agent than Lipoplatin™, although much less platinum was accumulated into DNA using this liposome. These results suggest that either oxaliplatin is distributed in DNA following a different and more efficient pathway than cisplatin when carried under their liposomal formulation, or oxaliplatin reacts with different sensitive targets in the cytoplasm. A third hypothesis could be that cisplatin DNA-adducts are removed less efficiently in the MMR deficient HCT116 cells than the oxaliplatin DNA-adducts resulting in a higher accumulation of cisplatin in DNA. One additional suggestion is that platinum drugs kill cancer cells by modulating signalling pathways that are different than those of DNA damage-induced apoptotic pathways [10]; in this case, Lipoxal™ could activate a more potent signal transduction regulatory pathway leading to apoptosis in cancer cells than Lipoplatin™.
In conclusion, incorporation of oxaliplatin into the liposome Lipoxal™ largely improved its accumulation in the HCT116 cells. However, for the same cytotoxic effect (IC₅₀), less platinum was bound to DNA when using Lipoxal™. Animal studies are required to determine the anti-cancer effect of Lipoxal™ and its potential advantage in reducing the systemic toxicity of oxaliplatin.

Acknowledgments

Canadian Institutes of Health Research (grant # MOP 81356). Léon Sanche, Benoit Paquette and Rami Kotb are members of the Centre de recherche Clinique-Étienne Lebel supported by the Fonds de la Recherche en Santé du Québec. Sanofi-Aventis Canada has offered a partial unrestricted grant to support this project.

References


CHAPTER III
ARTICLE NO. 2

To investigate the role of radiation sensitizing of platinum drugs, induction of DNA damage and cell cycle accumulation, we performed our study presented in article no.2.

The complete work is reported in the following article, entitled: "Synergism in concomitant chemoradiotherapy of cisplatin and oxaliplatin and their liposomal formulation in human colorectal cancer HCT116 model", by Thititip Tippayamontri, Rami Kotb, Benoit Paquette and Léon Sanche.

This article has been accepted to publish in Anticancer Research, vol. 32(10), 2012.

ANTICANCER RESEARCH 32: 4395-4404 (2012)

Synergism in Concomitant Chemoradiotherapy of Cisplatin and Oxaliplatin and their Liposomal Formulation in the Human Colorectal Cancer HCT116 Model

THITITIP TIPPAYAMONTRI1-2, RAMI KOTB1, Benoît PAQUETTE1,2 and LEON SANCHE1,2

1Department of Nuclear Medicine and Radiobiology. 2Center for Research in Radiotherapy. Faculty of Medicine and Health Sciences, Université de Sherbrooke.
3Department of Medicine, Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, QC, Canada
Résumé

Contexte: Nous avons choisi de tester l'effet de l'association chimio-radiothérapie à 8 h (le plus haut niveau de l'ADN-platine) et 48 h (le plus bas niveau de l'ADN-platine) afin de vérifier si l'irradiation au maximum de concentration ADN-platine pourrait améliorer la synergie. Méthodes: Inhibition de la croissance de la lignée humaine de cellules HCT116 (cancer colorectal) traités avec le cisplatine, l'oxaliplatine, le Lipoplatin™ et le Lipoxal™ combiné avec le rayonnements gamma a été déterminé par un essai de formation de colonies. La synergie a été évaluée par l'indice de combinaison. Résultats: Pour les 8 h et 48 h d'exposition au cisplatine ou Lipoplatin™ suivie d'une irradiation, les concentrations de médicament supérieures à l'CI50 a été jugée synergique, tandis qu'une plus faible concentration que l'IC50 était antagoniste. Pour l'oxaliplatine, l'exposition à une concentration supérieure à l'IC50 pendant 8 h était synergique, tandis que l'exposition à l'oxaliplatine (à n'importe quelle concentration) pendant 48 heures était antagoniste. Le Lipoxal™ améliore de façon significative la synergie par rapport à l'oxaliplatine libre. Tous les composés platinés testés sensibilisaient les cellules HCT116 soumises à l'irradiation en recrutant les cellules en phase G2. Conclusion: Les différentes concentrations de composés platinés et l'intervalle de temps entre l'administration du médicament et la radiothérapie pourrait donner des résultats différents dans la radiochimiothérapie.
Abstract

Background: We choose to test the effect of associating chemoradiotherapy at 8 h (the highest level of DNA-platinum) and 48 h (the lower level of DNA-platinum) to clarify if irradiation at the maximum DNA-platinum concentration could improve the synergism. Methods: Growth inhibition of the human colorectal cancer cell line HCT116 treated with cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™ plus gamma-radiations was determined by a colony formation assay. The synergism was evaluated using the combination index method. Results: For 8 h and 48 h exposure to cisplatin or Lipoplatin™ followed by irradiation, drug concentrations higher than IC₅₀ was found to be synergic, while a lower than IC₅₀ concentrations was antagonistic. For oxaliplatin, exposure to a concentration above IC₅₀ for 8 h was synergistic, while the exposure to oxaliplatin (at any concentrations) for 48 h was antagonistic. Lipoxal™ significantly improve synergism compared to its parent drugs. All platinum drugs tested sensitize radiation-treated HCT116 cells by inducing G₂ phase fraction. Conclusion: The difference of drug concentrations and time interval between drug administration and radiotherapy could give different results in chemoradiation therapy.
Introduction

The addition of chemotherapy to radiotherapy is expected to improve treatment outcome of numerous malignant diseases. This is evident in head and neck, lung, anal and rectal cancers (1-3), but for some other cancers the benefit of such association is less clear (ex. pancreatic cancer) (4). Some chemotherapeutic agents (as well as other drugs) have also been proposed to enhance the efficacy of radiotherapy, in addition to their potential anti-neoplastic effect.

In vitro, in vivo and clinical studies revealed the complexity of the interactions and showed promising results when platinum chemotherapeutic agents were combined with radiation (5, 6). Platinum salts, cisplatin and oxaliplatin have been shown to possess radiosensitizing properties (7-9) by means of increasing apoptotic cells death. In addition, the integration of platinum derivatives into DNA in close proximity to a radiation-induced SSB can act synergistically to make the defect significant more difficult to repair (10). Cisplatin and oxaliplatin appeared to be a particularly plausible choice for combination with radiation, since they are not considered to be radio-mimetic drugs (11).

However, the mechanism underlying such radiosensitisation of platinum drugs is not fully understood in most cases. Moreover, the effect of the relative time sequence of the combined treatment of chemotherapy and radiation has not been systematically studied. We recently observed the relationship between exposure time to various concentrations of different platinum salts and the platinum level in different intracellular compartments (cytoplasmic, nuclear and DNA-bound platinum) of HCT116 cells as a function of incubation time (12). Preclinical investigation on the concomitant interaction of platinum and radiation at a specific
time point might case some light on the clinical relevance of the optimal synergism and the reduction of systemic toxic effect for colorectal cancer treatment.

Although the wide range of applications as anticancer agent of cisplatin and oxaliplatin, severe side effects have been reported such as neurotoxicity, nephrotoxicity, ototoxicity and retinopathy (13). In addition, colorectal cancers have been reported for the intrinsic resistance to cisplatin and chronic resistance to oxaliplatin (14, 15), which has been observed in the reduction of the intracellular accumulation of free platinum (16). These severe side effects and cancer cells resistance to cisplatin and oxaliplatin provoke a limitation of their therapeutic efficiency in colorectal cancer treatment. In order to enhance its efficacy and reduce systemic toxicity, the drug is encapsulated in liposomal formulation.

Lipoplatin™ and Lipoxal™, the liposomal formulation of cisplatin and oxaliplatin, have been designed with the aim of reducing systemic toxicity, while simultaneously improving the circulation half-life time of the encapsulated drug that is slowly released as liposomes degrade (17, 18). In addition, the liposomal formulation has been suggested to bypass the uptake and efflux transporters, and potentially increase the delivery of free-platinum drugs to the tumor cells. Liposomal drugs have a better tolerance profile and are highly accumulated in the tumor, properties that promise an optimal radiosensitzation. A Phase I/II study in advanced gastric cancer showed that Lipoplatin™ radio-chemotherapy is feasible, with minor hematological and nonhematological toxicity (19).

In the current work, we describe the effect of radiotherapy at different times of exposure to these platinum salts (which implies different concentrations of platinum drugs). Another purpose was to compare the synergistic effects between free-platinum formulation, cisplatin
and oxaliplatin, with their liposomal formulation, Lipoplatin™ and Lipoxal™. The interaction of platinum and radiation was investigated with respect to colonies formation, combination index and apoptosis. From these data, we assessed the impact of these timings on the synergism between chemo-radiotherapies.

Materials and Methods

Cell lines and drugs

The HCT116 colorectal carcinoma cell line obtained from American Type Culture Collection is a p53 wild type. Cells were routinely cultures in modified Eagle’s medium (MEM) (Sigma, Oakville, Canada) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM Sodium-Pyruvate, 100 units/ml penicillin and 100 μM streptomycin in a fully humidified incubator at 37°C in an atmosphere containing 5% CO₂. Cisplatin and oxaliplatin were purchased from Sigma-Aldrich. Lipoplatin™ and Lipoxal™, the liposomal formulation of cisplatin and oxaliplatin, respectively, were generously provided from Regulon Inc. (Athena, Greece). All drugs were diluted to the given concentrations in culture medium immediately before to use in FBS-free MEM.

Clonogenic assay

The cytotoxicity of platinum and ionizing radiation against HCT116 cell line was assessed by colony formation assay. For chemoradiation combination therapeutic studies on HCT116 cells, we performed with three groups of treatment: 1) the control group treated with four representative platinum-based drugs only at five concentrations, 2) the group treated with ionizing radiation at four doses of radiation, 3) the group treated with a combination of drugs and radiation, at a constant of the IC₅₀ and LD₅₀ values (IC₅₀ referred to, and LD₅₀ referred to
the dose of drugs or radiation causing a 50% reduction of cell growth compared with untreated cells). All the experiments began by growing the 1000 cells from a single-cell suspension in 100-mm cell culture dishes containing 10 ml of culture medium and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. After the culture medium was removed, the cells were washed twice with PBS before following treatments.

For chemotherapy alone, cells were treated with platinum compounds at appropriate concentrations and further incubated for the desired period of 8 h and 48 h. Thereafter, the drugs were removed and the cells were incubated for another 7 days to allow the formation of colonies. Cells were fixed and stained with 0.1% crystal violet, after washing with tap water. Colonies containing more than 50 cells were counted manually to calculate the clonogenic survival fractions. Each experiment was performed at least of 3 times using triplicate cultures for each drug concentration. In addition, the cytotoxicity of platinum drugs alone was examined in order to determine the IC₅₀ values, i.e. the concentrations of drugs causing a 50% reduction of cell growth compared with untreated cells.

For radiation treatment, cells were irradiated with gamma-rays (γ-rays) during exponential cell growth using a ⁶⁰Co unit at a dose rate of 1.64 Gy/min. Cells were maintained in MEM supplemented with 10% FBS during all the radiation exposures, which were performed at room temperature. Dose response curves were established for the HCT116 cells using a total with 4 single doses of 1, 2, 3 and 5 Gy.

For concomitant chemoradiotherapy, cells were treated with the platinum compound at appropriate concentrations, which were the same concentrations as used to study the cytotoxicity of chemotherapy alone. Thereafter, the drug was removed and the cells were
further incubated for the desired periods. We selected exposure time of 8 and 48 h as they were previously shown to correlate with the highest and lowest levels of DNA-platinum adducts, concentration in the nucleus, respectively (12). Then, the cells were irradiated with γ-rays at the LD$_{50}$ value (i.e., at a single dose of 2.3 Gy, obtained from the results of Fig. 1). This figure shows the cell survival fraction decreased as a function of radiation dose. The 2.3 Gy value was obtained on platinum-free HCT116 cells and corresponds to the radiation dose which resulted in a reduction of 50% of the colonies formation.

After treatment with either radiation alone or concomitant chemoradiotherapy, the cells were incubated for additional 7 days (to allow the formation of colonies) before cells fixation and counting colonies formation were performed as described above. Each experiment was performed at least of 3 times using triplicate cultures for each drug concentration.

The result of colonies formation assays from different groups (chemotherapy alone, radiation alone and combined treatments) were used to calculate the combination index (CI) in order to assess the level of synergy as described in the following section.

**Determination of combination index: Antagonism/Synergism/Additivity**

Evaluation of the nature of activity of platinum complexes in combination with radiation is mutually non-exclusive modalities, since the plots for drug, radiation and their combination are not parallel to each others. This evaluation and the CI calculation with respected to cell survival were performed according to the method described by Chou and Talalay (20). The CI was evaluated for each drug at 8 and 48 h of incubation. We also sought to determine whether the synergism would be reached at platinum drug dose equivalent, lower and higher than that of respective IC$_{50}$. A lower concentration was defined as the average of
minimum tested concentration to the concentration at IC\textsubscript{50} values, and while a higher concentration defined as the average of concentration at IC\textsubscript{50} values to maximum tested concentration.

Mathematical evaluation of the data was carried out by computerized analysis of the median dose effect (IC\textsubscript{50}) and the combination index as well as linear correlation coefficient (r). According to the CALCUSYN software of BIOSOFT (Ferguson, MO) developed by Chou (21), the CI is expressed by the relationship where each term represents,

\[
CI = \frac{(Pt)}{(Ptx)} + \frac{(IR)}{(IRx)} + \frac{(Pt)(IR)}{(Ptx)(IRx)}
\]

the denominator, (Ptx) is for the concentration of platinum compound “alone” that inhibits colonies formation at x%, and (IRx) is for the dose of radiation alone that inhibits colonies formation at the same x%. In the numerator, (Pt) + (IR) “in combination” also inhibit the colonies formation also at the same x%. CI values <1.0, and = 1.0, and >1.0 indicate synergistic, additive, and antagonistic effects, respectively.

**Cell cycle and apoptosis analysis**

Cells were treated with platinum drugs at concentrations corresponding to their specific IC\textsubscript{50}, as determined by the clonogenic assay. After 8 h and 48 h of platinum drug exposure with or without radiation treatment, both adherent and floating cells were washed and allowed to grow for an additional 48 h in culture medium. Cells were harvested by trypsinization, pooled with the culture medium that contained floating cells, and collected by centrifugation (3 min, 1300 rpm). The cell pellets were resuspended in 0.5 ml of PBS and fixed with 1.5 ml of ice-cold 95% ethanol. Before analysis, 100 µl of FBS was added. The cell pellets were re-
suspended in 2 ml PBS and collected the pellet by centrifugation (3 min, 1300 rpm). The 200 μl of staining solution containing 0.5 mg/ml of RNase A and 5 μg/ml of propidium iodide was added, and the suspension was incubated at 37°C for 30 min. The samples were analyzed using FACScan (Becton Dickinson). The cells in the subdiploid (sub-G1/G0) region of the histogram are classified as death cell in apoptosis assay. For each sample 10,000 events were analyzed. These analyses were performed at least three separate cultures.

Statistical analysis

Results were expressed as the mean ± SD of three experiments performed in triplicate. P < 0.05 was considered statistically significant (two-tailed paired Student's t-test).

Results

Cytotoxicity potential:

Clonogenic assay for platinum-drugs chemotherapy

The survival of HCT116 clonogenic cells as a function of platinum-based drug concentration for four tested drugs on cells at 8 and 48 h incubation are shown in Figure 2 and 3, respectively, for the four drugs tested. The IC50 values for all of the drugs tested in HCT116 cells for 8 and 48 h are summarized in Figure 4. After 8 h incubation of cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™, the IC50 values were observed at 4.65 μM, 2.29 μM, 41.62 μM and 5.61 μM, respectively. These IC50 values of four tested drugs were reduced about 6.4-, 35.8-, 9.95- and 13.7-times, respectively, when the incubation time was increased to 48 h.
Clonogenic assay for radiation treatment

According to the clonogenic assay, the cell survival fraction decreased as a function of radiation dose (Figure 1). The LD$_{50}$ for the irradiated HCT116 cells was 2.30 ± 0.98 Gy. This value was further used in combination with platinum drugs at different concentrations to measure the CI.

![Figure 1](image)

**Figure 1.** Dose response curve for radiotherapy alone on HCT116 cells. Cells were irradiated with gamma-rays (γ-rays) during exponential cell growth using a $^{60}$Co unit at a dose rate of 1.64 Gy/min. Each point represents mean of 3 independent experiments and the bars indicate the standard deviation (S.D).

Clonogenic assay for chemoradiation combination

Similar plot of clonogenic cell survival were constructed for all chemoradiation combinations. The results are shown in the same graph as those of the platinum-drug treatment alone at 8 h and 48 h incubation in Figure 2 and 3. The IC$_{50}$ values for all of the drugs tested combination with radiation at LD$_{50}$ dose in HCT116 cells for 8 and 48 h are summarized in Figure 4. The platinum concentration needed to obtain the IC$_{50}$ was considerably reduced
where the tested drugs were combined with radiation. Theses IC\textsubscript{50} values were reduced about 11.4-, 7.3-, 44.2- and 10.89-times for combination of radiation and cisplatin, oxaliplatin, Lipoplatin\textsuperscript{TM} and Lipoxal\textsuperscript{TM}, respectively, when increased the time of incubation to 48 h.

Figure 2. Response curves for chemoradiotherapy on HCT116 cells at 8 h incubation time. (□) Cells were exposed to various concentrations of each platinum derivative alone, or (■) in combination with gamma radiation (2.3 Gy). The concentration of the drugs is based on the IC\textsubscript{50} values, which were previously obtained from the clonogenic assay at 8 h incubation. Each point represents mean of 3 independent experiments and the bars indicate the S.D.
Figure 3. Response curves for chemoradiotherapy on HCT116 cells at 48 h incubation time. (□) Cells were exposed to various concentrations of each platinum derivative alone, or (■) in combination with gamma radiation (2.3 Gy). The concentration of the drugs is based on the IC$_{50}$ values, which were previously obtained from the clonogenic assay at 48 h incubation. Each point represents mean of 3 independent experiments and the bars indicate the S.D.
Figure 4. The IC_{50} values of cisplatin, oxaliplatin and their liposomal formulation (Lipoplatin™ and Lipoxal™) in combination with radiation. A for 8 h of drug incubation with or without radiation, and B for 48 h of drug incubation with or without radiation.
Concomitance effect of platinum compounds with radiation

The effect of adding radiation to cells treated at platinum drug concentration equivalent to, lower or higher than their respective IC$_{50}$, in the time interval of 8 h and 48 h platinum drug incubation are shown in Figure 5A and 5B.

**Cisplatin:** With a similar trend after exposure for 8 h or 48 h, the treatment with cisplatin at IC$_{50}$ showed only an additive effects (CI = 1.02 and 1.05). With a dose lower than IC$_{50}$, the combination with radiation showed an antagonistic (less than additive) effect (CI = 1.22 and 1.22), while a synergism was only observed with a concentration above IC$_{50}$ (CI = 0.74 and 0.70).

**Oxaliplatin:** 8 h exposure to oxaliplatin at or below IC$_{50}$ showed only an additive effect (CI = 0.99 and 1.05), but a synergism was noted when a dose above IC$_{50}$ was used (CI = 0.87). Exposure to oxaliplatin for 48 h (at, above or below IC$_{50}$) resulted in an antagonistic effect (CI = 1.22, 1.14 and 1.4, respectively).

**Lipoplatin:** The treatment with Lipoplatin at IC$_{50}$, for 8 h or 48 h, showed only an additive effect (CI = 0.98 and 0.94). Exposure to a concentration below IC$_{50}$ for 8 h showed an additive effect (CI = 1.06), while a similar concentration for 48 h exposure resulted in an antagonistic effect (CI = 1.19). Treatment with a concentration higher than IC$_{50}$ resulted in a synergism after 8 h or 48 h of exposure (CI = 0.77 and 0.77).

**Lipoxal:** The treatment with Lipoxal at, above or below IC$_{50}$ value for 8 h showed a synergism (CI = 0.88, 0.89 and 0.77, respectively); but this gave only an additive effect with 48 h (CI = 1.04, 1.09 and 1.01, respectively).
Figure 5. The combination index (CI) of chemoradiotherapy on HCT116 cells at A) 8 h and B) 48 h incubation time. Column (■), (□) and (▲) indicate CI values at the concentration of platinum drugs lower, higher and equal to the IC$_{50}$ value.
Cell cycle analysis

The cell cycle progression of HCT116 cell exposed to four platinum drugs for 8 h and 48 h of incubation time with and without radiation treatment were observed as shown in the Figure 6A and 6B. Radiation treatment increased the accumulation of the cells in the G₀/G₁ and G₂/M (1.15- and 1.22-time), while decreased the S phase fraction (1.63-time) compared to the control group (57.33 ± 3.26%, 29.77 ± 3.18%, 13.03 ± 1.02% for G₀/G₁, S and G₂/M phase, respectively).

Platinum chemotherapy: After 8 h incubation, the cell accumulation of the G₀/G₁, S and G₂/M phase were observed for cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™, respectively, at 56.88 ± 1.58%, 50.57 ± 1.06%, 47.32 ± 3.00% and 57.14 ± 5.86%, respectively; 22.45 ± 0.81%, 23.09 ± 1.98%, 35.10 ± 1.31% and 28.32 ± 3.86% respectively; and 20.67 ± 1.01%, 26.38 ± 1.48%, 17.58 ± 1.73% and 14.29 ± 2.36%, respectively. These values of cell accumulation in the G₀/G₁ were increased about 1.4-, 1.46-, 1.68- and 1.29-times, respectively, when the incubation time was increased to 48 h. For the cell accumulation in the S and G₂/M phase were reduced about 2.30-, 1.48-, 3.88- and 1.47-times, respectively; and 1.97-, 2.56-, 1.70- and 1.32-times, respectively, when the incubation time was increased to 48 h.

The chemoradiation combination: After combined radiation treatment with cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™ administration, the cells accumulation in the G₀/G₁ phase for 8 h was increased, while the slightly reduced of cell accumulation was observed after 48 h incubation. The reduction of the cells accumulation in the S phase after combined drug and radiation, no substantial change was observed after the combination at 48 h of drug incubation. The similar trend of the increasing of the cells in the G₂/M phase for 8 h and 48 h drug incubation was observed.
Induction of apoptotic cells

To investigate whether the induction of apoptosis cells after the combined treatment, the cells in the subdiploid (sub-G1/G0) phase were analysed (Figure 7A and 7B).

Platinum-drug chemotherapy: After 8 h incubation of cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™, the percentage of the apoptotic cells were observed at 1.54%, 1.68%, 0.76% and 1.70%, respectively. These percentages of four tested drugs were reduced about 10.3, 8.0, 1.0 and 1.2-times of magnitude, respectively, when the incubation time was increased to 48 h.

Chemoradiation combination: The percentage of apoptotic cells were considerably induced where HCT 116 cells were incubated to the tested drugs for 8 h prior the radiation treatment. The apoptotic cells were reduced about 1.1-, 2.2-, 1.6- and 1.3-times for combination of radiation and cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™, respectively, when increased the time of incubation to 48 h.
Figure 6. Flow cytometric measurement of cell-cycle distribution of the HCT116 cell after cisplatin, oxaliplatin, Lipoplatin™ and Lipoxal™ administration for A) 8 h and B) 48 h incubation, followed by irradiation at 2.3 Gy. The concentration of the drugs is based on the IC_{50} values, which were previously obtained from the clonogenic assay at 8 h or 48 h incubation. Mean of three experiments is shown.
Figure 7. Induction of apoptotic cell death after treatment with platinum drugs and radiation. The number of apoptotic cells was quantified by PI staining and flow cytometric analysis in HCT116 cells treated with A) 8 h and B) 48 h incubation of platinum drugs alone or in combination with radiation at 2.3 Gy. The concentration of the drugs is based on the IC$_{50}$ values, which were previously obtained from the clonogenic assay at 8 h or 48 h incubation. Mean of 3 independent experiments is shown and the bars indicate the S.D.
Discussion

Despite their narrow therapeutic index and cumulative toxicity, platinum compounds are widely used as chemotherapeutic agents. Almost all current cisplatin-based chemoradiotherapy protocols consist of daily administration of radiotherapy, with intermittent administration of the agent. This implies that the tumor and healthy tissues are exposed to radiotherapy after different periods of administration of cisplatin, and that the drug level varies overtime. The same scheme is also found in most protocols associating oxaliplatin and 5-FU regimens in the chemoradiotherapy treatment of rectal cancer.

The cytotoxicity of platinum-based drugs is dose and time-dependent (5, 22). An effective combination of radiation and chemotherapy is expected to lead to synergism or at least yield an additive effect on tumor cells. Bentzen and co-worker have been suggested that for maximal cytotoxic enhancement the drug must be present at the time of irradiation to modify the initial stage of radiation-induced cell killing or repair (23). Adequate information concerning the pharmacokinetics of each drug used as well as the intra-tumor drug distribution could therefore help optimizing the treatment schedule.

We previously studied the kinetic of cisplatin and oxaliplatin uptake and that of their liposomal formulation uptake in human colorectal cancer cell line HCT116 (12) to determine the amount of platinum in different tumor cell compartments and DNA-bound platinum at different time intervals after administration. For the free cisplatin and oxaliplatin, the rapid initial accumulation in DNA reached respectively 0.16 ng Pt/μg DNA and 0.32 ng Pt/μg DNA after 8 h incubation. The levels of platinum DNA adducts were progressively reduced as a function of time down to a minimum at 48 h. More than 90% of the drugs were accumulated in the cytoplasm after 48 h incubation with the cells. Lipoplatin™ and Lipoxal™ resulted in a
generally more than 10-fold lower distribution of platinum in DNA compared to their free formulation. The results of cellular uptake and cytoplasm / DNA distribution of cisplatin and oxaliplatin and their liposomal formulation in HCT116 suggested the point of maximum DNA-platinum adducts formation at 8 h.

In addition, the radiosensitivity is a function of the cell cycle. The radiosensitive is pronounced when the cells are in the G0/G1 and G2/M phase, while cells are radioresistant when the cells are in the S phase. The G1 arrest was a majority of the cell cycle analysis showed that the majority of the cells after drug treatment for both 8 h and 48 h incubation with and without radiation were accumulated in the G1 phase. Our results are similar to the previous studied (24-26). Moreover, the cell accumulation in the G2/M phase which is the most radiosensitive phase, were mostly observed after 8 h of drug incubation with and without radiation, while no significantly change was observed after 48 h incubation. These results accompanied by a higher proportion of apoptotic cells after 8 h incubation than after drug exposure for 48 h incubation. Thus, we chose to test the effect of associating radiotherapy and chemotherapy at 8 h and 48 h to clarify if the maximum DNA-Pt could improve synergism.

Clonogenic assays after 8 h of incubation indicated that the concentration of cisplatin and oxaliplatin needed to obtain the same level of cytotoxicity was approximately 3-10 times lower than their liposomal formulation. When the incubation period is increased to 48 h, there were still about a factor of two differences in the IC50 values between cisplatin and oxaliplatin compared to their liposomal formulations. This confirms our initial results on the pharmacokinetics of platinum drugs (12) where the cells were less sensitive to the liposomal-platinum formulation than free-platinum formulation. For all tested drugs, whether after 8 h or
48 h of incubation, the association of radiotherapy reduced the IC\textsubscript{50} and increased the efficiency of HCT116 cells killing by factors of 3 to 7.

As each of these drugs remain for long time in the target tissues, while the patient is given radiotherapy daily, difference of drug concentrations and time interval between drug administration and radiation treatment could cause a difference in the combined effect. Our results confirm our hypothesis that radiotherapy at various times after injection of the platinum drugs at different concentrations could lead to different outcomes. For cisplatin, a drug concentration higher than IC\textsubscript{50} is synergic, while a lower than IC\textsubscript{50} concentration is antagonistic. Although, cisplatin showed a great improvement in synergism at high concentrations, its severe toxicity is of considerable concern. For oxaliplatin, exposure to a concentration above IC\textsubscript{50} for 8 h is synergic, while the exposure to oxaliplatin (at any concentrations) for 48 h is antagonistic. Lipoplatin\textsuperscript{TM} showed a similar trend as observed with cisplatin, but Lipoxal\textsuperscript{TM} differed significantly from its parent drugs. These results also suggested that time-dependence on irradiation are more important to reach the synergism in the case of oxaliplatin and Lipoxal\textsuperscript{TM} than for cisplatin and Lipoplatin\textsuperscript{TM}.

Several potential mechanisms of platinum-mediated radiation sensitization have been reported (5, 10, 27) to rationalize the synergic and additive effects of chemoradiation treatment. Zheng and co-worker clearly demonstrated that there is a significant increase of single and double strand breaks of DNA when DNA is irradiated in the presence of cisplatin (10). In addition, it has been proposed that ionizing radiation caused higher uptake rate, probably due to an increased permeability of the cells membrane (28, 29). Some authors suggested that a sufficient number of platinum atoms must accumulate in the cancer cell to reach a radiosensitizing effect. Gabriel and co-workers showed the relationship between cell uptake
and radiosensitizing potential (30). Liposomal formulation showed promising results in order to increase the level of cell uptake, compared to their free platinum form. High accumulation of liposomes in the cancer cells could be considerable value to increase the benefit of chemotherapy when it is combined with radiotherapy.

An antagonistic effect was proposed to be associated with the efficiency of DNA repair, as well as, alterations in cell oxygenation and the cellular levels of thiol groups (31, 32). The prolonged time of drug incubation could lead to the reduction of platinum accumulation and Pt-DNA adducts. An antagonistic effect was clearly observed when HCT116 cells were treated with oxaliplatin and Lipoxal™ at time interval of 48 h before irradiation. Importantly in the clinical treatment, typical radiation treatment schedule is 2 Gy irradiations each day for 5 days a week (33), irradiation at the time point that displayed low level of platinum concentrations in the cell would lead to antagonistic effect.

Conclusion

The present study provides information about the schedule dependence of the synergism of platinum-based drugs and radiation. Confirmation of these data and their validation in animal models can have a tremendous impact on designing future platinum based chemoradiotherapy protocols. Associating radiotherapy to the time intervals of maximum synergism could improve efficacy and limit toxicity of chemotherapeutic agents.

Acknowledgments

This research was financed by the Canadian Institute of Health Canada (grant # 81356), Léon Sanche, Benoit Paquette and Rami Kotb are members of the Centre de recherche
Clinique-Étienne Lebel supported by the Fonds de la Recherche en Santé du Québec. Sanofi-Aventis Canada has offered a partial unrestricted grant to support this project.

References


CHAPTER IV
ARTICLE NO. 3

In this chapter, we investigated the synergism of combined oxaliplatin and its liposomal formulation plus radiation treatment using an animal model. This work was performed to determine radiosensitizing activity of platinum drugs in an animal model.

The complete work is reported in the following article, entitled: “New therapeutic possibilities of combined treatment of radiotherapy with oxaliplatin and its liposomal formulations (Lipoxal™) in colorectal cancer using nude mouse xenograft”, by Thititip Tippayamontri, Rami Kotb, Benoit Paquette and Léon Sanche.

This article has been submitted to Radiation Research, 2012.
New therapeutic possibilities of combined treatment of radiotherapy with oxaliplatin and its liposomal formulations (Lipoxal™) in colorectal cancer using nude mouse xenograft

Running title: Chemoradiotherapy for colorectal cancer

Thititip Tippayamontri,a,b Rami Kotb,c Benoit Paquette,a,b and Léon Sanche,a,b

aDepartment of Nuclear Medicine and Radiobiology, bCenter for research in radiotherapy, Faculty of Medicine and Health Sciences, Université de Sherbrooke, cDepartment of Medicine, Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, Québec, Canada.

Corresponding author:
Prof. Benoit Paquette, Faculty of Medicine and Health Science, Université de Sherbrooke, 3001, 12e Avenue North, Sherbrooke, Quebec, Canada, J1H 5N4.
Tel. (1) (819) 820-6868, ext. 14767
Fax: (1) (819) 564-5442
E-mail address: Benoit.Paquette@USherbrooke.ca

ACKNOWLEDGMENTS: This research was financed by the Canadian Institute of Health Research (grant # 81356). Léon Sanche, Benoit Paquette and Rami Kotb are membres of the Centre de recherche Clinique-Étienne Lebel supported by the Fonds de la Recherche en Santé du Québec. Sanofi-Aventis Canada has also offered a partial unrestricted grant to support this project.

Même si l'ADN est considéré comme la cible principale pour la radiosensibilisation menant à la mort cellulaire, la période d'incubation suivant l'administration d'un compose platiné menant à une accumulation maximum à l'ADN et un meilleur effet concomitant n'est pour le moment pas bien déterminée pour une application clinique. Dans cet ouvrage, nous explorons le lien entre le niveau d'adduits platine-ADN menant à un effet concomitant potentiel et différent temps d'attente entre l'administration de composé platiné et la radiothérapie. Pour augmenter l'efficacité anticancéreuse tout en surmontant ces effets secondaires, nous avons également évalué une formulation liposomale de l'oxaliplatine, le Lipoxal™. Des études ont été effectuées dans le carcinome colorectal humain HCT116 implanté chez la souris nu/nu. La quantité de platine dans la tumeur, dans l'ADN tumoral, dans des tissus normaux et dans le sang a été mesurée par un spectromètre de masse couplé au plasma inductit. L'effet concomitant avec le rayonnement a été évaluée à 4, 24, et 48 h après l'injection de drogue et lorsque les médicaments ont été administrés immédiatement après la radiothérapie. Le retard de croissance tumorale obtenu est rapporté et corrélé à l'analyse d'apoptose. Le retard de croissance tumorale obtenu est rapporté et corrélé à l'analyse d'apoptose. Tandis que la quantité de platine dans le tissu tumoral atteint un pic initial 4h suivant l'administration et diminue par la suite avec le temps, le niveau d'adduits platine-ADN oscille en deux pics après l'administration. En effet, le niveau d'adduit platine-ADN atteint un premier pic à 4 h suivi d'une diminution progressive pour atteindre un premier nadir à 24 h suivi d'une nouvelle augmentation pour atteindre un pic plus élevé à 48 h avant de rebaisser, sauf pour Lipoxal™. Un important potentiel radiosensibilisant est observé lorsque la radiothérapie est effectuée 4 et 48 h après l'injection de médicaments, ce qui correspond à la pointe de concentration d'adduits platine-ADN. Ces résultats ont été associés à un plus grand nombre de cellules apoptotiques. À l'inverse, un effet antagoniste a été observé lorsque la radiothérapie a été délivrée 24 heures après l'injection d'oxaliplatine, ce qui correspond à des niveaux inférieurs d'adduit platine-ADN. Un effet concomitant a été obtenu avec le Lipoxal™.

Although DNA is considered the main target of radiosensitizing activity leading to cancer cell death, there are limited number of publications that studied on the beneficial of adding radiotherapy at the optimal time at which the platinum is bound to the DNA of tumor cells. Here, we explored relationships between different levels of platinum-DNA adducts and the potential of the concomitant effect after different schedules of combined treatment. To further increase the anticancer effectiveness while overcoming the side effects of oxaliplatin, we also evaluated its liposomal formulation, named Lipoxal™. Studies were performed with the human colorectal carcinoma HCT116 implanted in nude mice. The amount of platinum in the tumor, tumoral DNA, and in normal tissues and blood was measured in function of time using an Inductively Coupled Plasma Mass Spectrometer. The concomitant effect with radiation was assessed at 4, 24, and 48 h after drug exposure. The tumor growth delay was reported and correlated to the apoptosis analysis. While the amount of platinum in tumor tissues reached an initial peak at 4 h after drug exposure and declined over time, the concentration of platinum-DNA adducts was more varied and two maxima were observed at 4 h and 48 h after drug administration, excepted for Lipoxal™. The concomitant effect was observed when radiotherapy was performed at 4 h and 48 h after oxaliplatin administration, which were related to maximum levels of oxaliplatin-DNA adducts. These results were associated with an increase of apoptotic cell death. Conversely, no advanced improvement of the concomitant effect was observed when radiotherapy was delivered at 24 h after oxaliplatin administration, which was related to the lower level of platinum-DNA adducts. The concomitant effect was obtained with Lipoxal™ for each of combined treatment schedules.
INTRODUCTION

Concomitant chemoradiotherapy is proposed to improve the treatment effectiveness in several malignant diseases, including rectal cancer, advance head and neck squamous cell carcinoma and cervical cancer (1-4). The efficacy of concurrent chemotherapy with radiation treatment is often increased by using chemotherapeutic drugs as radiosensitizers (5). The common chemoradiation treatment for colorectal cancer is the combination of 5-fluorouracil (5-FU), leucovarin, oxaliplatin and radiation (6).

While the increased treatment efficacy of combined treatment, recent completed clinical phase III trials (ACCORD-0405, STAR-01 and NSABPR-04) that added oxaliplatin to 5-FU backbone or capcitabine have failed to improve results of combined treatment (7-9), with the exception of the German CAO/ARO/AIO-04 study (10) when added oxaliplatin to 5-FU plus radiation. In addition to increase efficacy of combined treatment, some difficulties in managing side effects from this combined treatment are still commonly found in clinical practices, such as hematologic and gastrointestinal toxicity (11). Since limited number of publications on the use of oxaliplatin in combination with radiation, Flatmark and Ree have suggested the need of initial testing radiosensitizing activity of oxaliplatin alone in preclinical setting prior the clinical use as a local radiosensitizer for radiation (12). Moreover, the radiosensitizing activity, and an accurate scheduling of combined treatment to achieving the benefit of drug-radiation interactions while maximizing tumor responses have to be determined.

The role of oxaliplatin as radiosensitizers has been recently described (6, 13). Like other platinum compounds, the main mechanism of antineoplastic and radiosensitizer activity of oxaliplatin is thought to occur mostly via the formation of platinum-DNA adducts (14, 15). The oxalate bidentate ligand of oxaliplatin preferentially reacting with the highly nucleophilic
N7 position on guanine or adenine, and form coordinated covalent bonds. Oxaliplatin can bind to two sites in DNA with the following order of preference: -GG- > -AG- > -GA. The resulting biadducts composed of approximately 60-65% 1,2-intrastrand GG, 25-30% 1,2-intrastrand AG, 5-10% 1,3-intrastrand GXG and 1-3% interstrand GG (16). Platinum binds to DNA and causes a critical structure change in the DNA such as a bend of 45 degree, unwinding of DNA and causing disrupting of the purine bases. The formation of platinum-DNA adducts which interfere with cellular repair and DNA replication, and which therefore trigger a chain of cell regulatory events, ultimately leading to cell death (17).

Although the most favorable concomitant effect would be occurred when radiosensitizers reach the DNA, the activity of radiosensitizers is often referred to the concentration of drugs accumulation in the tumor tissue (18-20). To date very few number of preclinical publications studied on combined treatment of oxaliplatin and radiation for colorectal cancer. Also the information underlying the assessment of radiotherapy at the maximum oxaliplatin-DNA adducts is not yet clarified in both preclinical and clinical setting. In this study we hypothesized that the potential of combined treatment possibly depend on the interaction of radiation at different times after drug administration, which were referred to difference levels of platinum-DNA adducts.

Previous studies demonstrated efficacy of oxaliplatin in the treatment of colorectal cancer, however the antitumor activity of oxaliplatin may be limited by its severe systemic side effects, such as neurotoxicity, haematological and gastrointestinal toxicity (21). In recent years, liposomal oxaliplatin (Lipoxal™) was developed to reduce the systemic toxicity of oxaliplatin, while attempting to improve the anticancer efficiency (22). As a single agent, Lipoxal™ has produced significant cytotoxicity in human colorectal cancer cells and showed adequate
effectiveness in pretreated patients with colorectal cancer in phase I study (23). Lipoxal™ is currently under study for phases II for colorectal cancer (22). The combination of radiation and Lipoxal™ is an interesting preclinical research subject that might shed some light on new clinical treatment strategies. To date, there is not sufficient data concerning the pharmacokinetic, anticancer activity, and combined treatment efficacy of Lipoxal™ comparative to its parent compound (oxaliplatin).

In the present work, variations of platinum accumulation with incubation time in blood, different normal and tumor tissues compartments, as well as platinum-DNA were measured. Therefore, we explore relationships between different levels of platinum-DNA adducts and the potential of the concomitant effect after combined treatment of oxaliplatin and Lipoxal™ plus radiation. The concomitant effects were evaluated with respect to tumor growth delay and apoptosis analysis.
MATERIALS AND METHODS

Cell line

The HCT116 colorectal carcinoma cell line was obtained from the American Type Culture Collection. Cells were routinely cultured in modified Eagle's medium (MEM) (Sigma, Oakville, Canada) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 1mM sodium pyruvate, 100 units/mL penicillin and 100 µM streptomycin in a fully humidified incubator at 37°C and 5% CO₂.

Animal xenograft model

All experiments were performed with outbred male nu/nu nude mice at 4-6 weeks of age (Charles River, USA). The animals were maintained in our conventional animal facility, under specific pathogen-free conditions. Housing and all procedures involving animals manipulation were performed according to the protocol approved by Université de Sherbrooke Animal Care and Use Committee. HCT 116 tumor cells were inoculated by subcutaneous (s.c.) injection into each rear flank with 0.1 mL of cell suspension containing 2 x 10⁶ cells. Tissue culture medium without serum was used as injection vehicle. Tumor measurement began 1 week post-injection and continued twice weekly through the course of the experiment. Tumor volumes were measured with a caliper and calculated by the formula: V (mm³) = π/6 x a (mm) x b² (mm²), where a and b referred to the largest and smallest perpendicular tumor diameters, respectively. The pharmacokinetic study began when tumor volumes reached the range of 100-300 mm³, with an average tumor volume of approximately 200 mm³. The tumor-bearing animals were randomized into different groups of three to five animals. For all procedures
(implantation, chemotherapy, radiotherapy and euthanasia) nu/nu nude mice were anesthetised with an intraperitoneal injection of ketamine/xylazine (87/13 mg/mL) at 1 mL/kg.

**Drug preparation**

Oxaliplatin was obtained through the pharmacy of the Centre Hospitalier Universitaire de Sherbrooke. Lipoxal™, the liposomal formulation of oxaliplatin, was generously provided by Regulon Inc. (Athens, Greece).

**Pharmacokinetics studies**

Three groups of mice were studied, including: 1) oxaliplatin, 2) Lipoxal™ and 3) control group. The tested drugs were dissolved in 5% dextrose for a final concentration of 1 mg/mL to be given as a single dose of 10 mg/kg intravenously (i.v.) via the tail vein. Animals in the control group received an injection of only 5% dextrose. Three to five animals from each group were sacrificed at 4, 24, 48, 72, and 96 h post-injection. A sufficient blood sample was obtained via cardiac puncture for later whole blood and serum platinum assessments. Tumor and different normal tissues were taken to determine platinum accumulation in the cytoplasm, the nucleus as well as in DNA extractions.

**Determination of cytoplasmic and nuclear platinum accumulation**

Samples were processed with the nuclear and cytoplasm extraction kit Activemotif (ActiveMotife North America). Briefly, tumors were weighed and diced into very small pieces using a razor blade. Three mL of ice cold 1X hypotonic buffer supplemented with 3 μL of 1 M dithiothreitol and 3 μL of detergent were added per gram of tissue, homogenized, incubated on ice for 15 min. and then centrifuged for 10 min. at 850 x g at 4°C. The hypotonic buffer and
detergent were supplied in the kit. Subsequently, the supernatants corresponding to the cytoplasmic fraction were transferred into pre-chilled microcentrifuge tubes, while the pellets were gently resuspended in 500 μL 1X hypotonic buffer, transferred to a pre-chilled microcentrifuge tube, and incubated on ice for 15 min. The detergent (25 μL) was added and vortex for 10 sec at the highest setting. The samples were centrifuged for 30 sec at 14,000 x g in a microcentrifuge pre-cooled at 4°C and these second supernatants were combined with the first ones. The resulting pellets corresponded to the nuclear fraction.

Quantification of DNA-bound platinum

DNA was extracted according to a salting-out procedure (24). Briefly, the tumor was weighed and diced into very small pieces, and homogenized with a pre-chilled Dounce homogenizer. On ice, 3 mL of a lysing buffer containing 10 mM Tris-HCl, 400 mM sodium chloride and 2 mM EDTA were added. Then, 0.1 mL sodium dodecylsulfate (SDS 20%) and 0.5 mL proteinase K (10 mg/mL, DNase free) were added and incubated overnight at 37°C. DNA was precipitated by adding 1.2 mL of 5 M sodium chloride. The tube was gently agitated for 1 min, centrifuged at 2500 x g for 15 min and then the supernatant was transferred to another tube. DNA was precipitated with 2.5 vol. of 95% ethanol, the tube was gently inverted for 30 s, and DNA was spooled out and air-dried briefly. DNA was dissolved in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and RNase A (0.05 mL of a 10 mg/mL solution) was added and incubated for 1 h at 37°C. DNA was precipitated a second time with ethanol as described above and re-dissolved in TE buffer. The amount of DNA extracted was evaluated by measuring the absorbance of the DNA solution at 260 nm (A260) with a spectrophotometer (Synergy HT, BIO-TEX) and calculated using the following equation: A260 x 50 (μg/mL).
Platinum quantification using an Inductively Coupled Plasma Mass Spectrometer

The quantities of platinum accumulated in the cytoplasm, nucleus and DNA of tumor tissue samples were determined with an inductively coupled plasma mass spectrometer (ICP-MS) (ELAN DRC-II, PerkinElmer). Briefly, samples were treated with 23% nitric acid, and 8% H$_2$O$_2$. The solutions were then injected in the ICP-MS to quantify the platinum concentration. As internal controls, platinum and thallium (m/z 195 and 205, respectively) were quantified in untreated animals. Cytoplasm and nucleus-uptake of platinum of tumor tissue samples were expressed as ng of platinum per g of cytoplasm or nucleus. Concentration of platinum in whole blood and in serum was expressed as µg of platinum per mL of whole blood and serum. DNA-Pt adducts were expressed as ng of platinum per µg of DNA. Muscle, liver and kidney uptake was expressed as ng of platinum per g of tissue.

Chemotherapy treatment

The tested drugs were dissolved in 5% dextrose for a final concentration at 1 mg/mL to be given as a single dose of 10 mg/kg intravenously (i.v.) via the tail vain. Then, platinum drugs were further incubated for 4, 24 and 48 h prior the radiation treatment, as shown in Figure 3. The selected drug concentration of oxaliplatin and Lipoxal™ did not induce the appearance of any side effects when combined with radiation treatment, as no significant decrease in body weight was observed between time of initiation and termination of treatment (Table 1). Animals in the control group received an injection of only 5% dextrose.
Table 1. Change in body weight of male nu/nu nude mice treated with i.v.\(^a\) oxaliplatin or Lipoxal\(^\text{TM}\) in combination with GK\(^b\) irradiation

<table>
<thead>
<tr>
<th>Platinum drugs</th>
<th>Body weight (g) (n = 3-5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before the treatment</td>
<td>After the treatment</td>
</tr>
<tr>
<td>Control(^c)</td>
<td>21.57±1.00</td>
<td>27.07±2.25</td>
</tr>
<tr>
<td>Oxaliplatin (48 h)-IR</td>
<td>27.37±1.14</td>
<td>31.03±1.18</td>
</tr>
<tr>
<td>Lipoxal(^\text{TM}) (48 h)-IR</td>
<td>27.60±0.95</td>
<td>30.00±0.56</td>
</tr>
</tbody>
</table>

\(^a\) i.v. = intravenous injection of single concentration 10 mg/kg of platinum drug

\(^b\) GK = Gamma Knife irradiation of single dose 15Gy

\(^c\) Control group = no chemotherapy and radiation treatment

Chemotherapy was given 48 h initially followed by radiotherapy

Tumor irradiation

To prevent the damage repair that may occur after fractionated radiation treatment (25), a single dose of radiation treatment was used in this study. Mice were anesthetised and positioned in our home made stereotactic frame designed for irradiation with a 4C Gamma Knife (Elekta Instruments AB). The radiation treatment (15 Gy with a dose rate of 3.6 Gy/min) with 8 mm collimators was delivered at predetermined coordinates targeting the tumor (Fig.3). The radiation dose of 15 Gy was taken from a previous study (26) as the dose that induced a temporary tumor growth delay and not led to a complete tumor cure. The tumors were irradiated with a single dose of radiation at one side of the rear flank, whereas the other side was kept as control for non-irradiated tumor.
A) Pharmacokinetics study

- 2 weeks
  (~125mm³)

Blood, Serum, Normal and Tumoral tissues
pharmacokinetics

s.c. implantation   i.v. injection
HCT116 cells       oxaliplatin/Lipoxal™
10 mg/kg

B) Chemoradiation treatment

- 2 weeks
  (~125mm³)

H&E, Apoptosis

Determination synergistic effect

s.c. implantation   i.v. injection
HCT116 cells       oxaliplatin/Lipoxal™
10 mg/kg

15Gy GK irradiation

C) Gamma Knife irradiation

Figure 1. The experiment setup for chemoradiotherapy in nu/nu nude mice model. A) represent the pharmacokinetics study scheme, B) represent the chemotherapy was given initially 4, 24, and 48 h followed by radiotherapy and C) represent a) the homemade stereotactic frame and b) the coordinate taking from program GKElecta. GK = Gamma Knife irradiation; s.c. = subcutaneous implantation; i.v. = intravenous injection. The dose of chemotherapy is 10 mg/kg of oxaliplatin and Lipoxal™; the single dose of radiation at 15Gy.
Determination of combination indices: Synergistic/Additive/Antagonistic

In each group of animals, the relative tumor volume was expressed as $V_t/V_0$ ratio where $V_t$ is the mean tumor volume on a given day during the treatment and $V_0$ is the mean tumor volume at the beginning of the treatment. The details for antitumor activity after chemoradiotherapy have been modified from a previous study (27). Briefly, the combined treatment activity was evaluated by the time to reach a tumor volume that was five times greater than the initial volume [5Td]. The expected 5Td of combined treatment was calculated according to the following formula: Expected 5Td = Mean control + (mean drug alone – mean control) + (mean radiation alone – mean control). The effect of combined treatment (synergistic/additive/antagonistic) was assessed by calculating the ratio of the observed 5Td [O5Td] divided by that of the expected 5Td [E5Td]. Previous study on the effect of combined treatment reported an additivity of combined treatment at the range of 0.9-1.1 (28). Then in this study, the ratio values of [O5Td]/ [E5Td] is > 1.1, the combination is indicate a synergistic; 0.9 to 1.1 the combination is additive; and < 0.9 the combination is antagonistic.

Apoptosis analysis

Groups of three nu/nu nude mice bearing HCT116 tumors were treated with platinum drugs for 24 or 48 h before radiation treatment, as described. Two days after irradiation, mice were euthanized and tumors were immediately removed, fixed in 10% buffered formalin and embedded in paraffin. Tumor sections of 4 μm were taken at three different levels. Analysis of apoptotic cells within tumor tissue was done by using a commercially available ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore), following the manufacturer's protocol. Briefly, formalin-fixed, paraffin-embedded tissue slides were rehydrated using xylene to alcohol washings, followed by a hydrogen peroxide–methanol quench. The samples were

93
treated with 20 µg/mL of proteinase K for 15 min at room temperature. After washing and incubation with equilibration buffer for 5 min, TdT (33 µL of TdT enzyme in 77 µL of reaction buffer) was incubated on the slides for 1 h at 37°C. After applying stop solution for 10 min and washing, the samples were incubated with anti-digoxigenin peroxidase conjugate (65 µL/cm² of specimen surface area) for 30 min at room temperature. Slides were developed with a 1:20 dilution of diaminobenzidine (3,3'-diaminobenzidine) substrate, counterstained with methyl green, dehydrated, and coverslipped. TUNEL-positive apoptotic nuclei were detected with a fluorescent microscope. Positive control slides were prepared by nicking DNA with DNaseI and treated similarly to the sample (data not shown). Samples treated similarly but without enzyme treatment served as negative controls. To determine the amount of cellular area stained with 3,3'-diaminobenzidine (positive for apoptosis) and the amount of tumor area counterstained with methyl green (negative for apoptosis), the tumor sections were scanned with the Olympus FSX100 microscope and the values were quantified using the Adobe Photoshop CS5 analysis software. Briefly, the percentage of the apoptotic cells was calculated as the quotient of 3,3'-diaminobenzidine-positive area over the total area (3,3'-diaminobenzidine positive + methyl green negative), times 100 (29).

**Statistical analysis**

The mean ± SD were calculated. P< 0.05 was considered statistically significant (two-tailed paired Student’s t-test).
RESULTS

Platinum concentration in whole blood, serum, tumor and normal tissues

After i.v. administration, the total blood levels of oxaliplatin or Lipoxal™ declined steadily, with a tendency for later stabilisation or even slight increased levels. These changes were more pronounced for oxaliplatin than Lipoxal™, and for whole blood than serum levels. Evolutions of total blood and serum levels are given in Figure 2A, B. Serum levels were about 10% of the corresponding whole blood levels. Figure 2C shows the accumulation of platinum in tumor tissues after oxaliplatin and Lipoxal™ administration. At 4 h, the concentration of platinum in tumor tissues after treatment with oxaliplatin was about two times higher compared to the Lipoxal™ group. For both drugs, the total tumour platinum content declined gradually over time. Similar results were obtained in muscle and kidney tissues (Figure 2D and E). Oxaliplatin and Lipoxal™ exhibited similar levels of platinum accumulation in liver tissues (Figure 2F).
Figure 2. Pharmacokinetic profiles of platinum concentration presented in blood, tumor tissues and normal tissues. The platinum accumulation in A) whole blood, B) serum, C) muscle, D) tumor, E) liver, and F) kidney, was obtained after a single i.v. injection at 10 mg/kg oxaliplatin or Lipoxal™ in colorectal cancer HCT116 tumor nude mice. The amount of platinum was measured by ICP-MS. Each point represents the mean ± SD of the concentration in 3-5 mice.
Platinum accumulation in cytoplasm, nucleus of tumor cells and tumoral DNA-bound platinum

The time profiles of platinum accumulation in the cytoplasm and nucleus of tumor cells are shown in Figure 3A and 3B. After peaking initially at 4 h, the amount of platinum in the cytoplasm and the nucleus declined gradually over time for both oxaliplatin and Lipoxal™. The level of tumoral DNA-bound platinum showed similar kinetics only in the case of Lipoxal™. For oxaliplatin, the level of tumoral DNA-bound platinum reached a first peak at 4 h, and then gradually declined to reach a first nadir at 24 h, then rose again to reach a second and higher peak at 48 h before re-declining. Even at 72 h and 96 h, levels of tumoral DNA-bound platinum were found to be closer to those at 4 h (Figure 3C).

Tumor growth delay

The tumor growth delay after nu/nu nude mice were treated initially with chemotherapy for 4, 24 and 48 h and followed by radiotherapy is shown in Figure 4. Efficiency of combined treatment is reported in Table 1. A single oxaliplatin or Lipoxal™ administration induced a significant tumor growth delay compared to non-treatment animals \( (p < 0.05) \). Treatment with radiation alone had considerable effects compared to the control group \( (p < 0.01) \). When oxaliplatin or Lipoxal™ were combined with the radiation treatment, the tumor growth delay increased significantly compared to the control group \( (p < 0.01) \). In addition, no significant decreased in body weight of animals was observed between time of initiation and termination of treatment (Table 2), suggesting the absence of additive toxicity of these combined treatment.

Figure 4A, combined treatment of oxaliplatin administration for 4, 24 and 48 h prior radiation treatment induced a dramatic reduction in the tumor growth \( (5T_{dox4h} = 25 \pm 1.41 \text{ days}; 5T_{dox24h} = 23 \pm 2.5 \text{ days}; 5T_{dox48h} = 39 \pm 2 \text{ days}, \text{respectively}) \) compared to oxaliplatin alone.
(5T_{d_{ox \text{ alone}}} = 16 \pm 0.71, p < 0.01). Similar enhancement of tumor growth delays was observed after combined treatment with Lipoxal™ for 4, 24 and 48 h of drug incubation plus radiation (Figure 4B), (5T_{d_{lipox4h}} = 39 \pm 1.41 \text{ days}; 5T_{d_{lipox24h}} = 32 \pm 1 \text{ days}; 5T_{d_{lipox48h}} = 35 \pm 0.5 \text{ days}, respectively) compared to Lipoxal™ alone (5T_{d_{lipox \text{ alone}}} = 14.5 \pm 0.71, p < 0.01). The significant different of tumor growth delay between the group of drug treatment alone and combined treatment was observed after 7 days and 5 days for oxaliplatin and Lipoxal™, respectively.

Oxaliplatin and Lipoxal™, in combination with radiation showed a greater efficacy than with radiation treatment alone (5T_{IR \text{ alone}} = 19 \pm 1.00, p < 0.01), excepted for the combination of radiation at 24 h after oxaliplatin administration (p = 0.08). Significant differences of tumor growth delay were observed 20 days after combined treatment between 48 h of oxaliplatin administration and 4 h or 24 h of oxaliplatin administration. For Lipoxal™, the tumor growth delay showed no significant different among each treatment schedules.
Figure 3. Time dependence of platinum concentration in A) cytoplasm, B) nucleus and C) platinum-DNA adducts obtained after a single i.v. injection at 10 mg/kg oxaliplatin or Lipoxal™ in colorectal cancer HCT116 tumor nude mice. The amount of platinum bound to DNA was measured by ICP-MS. Each point represents the mean ± SD of the concentration in 3-5 mice.
Figure 4. The tumor response after nu/nu nude mice were treated initially with chemotherapy and followed by radiotherapy. A and B show the combination of radiation with oxaliplatin and Lipoxal™, respectively. Treatments were administered with a single dose of platinum drugs of 10 mg/kg; and a single dose of radiation of 15Gy. Each symbol represents the mean of the results obtained with 5 mice and the bars ± S.D. (p ≤ 0.05).
Table 2. *In vivo* efficacy of platinum-based chemotherapy alone and in combination with radiation treatment in the HCT116 human colorectal cancer s.c. flank xenografts grown in male nu/nu nude mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5Td(^a) (days)</th>
<th>TGD(^b)</th>
<th>%TGD(^c)</th>
<th>Ratio of 5Td/ETd(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.5 ± 0.71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR alone</td>
<td>19 ± 1.00</td>
<td>8.5 ± 1.00*</td>
<td>80.95</td>
<td></td>
</tr>
<tr>
<td>Oxaliplatin alone</td>
<td>16 ± 0.71</td>
<td>5.5 ± 0.71*</td>
<td>52.38</td>
<td></td>
</tr>
<tr>
<td>Lipoxal(^{TM}) alone</td>
<td>14.5 ± 0.71</td>
<td>4.0 ± 0.71*</td>
<td>38.10</td>
<td></td>
</tr>
<tr>
<td>Oxaliplatin (4h)-IR</td>
<td>25 ± 1.41</td>
<td>14.5 ± 1.41**</td>
<td>138.10</td>
<td>1.07 ± 0.03(^{y})</td>
</tr>
<tr>
<td>Oxaliplatin (24h)-IR</td>
<td>23 ± 2.50</td>
<td>12.5 ± 2.50**</td>
<td>119.05</td>
<td>0.92 ± 0.06(^{y})</td>
</tr>
<tr>
<td>Oxaliplatin (48h)-IR</td>
<td>39 ± 2.00</td>
<td>28.5 ± 2.00**</td>
<td>271.43</td>
<td>1.49 ± 0.08(^{y})</td>
</tr>
<tr>
<td>Lipoxal(^{TM}) (4h)-IR</td>
<td>39 ± 1.41</td>
<td>28.5 ± 1.41**</td>
<td>271.43</td>
<td>1.76 ± 0.09</td>
</tr>
<tr>
<td>Lipoxal(^{TM}) (24h)-IR</td>
<td>32 ± 1.00</td>
<td>21.5 ± 1.00**</td>
<td>204.76</td>
<td>1.33 ± 0.07</td>
</tr>
<tr>
<td>Lipoxal(^{TM}) (48h)-IR</td>
<td>35 ± 0.50</td>
<td>24.5 ± 0.71**</td>
<td>233.33</td>
<td>1.54 ± 0.07(^{y})</td>
</tr>
</tbody>
</table>

\(^{a}\) 5Td; Time necessary to reach a tumor volume five times greater than the initial volume  
\(^{b}\) TGD is 5Td minus Control group (C) (Control group = no chemotherapy and radiation treatment)  
\(^{c}\) %TGD = (5Td - C)/C x 100%  
\(^{d}\) Ratio of 5Td/ETd obtained by dividing the 5Td by the expected 5Td of the combination.  
ETd (Expected of combination) = Mean Control + (Mean drug alone- Mean Control) + (Mean radiation - Mean Control)  
Ratio of Td/ETd obtained by dividing the observed Td by the expected Td of the combination.  
A ratio = 0.9-1.1, >1.1, <0.9 indicates additivity, synergism and antagonism  
Statistically significant (P<0.05), * compared to the control group, ** compared to both either chemotherapy or radiation treatment alone group, and * compared to combined treatment group at 24 h of drug administration before irradiation.
Combination index

Figure 5 shows a combination index after chemoradiation treatment. When combined radiation at 4 h, 24 h and 48 h of oxaliplatin administration, the combination index (CI) was 1.07 ± 0.03, 0.92 ± 0.06, 1.49 ± 0.08, respectively.

In case of Lipoxal™ combined with radiation, the CI was 1.76 ± 0.09, 1.33 ± 0.07 and 1.54 ± 0.07 at time intervals of 4, 24 and 48 h of drug incubation, respectively, suggesting a synergic effect for every time schedules.

Induction of apoptosis

Induction of apoptosis at different time intervals between drug injection and radiation treatment is shown in Figure 6A. The percentage of apoptotic cells (Figure 6B) was 0.94 ± 0.38 % and 2.86 ± 0.89 % in the untreated group and in the radiotherapy alone group, respectively. Compared to treatment with oxaliplatin alone (1.59 ± 0.31 %) (p < 0.05), combined treatment of oxaliplatin with radiation significant increased the percentage of apoptotic cells to 3.89 ± 1.05 % and 12.53 ± 2.35 % for 24 h and 48 h drug administration prior radiation treatment, respectively. For Lipoxal™, the percentage of apoptosis cells was of 1.78 ± 0.69 %, 6.41 ± 1.72 % and 16.32 ± 4.81 % for Lipoxal™ alone and when combined with radiation at 24 and 48 h of drug incubation, respectively.
Figure 5. The combination index of chemoradiotherapy in nude mice model. Animals were treated initially with oxaliplatin (■) or Lipoxal™ (□) chemotherapy followed by radiotherapy. *, statistically significant (P<0.05) from the combined treatment at 24 h of drug administration group.
Figure 6. A) TUNEL assay after chemoradiotherapy. Animals were irradiated at 24 and 48 h of drug administration. Treatment was administered with a single dose of oxaliplatin or Lipoxal™ of 10 mg/kg; and a single dose of radiation of 15Gy. B) Percentage of apoptosis cells after chemoradiotherapy. Apoptotic Fig.s were counted in 10 random fields per tumor (n=3) (Mean ± S.D.). Magnification, X40. Statistically significant (P<0.05) * compared to the control group; ** compared to both radiation treatment and the control sample; *** compared to both from
DISCUSSION

The optimal chemoradiotherapy schedules for colorectal cancer using oxaliplatin-based therapy are being actively investigated. However, preclinical evidences of oxaliplatin as a radiosensitizer for colorectal cancer treatment are limited and remain undetermined. The radiosensibilizing activity of platinum-based drugs has been previously suggested involving two successful mechanisms of interaction between drug and radiation; firstly, by inducing additional DNA damage or modifying radiation induced DNA damage, and secondly, by inhibiting post-irradiation DNA damage repair (30).

After a single dose injection of chemotherapeutic agents, the platinum concentration in tumor tissues is declined gradually over time after drug administration. However, the amount of platinum drugs binding to tumoral DNA was varied with the time. Additionally, Pieck and co-workers have reported a characteristic time course of oxaliplatin-DNA adducts in white blood cells of cancer patients, which was not correlated to platinum pharmacokinetics (31). Therefore, this raises the concern of whether or not the binding of drug to DNA during irradiation interferes with the degree of inhibiting post-irradiation DNA damage repair, and subsequent to an efficacy of the concomitant effect.

The variation of platinum-DNA adducts may be explained by different types of binding to DNA. The initial peak at 4 h may suggest the formation of platinum-DNA mono-adducts (Pt-dG or Pt-dA), which rapidly removed by the nucleotide excision repair system, leading to the reduction of the amount of platinum-DNA adducts. At 24 h of oxaliplatin administration, most of DNA mono-adducts have possibly been repaired, and thus minimizing the amount of platinum binding to DNA. Therefore, the exposure of oxaliplatin at 4 h and 24 h followed by
radiotherapy had no impact on the HCT116 xenografts, as only a lower degree of the concomitant effect was observed. The time interval between these two modalities appeared to be important since a profound concomitant effect was observed at 48 h of drug incubation. This may explain by the formation of platinum-DNA adducts at the later time after drug administration. The platinum-DNA adducts which are formed directly as bi-functional adducts or rearranged from mono-functional adducts (Previous measurement of the rate constant for oxaliplatin mono-adduct to di-adduct conversion was about 0.00000576 s⁻¹ (32)), kept increasing upto 48 h after drug administration. The interstrand cross links are known to contribute a significant drug activity and are more vulnerable to DNA repair systems (33-35). Therefore, a maxima formation of platinum-DNA adduct especially as interstrand cross links might be occurred after 48 h of oxaliplatin administration, leading to a maximum effect of radiosensitivity of oxaliplatin. This may contribute to a better understanding of radiosensitizing activity of oxaliplatin, and explain the wide variation of different clinical reports which describing the efficacy of oxaliplatin as a radiosensitizer (13, 35-37). In addition, the amount of platinum-adducts after drug administration may be considered as an excellent indicator to evaluate the concomitant effect of combined treatment.

Moreover, the induction of apoptotic cell death after combined treatment was similar to the previous study (38), the number of apoptotic cells was significant increased after combined treatment of oxaliplatin or Lipoxal™ with radiation treatment compared to untreated mice. Chater and co-workers reported the reduction of the inhibitor of apoptosis protein after combined treatment, which was not observed for either chemotherapy or radiation treatment alone (38). In addition, Soini and co-workers suggested the improvment of platinum
distribution to DNA after radiotherapy may contribute to a higher DNA fragmentation, subsequent to inducing high apoptosis and cell shrinkage (39).

The ability of liposomes to extravasate through leaky tumor vessels was suggested to contribute to selective localization of liposomal oxaliplatin in tumor tissues (40). However our results shown that the encapsulation of oxaliplatin in liposome did not improve its accumulation in tumor cells, neither its fraction bound to DNA. In this reason, the higher concentration of Lipoxal™ can be used compared to oxaliplatin. In addition, low severe side effects observed with Lipoxal™ may suitable for the combination with radiation to produce a high potential of the concomitant effect.

The radiosensitizer role of Lipoxal™ was previously evaluated in terms of a cellular response for systemic cytotoxicity and the potential of a synergistic effect with radiation treatment (41). Previous in vivo studies of glioblastoma treated with combined Lipoxal™ and radiation showed promising results of animal survival (42), however, little is known about the mechanisms of this improved outcome. In our experimental studies, applied radiation treatment at different time intervals of Lipoxal™ administration, a slight difference in antitumor efficiency was observed. However, the lowest concomitant effect was observed when radiation administered 24 h after Lipoxal™ treatment.

Although the encapsulation of oxaliplatin in liposome gave a smaller amount of platinum-DNA adducts compared to its free form, a high level of the concomitant effect of combined treatment was observed. As shown in our results, Lipoxal™ administration alone or combined with radiation induced a larger number of apoptotic cells than oxaliplatin. The different mechanisms of interaction between Lipoxal™ and radiation remain to be clarified.
Previous study has suggested the binding of platinum drugs to cellular proteins via sulfur atoms in the cysteine and/or methionine residues may affect the activity of enzymes, receptors, and other proteins (43). Moreover, platinum drugs can induce a high level of mitochondrial reactive oxygen species which would further interfere with vital cellular functions, leading to cell death (44). Therefore, these data may apply to further investigations on the concomitant effect after combined treatment between Lipoxal™ and radiation both in experimental systems and in the clinical setting.

With respect to improving combined treatment outcomes of oxaliplatin in clinical radiotherapy, our results demonstrated that the potential of concomitant effects between radiation and oxaliplatin depended on the exposure schedule of both modalities. This correlates considerably with platinum levels in different cellular compartments, mainly with platinum-DNA adducts. The high degree of concomitant effects after Lipoxal™ plus radiation treatment was observed, further evaluation of the radiosensitizing activity of Lipoxal™ for clinical trials should be determined. A better understanding of the mechanisms of synergism between radiotherapy and oxaliplatin could lead to the design of new and more efficient colorectal cancer chemoradiation treatment protocols.
References


109


Designing platinum compounds in cancer: structures and mechanisms. Cancer Therapy. 2007; 5: 537-83


24. Paquette B and Little JB. In vivo enhancement of genomic instability in minisatellite sequences of mouse C3H/10T1/2 cells transformed in vitro by X-rays. Cancer Res. 1994; 54: 3173-8


CHAPTER V

ARTICLE NO.4

In this chapter, we investigated the concomitant effect of combined cisplatin and its liposomal formulation plus radiation treatment using animal model. This work was performed to confirm our previous results from \textit{in vitro} studies.

This work is presented in the following article, entitled: "Efficiency of cisplatin and Lipoplatin\textsuperscript{TM} for the concomitant treatment with radiation of a colorectal tumor in \textit{nu/nu} nude mouse", by Thititip Tippayamontri, Rami Kotb, Benoit Paquette and Léon Sanche.

This article has been accepted to publish in Anticancer Research, 2013.
Efficiency of cisplatin and Lipoplatin™ for the concomitant treatment with radiation of a colorectal tumor in nu/nu nude mouse

Short title: Synergism of cisplatin and Lipoplatin™ with radiation

Thititip Tippayamontri, Rami Kotb, Benoit Paquette and Léon Sanche

Department of Nuclear Medicine and Radiobiology, Center of Radiotherapy Research, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada.

Department of Systemic Therapy, BC Cancer Agency's Vancouver Island Centre, Victoria, BC, Canada.

Correspondence to: Professor Léon Sanche, Department of Nuclear Medicine and Radiobiology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001,12th Avenue North, Sherbrooke, QC, Canada, J1H 5N4
Tel. 1-819-820-6868, ext. 14601, Fax: 1-819-564-5442, e-mail: Leon.Sanche@USherbrooke.ca

Key Words: Radiotherapy, chemotherapy, concomitant therapy, colorectal cancer, cisplatin, Lipoplatin™.
Résumé

Dans le but de développer de plus efficaces protocoles de traitement utilisant la concomitance chimio-radiothérapeutique, nous avons cherché les conditions de cédule temporelle menant à une radiosensibilisation optimale. Nous avons proposé que la concomitance soit maximale lorsque le niveau maximal de liaison de platine à l'ADN serait atteint. D'autre part, les effets toxiques causés par le cisplatine aux tissus sains peuvent empêcher l'atteinte de son accumulation optimale à l'ADN. Pour pallier à cet inconvénient, le Lipoplatin™, la formulation liposomale du cisplatine, a été inclus dans cette étude. Nous avons défini la "fenêtre Platinum" en étudiant la pharmacocinétique et la distribution intracellulaire temps-dépendante du cisplatine et du Lipoplatin™ dans le carcinome colorectal humain HCT116 implantées chez des souris nu/nu. L'effet concomitant a été évalué lorsque le rayonnement (15 Gy) a été donné 4, 24, et 48 h après l'administration du médicament, temps correspondant aux variations maximum d'adduits platine-ADN. Le retard de croissance tumorale résultant est reporté et corrélé à des analyses d'apoptose. La meilleure concomitance et le plus haut taux d'apoptose ont été observés lorsque le rayonnement a été donné à 4 h ou 48 h après l'injection de drogues. Ces temps correspondent aux délais pour une liaison maximale de platine à l'ADN de la tumeur et sont probablement dus à une augmentation immédiate de produits létaux générés par les radiations ionisantes. Ces données améliorent notre compréhension des mécanismes de radiosensibilisation induite par le platine et peuvent avoir un impact significatif sur la conception de protocoles de traitement plus efficaces.

Mots-clés: cisplatine; Lipoplatin™; pharmacocinétiques; radiation; tumeur colorectale
Abstract

Background: Optimal conditions for efficient concomitant chemoradiation treatment of colorectal cancer with cisplatin still need to be better defined. In addition, intolerance of healthy tissue to cisplatin prevents the full exploitation of its radiosensitizing potential. A liposomal formulation of cisplatin, Lipoplatin™, was proposed to overcome its toxicity. Using an animal model of colorectal cancer, we determine the platinum window, defined by studying the pharmacokinetics and time-dependent intracellular distribution of cisplatin and Lipoplatin™. Materials and Methods: In nude mice bearing HCT116 human colorectal carcinoma treated with cisplatin or Lipoplatin™, the platinum accumulation in blood, serum, different normal tissues, tumor and different tumor cell compartments was measured by inductively coupled plasma mass spectrometry. Radiation treatment (15 Gy) was given 4, 24, and 48 h after drug administration and was correlated to the amount of platinum–DNA adducts in the cancer cells. The resulting tumor growth delay is reported and correlated to apoptosis analysis. Results: The greatest effects and highest apoptosis were observed when radiation was given at 4 h or 48 h after drug injection. These times correspond to the times of maximal platinum binding to tumor DNA. An enhancement factor (ratio of group treated by combined treatment compared to chemotherapy alone) of 13.00 was obtained with Lipoplatin™, and 4.09 for cisplatin when tumor irradiation was performed 48 h after drug administration. Conclusion: The most efficient combination treatment of radiation with cisplatin or Lipoplatin™ was observed when binding of platinum to DNA was highest. These results improve our understanding of the mechanisms of platinum-induced radiosensitization and should have significant impact on the design of more efficient treatment protocols.
Introduction

Cisplatin is a chemotherapeutic agent, which is also widely used as radiosensitizer (1-6). Addition of cisplatin to radiotherapy has been approved as a standard procedure because improved treatment has been achieved in a number of randomized trials compared with radiotherapy alone. However, in some other studies, concurrent radiation treatment with cisplatin administration did not result in better efficacy (3, 6-9). The explanation for the latter results is mainly associated with severe side-effects of either chemotherapy or radiotherapy, which may limit the escalation of anticancer drug concentrations, as well as radiation doses. In addition, there is still no general consensus for the optimal timing between drug administration and radiation treatment. Therefore, the precise determination of this timing appears a promising objective for improving the efficacy of the combined treatment without added toxicity. Optimal timing requires determination of platinum drug concentration in the tumor during the course of radiation treatment in order to assess the time evolution of radiosensitization (10, 11).

DNA damage induced by cisplatin-based radiosensitization has been proposed to be mainly responsible for cytotoxicity (12). This toxicity is related to the formation of cisplatin-DNA adducts, which include intra- and interstrand cross links of two guanosine nucleotides (GG) or adenosine guanosine nucleotides (AG), and monobifunctional binding to guanosine (12). Different mechanisms underlying the antitumor effect of concurrent radiation and cisplatin treatments have been proposed (13). Radiation induces single-and-double-strand DNA breaks and DNA base damage, while cisplatin forms adducts with DNA. The presence of cisplatin-DNA adducts can reduce the repair of sublethal and potentially lethal DNA damage (14). Moreover, ionizing radiation preferentially induces DNA damage where cisplatin is
located (15). In a previous in vitro study, we showed that a high level of cisplatin-DNA adduct formation appeared to be associated with better treatment effect in human HCT116 colorectal cancer cells (16). However, there are still some inconsistencies in the literature regarding the relationship between the efficacy of anticancer platinum therapy and cisplatin-DNA adduct formation (17), and it is not yet clear how to assess, in the clinic, the information underlying the efficacy of radiotherapy at maximum cisplatin-DNA adduct concentration in the cancer cells.

Lipoplatin™ is a new liposomal formulation of cisplatin. It is currently being developed, and aims to reduce the systemic toxicity of cisplatin, while improving its accumulation in primary tumor tissue (18). A phase I/II study in advanced gastric cancer shows that chemoradiotherapy with Lipoplatin™ is feasible, with minor toxicity (19). This suggests that the therapeutic index of Lipoplatin™ could be larger than that of cisplatin. In our previous report (20), we showed that distribution of cisplatin in different tumor cell compartments was affected by its encapsulation in liposomes. Therefore, studying the mechanisms of its combination with radiotherapy is important in defining the role of Lipoplatin™ as a radiosensitizer and also to confirm our hypothesis for this formulation.

In the present study, we determined the correlation between different cellular levels of DNA-platinum adduct as a function of time after administration of the drug and the efficacy of combined treatment with ionizing radiation. The investigations were performed using nude mice implanted with the human colorectal xenografts of HCT116. We also assessed the variation of platinum concentrations in blood and in different normal tissues. The results of this study should provide useful information enabling suitable scheduling of chemoradiotherapy treatment of colorectal cancer.
Materials and methods

Cell line. The HCT116 colorectal carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were routinely cultured in modified Eagle's medium (MEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μM streptomycin in a fully humidified incubator at 37°C and with 5% CO₂.

Animal xenograft model. All experiments were performed with outbred male nude mice at 4-6 weeks of age (Charles River Laboratories, Saint-Constant, QC, Canada). The animals were maintained in our animal facility, under specific pathogen-free conditions. Housing and all procedures involving animals were performed according to the protocol approved by the Université de Sherbrooke Animal Care and Use Committee (protocol number: 235-10B). HCT116 tumor cells (2X10⁶, 0.1 ml) were inoculated subcutaneously (s.c.) into each rear flank. Tumor measurements began one week post-injection and continued biweekly. Tumor volumes were calculated with the formula: \( V = \frac{1}{6} \pi \times a \times b^2 \) (mm³), where \( a \) and \( b \) were the largest and smallest perpendicular tumor diameters, respectively. The pharmacokinetic studies began when tumor volumes reached the range of 30-60 mm³. The tumor-bearing animals were randomized into different groups of three to five animals each. For all procedures (implantation, chemotherapy and radiotherapy), nude mice were anesthetised with an intraperitoneal injection of ketamine/xylazine (87/13 mg/ml) at 1 ml/kg.

Drug preparation. Cisplatin was obtained through the pharmacy of the Centre Hospitalier Universitaire de Sherbrooke. Lipoplatin™ was generously provided by Regulon Inc. (Athens, Greece). All drugs were diluted to the given concentrations in a solution of 5% dextrose immediately before use.
Pharmacokinetic studies. Drugs were dissolved in 5% dextrose for a final concentration of 1 mg/ml to be given as a single dose of 10 mg/kg into the mice tail vein. Three groups of mice were studied with the following treatment: i) cisplatin, ii) Lipoplatin™, and iii) 5% dextrose alone, as a control group. Three to five animals from each group were sacrificed at 4, 24, 48, 72, and 96 h post-injection. Blood samples were obtained via cardiac puncture for whole-blood and serum platinum assessments. Tumors and samples of different tissues were taken to determine platinum accumulation in the cytoplasm, and the nucleus, as well as in DNA extractions.

Determination of cytoplasmic and nuclear platinum accumulation. Samples were processed with the nuclear and cytoplasm extraction kit Activemotif (ActiveMotif, Carlsbad, CA, USA). Briefly, tumors were weighed and diced into very small pieces using a razor blade. Three milliliters of ice-cold hypotonic buffer supplemented with 3 μl of 1 M dithiothreitol and 3 μl of detergent were added per gram of tissue and incubated on ice for 15 min, and then centrifuged for 10 min at 850 X g and 4°C. The hypotonic buffer and detergent were supplied in the kit. Thereafter, the supernatants corresponding to the cytoplasmic fraction were transferred into pre-chilled microcentrifuge tubes, while the pellets were gently resuspended in 500 μl of hypotonic buffer, and incubated on ice for 15 min. The detergent (25 μl) was added and then tubes vortexed. Samples were centrifuged for 30 s at 14,000 X g in a microcentrifuge pre-cooled at 4°C and these second supernatants were combined with the first ones. The resulting pellets corresponded to the nuclear fraction.

Quantification of DNA-bound platinum. DNA was extracted according to a salting-out procedure (21). Briefly, the tumors were weighed and diced into very small pieces, and homogenized with a pre-chilled Dounce homogenizer. On ice, 3 ml of a lysing buffer
containing 10 mM Tris-HCl, 400 mM sodium chloride and 2 mM EDTA were added. Subsequently, 0.1 ml of 20% sodium dodecylsulfate and 0.5 ml of proteinase K (10 mg/ml) were added and the samples incubated overnight at 37°C. DNA was precipitated by adding 1.2 ml of 5 M sodium chloride. The tube was gently agitated for 1 min, centrifuged at 2,500 X g for 15 min and then the supernatant was transferred to another tube. The DNA was precipitated with 2.5 vol. of 95% ethanol, spooled out and air-dried briefly. The DNA was then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and RNase A (0.05 ml of a 10 mg/ml solution) was added and incubated for 1 h at 37°C. The DNA was precipitated a second time with ethanol and re-dissolved in TE buffer. The amount of DNA extracted was evaluated by measuring its absorbance in the solution at 260 nm (A260) with a spectrophotometer (Synergy HT, BIO-TEX, Winooski, VT, USA) and calculated using the following equation: A260 X 50 (µg/ml).

Platinum quantification using an inductively coupled plasma mass spectrometer (ICP-MS). The quantity of platinum accumulated in tissue samples and in the cytoplasm, nucleus and DNA of tumor were determined with ICP-MS (ELAN DRC-II; PerkinElmer, Waltham, MA, USA) (22). Briefly, samples were treated with 23% nitric acid and 8% H2O2. The solutions were then injected in the ICP-MS to quantify the platinum concentration. As internal controls, platinum and thallium (m/z 195 and 205, respectively) were quantified in untreated animals.

Chemotherapy treatment. The tested drugs were dissolved in 5% dextrose for a final concentration of 1 mg/ml to be given as a single dose of 10 mg/kg into the tail vein. The platinum drugs were injected at 4, 24 and 48 h prior to radiation treatment. The selected drug concentration of cisplatin and LipoplatinTM did not induce the appearance of any side-effects
after combined treatment, as the body weight of all groups of animals significantly increased between initial and final time of combined treatment (Table I).

Table 1. Change in body weight of male nu/nu nude mice after combined treatment of cisplatin or Lipoplatin™ and Gamma Knife irradiation

<table>
<thead>
<tr>
<th>Platinum drugs</th>
<th>Body weight (g) (n=3-5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before the treatment</td>
<td>After the treatment</td>
</tr>
<tr>
<td>Control</td>
<td>21.57±1.00</td>
<td>27.07±2.25</td>
</tr>
<tr>
<td>Cisplatin (48 h)-IR</td>
<td>27.20±1.27</td>
<td>31.25±0.35</td>
</tr>
<tr>
<td>Lipoplatin™ (48 h)-IR</td>
<td>22.50±2.29</td>
<td>25.43±1.46</td>
</tr>
</tbody>
</table>

*a Control group = no chemotherapy and radiation treatment
i.v. = intravenous injection of single concentration 10 mg/kg of platinum drug
GK = Gamma Knife irradiation of single dose 15 Gy
p<0.05 is considered as statistically significant between body weight before and after the treatment

Tumor irradiation. Mice were anaesthetized and positioned in our in-house constructed stereotactic frame designed for the 4C Gamma Knife (Elekta Instruments AB, Stockholm, Sweden) (23, 24). The radiation treatment (15 Gy, dose rate of 3.6 Gy/min) using 8 mm collimators was delivered at predetermined coordinates targeting the tumor, which had a diameter of about 7 mm. Radiation was applied to one side of the rear flank, whereas the other side was kept as the non-irradiated control tumor.

Apoptosis analysis. Groups of three mice bearing HCT116 tumors were treated with platinum drugs at 24 or 48 h before the radiation treatment, as previously described. Two days after irradiation, tumors were removed, fixed in 10% buffered formalin and embedded in paraffin. Tumor sections of 4 μm were taken at three different levels. Apoptotic cells within tumor tissues were analyzed with a commercially available ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Temecula, CA, USA); following the manufacturer's protocol. Briefly,
formalin-fixed, paraffin-embedded tissue slides were rehydrated using xylene, rinsed with ethanol, and then incubated with a solution of 3% hydrogen peroxide. The samples were treated with 20 μg/ml of proteinase K for 15 min at room temperature. After washing and incubation with equilibration buffer included in the kit for 5 min, then 33 μl of terminal deoxynucleotidyl transferase (TdT) enzyme in 77 μl of reaction buffer included in the kit was incubated on the slides for 1 h at 37°C. After applying stop solution included in the kit for 10 min and washing, the samples were incubated with anti-digoxigenin peroxidase conjugate (65 μl/cm² of specimen surface area) for 30 min at room temperature. Slides were developed with a 1:20 dilution of diaminobenzidine (3,3′-diaminobenzidine) substrate, counterstained with methyl green, dehydrated, and coverslipped. Terminal deoxynucleotidyl transferase-mediated dUPT nick end labeling (TUNEL)-positive apoptotic nuclei were detected with a fluorescence microscope. A positive control slide was prepared by nicking DNA with DNasel and treated similarly (data not shown). Samples treated similarly but without enzyme treatment served as negative controls. To determine the amount of cellular area stained with diaminobenzidine (positive for apoptosis) and the amount of tumor area counterstained with methyl green (negative for apoptosis), the tumor sections were scanned with an Olympus FSX100 microscope (Olympus, Center Valley, PA, USA) and the values were quantified using Adobe Photoshop CS5 (Adobe Systems Canada, Ottawa, Ontario, Canada) analysis software. Briefly, the percentage of apoptotic cells was calculated as the quotient of the diaminobenzidine-positive area over the total area (diaminobenzidine positive + methyl green negative), multiplied by 100.

Statistical analysis. The mean±SD were calculated. p<0.05 was considered statistically significant (two-tailed paired Student’s t-test).
Results

Platinum concentration in whole blood, serum, tumor and normal tissues. The time-concentration profiles of platinum in the blood and serum are shown in Figures 1a and b. After i.v. administration (10 mg/kg) of cisplatin or Lipoplatin™, the initial maximum of platinum concentration in whole blood and serum was reached at the first assessment time point (4 h), followed by a rapid decline over the next 24 h. Thereafter, the decline was very slow. The platinum concentration in whole blood was about three times higher than that in the serum. While free cisplatin reached a higher peak level in kidney tissues than observed with the liposomal formulation (Figure 1c), both drugs exhibit similar levels of platinum accumulation in liver tissues at different time points, with no well-defined initial peak (Figure 1d). A higher level of platinum accumulation was observed in muscle tissues with cisplatin than with Lipoplatin™ (Figure 1e). Accumulation of platinum in the tumor tissue after administration of cisplatin, and Lipoplatin™ are shown in the Figure 1f. Over a sampling period of 96 h, the platinum concentration in tumor was about five times higher in the cisplatin-treated group than in the Lipoplatin™-treated group. For both drugs, the total tumor platinum content declined gradually over time.
Figure 1. Pharmacokinetic profiles of platinum concentration in a) whole blood, b) serum, c) kidney, d) liver, e) muscle, and f) tumor obtained after a single i.v. injection of 10 mg/kg cisplatin or Lipoplatin™, in nude mice bearing HCT116 colorectal tumor. The amount of platinum was measured by an inductively coupled plasma mass spectrometer. Each point represents the mean±SD of the concentration in 3-5 mice.
Platinum accumulation in cytoplasm and nucleus, and platinum-DNA adducts in tumor tissue.

The time profiles of platinum accumulation in the cytoplasm and nucleus of HCT116 tumor cells are shown in Figures 2a and b. The amount of platinum in the cytoplasm and the nucleus declined gradually over time for both cisplatin and Lipoplatin™. Cisplatin administration led to about 3- to 5-fold more platinum accumulation in the cytoplasm and the nuclei of cells of tumor tissues, respectively, than did Lipoplatin™. Regarding the level of DNA-bound platinum in the nuclei of tumor cells, for both drugs, the maximum was measured at 4 h, declining to reach a first nadir at 24 h, increasing again to reach a second peak at 48 h before declining again (Figure 2c). While the initial and later peak levels of DNA-bound platinum were much higher in the case of cisplatin, for both drugs, the level of DNA-bound platinum declined to a similar nadir level at 24 h.
Figure 2. Time dependence of platinum concentration in a) cytoplasm, b) nucleus and c) platinum-DNA adducts obtained after a single i.v. injection of 10 mg/kg cisplatin, or Lipoplatin™, in nude mice bearing HCT116 colorectal tumor. The amount of platinum bound was measured by inductively coupled plasma mass spectrometer. Each point represents the mean ±SD of the concentration in 3-5 mice.
Tumor growth delay. Figure 3 shows the tumor response when the drugs were injected before radiation treatment. Results in terms of the time necessary to reach five times the initial tumor volume (5Td) and the tumor growth delays (TGD = 5Td value for a treated group minus 5Td for the control group) are reported in Table II. A single cisplatin administration significantly induced a tumor growth delay compared to non-treated animals (p<0.05), whereas no significant improvement was observed when Lipoplatin™ was given alone. Treatment with radiation alone (15 Gy) led to a good tumor response, with a 5Td of 19±1.0 days compared to 10.5±0.71 days for the non-treated control group (p<0.01). The tumor response was further improved when cisplatin or Lipoplatin™ were combined with tumor irradiation (Figures 3a and b). A significant difference in TGD between the groups of single drug treatments and combined modalities was initially observed after eight and six days for cisplatin and Lipoplatin™, respectively. With both drugs, the TGD was better when the irradiation was delivered at 4 h or 48 h after drug administration compared to 24 h after drug administration (p<0.01). It is noteworthy that no improvement was observed when cisplatin was administered 24 h prior the radiation treatment, compared to the irradiated control. Indeed, the TGDs were similar for the group treated with radiation alone (8.5±1.00 days), and the group treated with cisplatin 24 h prior the radiation treatment (10.5±1.41 days, p=0.19) (Table II).
Figure 3. The tumor growth delay after initial treatment of nude mice bearing HCT116 colorectal tumor with a) cisplatin and b) Lipoplatin™ followed by radiotherapy. Tumor growth delay is reported as $V_t/V_0$ ratio where $V_t$ is the mean tumor volume on a given day during the treatment and $V_0$ is the mean tumor volume at the beginning of the treatment. A single dose of platinum drug of 10 mg/kg and a single dose of radiation of 15 Gy were administered. Each symbol represents the mean±S.D of the results obtained with five mice.
Enhancement factor (EF). The EF, which is the ratio of TGD measured with a group treated by radiation and chemotherapy compared to TGD obtained with chemotherapy alone, was calculated to better assess the effect obtained by radiation with cisplatin, and Lipoplatin™ at 4, 24 and 48 h after drug administration (Table II). When radiation was given at 4 h and 48 h after cisplatin administration, the EF was 3.73 and 4.09 respectively, suggesting an important improvement, while a modest EF of 1.91 was measured when radiation treatment was given 24 h after cisplatin administration. The encapsulation of cisplatin in a liposome, i.e. Lipoplatin™, resulted in a much more important effect when combined with tumor irradiation; the EF was 13.67, 10.33 and 13.00 at time intervals of 4, 24 and 48 h when tumor irradiation was combined with Lipoplatin™ after drug administration, respectively. As for cisplatin, the lowest EF with Lipoplatin™ was measured at 24 h post drug administration.

Induction of apoptosis. The number of apoptotic cells was scored in tumor sections after treatment (Figure 4). The percentage of apoptotic cells was very low in the untreated control group (0.94±0.38%) and the group treated with radiation alone (2.86±0.89%). When the tumor was irradiated 24 h after cisplatin administration, a modest but significant increase of apoptotic cells (3.48±0.69%) was measured compared to tumors treated with cisplatin alone (1.97±0.77%) (p<0.05). Incubation with cisplatin alone required 48 h before a significant increase in the number of apoptotic cells (13.40±2.81%) was observed compared to treatment with cisplatin alone or radiation alone (p<0.01). With a similar trend, a higher percentage (p<0.05) of apoptotic cells was observed when radiation was given 48 h after Lipoplatin™ administration (15.29±2.90%) compared to when given 24 h after drug administration (5.72±1.23%).
Table II. Efficacy of platinum-based chemotherapy alone and in combination with radiation treatment (IR).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5Td (days)</th>
<th>TGD (days)</th>
<th>EF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.5±0.71</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>IR alone</td>
<td>19±1.00</td>
<td>8.5±1.00*</td>
<td>---</td>
</tr>
<tr>
<td>Cisplatin alone</td>
<td>16±0.71</td>
<td>5.5±0.71*</td>
<td>---</td>
</tr>
<tr>
<td>Lipoplatin™ alone</td>
<td>12±1.29</td>
<td>1.5±1.29</td>
<td>---</td>
</tr>
<tr>
<td>Cisplatin + IR at 4h</td>
<td>31±1.25</td>
<td>20.5±1.25*,#</td>
<td>3.73</td>
</tr>
<tr>
<td>Cisplatin + IR at 24h</td>
<td>21±1.41</td>
<td>10.5±1.41*</td>
<td>1.91</td>
</tr>
<tr>
<td>Cisplatin + IR at 48h</td>
<td>33±2.00</td>
<td>22.5±2.00*,#</td>
<td>4.09</td>
</tr>
<tr>
<td>Lipoplatin™ + IR at 4h</td>
<td>31±2.12</td>
<td>20.5±2.12*,#</td>
<td>13.67</td>
</tr>
<tr>
<td>Lipoplatin™ + IR at 24h</td>
<td>26±1.52</td>
<td>15.5±1.52*,#</td>
<td>10.33</td>
</tr>
<tr>
<td>Lipoplatin™ + IR at 48h</td>
<td>30±1.29</td>
<td>19.5±1.29*,#</td>
<td>13.00</td>
</tr>
</tbody>
</table>

5Td, Time necessary to reach a tumor volume five times greater than the initial volume; TGD, tumor growth delay, is the 5Td value minus that for the control group (no chemotherapy or radiation treatment); EF, enhancement factor, calculated by dividing the TGD of the group treated by combined treatment with that of the group treated by chemotherapy alone.

Statistically significant at p<0.05 *compared to the control group, #compared to chemotherapy alone and radiation treatment alone.
Figure 4. Assessment of apoptosis after chemoradiotherapy of cisplatin and Lipoplatin™. a) Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive apoptotic nuclei assay after chemoradiotherapy. Animals were irradiated at 24 or 48 h after drug administration. A single dose of cisplatin or Lipoplatin™ of 10 mg/kg and a single dose of radiation (15 Gy) were administered. b) Percentage of apoptotic cells after chemoradiotherapy. Apoptotic cells were counted in 10 random fields per tumor (n=3) (mean±S.D.). Magnification, ×40. Statistically significant differences: \( p<0.05 \) *from the control group; **from both radiation-treated and the control group; ***from radiation alone, chemotherapy alone and the control group.


Discussion

For most clinical chemoradiotherapy protocols of weekly cisplatin plus five fractionated doses of radiation, time between the administration of cisplatin and tumor irradiation can change the anti-tumor response obtained by combining them. The present experiments were performed with the chemotherapeutic agents cisplatin and Lipoplatin™ to investigate the maximum concomitant effect between these agents and radiation at different times after drug administration. We also assessed the level of platinum in blood, serum, and different normal tissues with the aim to facilitate the transition to human trials where extraction of cancerous tissue samples is more difficult. To correlate the efficacy of combined treatments, we defined the platinum window of the maximum concomitant effect, which was associated with the maximum level of platinum-DNA adducts in tumor cells. Supporting the importance of platinum-DNA adducts, in clinical study with cisplatin chemotherapy Los and co-workers reported that cisplatin-induced DNA modifications were observed in human tumor biopsies, and were positively correlated with tumor remission (25).

It was clear from the EF values that cisplatin and its liposomal formulation (Lipoplatin™) can act as radiosensitizers. The EFs, the results of apoptotic analysis and the pharmacokinetics data showed that cisplatin and Lipoplatin™ were most efficient radiosensitizers when their binding to tumoral DNA was maximized. In contrast, we found less or even nearly no improvement in the combined effect of cisplatin and radiotherapy, when binding of cisplatin to tumoral DNA was minimal (i.e. 24 h after drug injection).

While the initial high DNA-platinum peak at 4 h is explained by drug influx into tumor cells, the reasons for the low level of adduct at 24 h and the second DNA-platinum peak at 48 h are unclear. However, this observation leads to the following hypothesis. At 4 h, the majority
of DNA-platinum adducts are intrastrand cross-links binding as monoaucts. These monoaucts are rapidly excised by the nucleotide excision repair system, leading to the reduction of DNA-platinum binding (26). Twenty-four hours after administration of cisplatin, most of the DNA monoaucts disappear, while the repair system becomes more saturated with repair-resistant DNA-platinum interstrand cross links, which would maximize at 48 h.

There are many mechanisms that can enhance the effects of radiation (27, 28). Among these, essentially two mechanisms may explain the radiosensitizing properties of cisplatin. Previous in vitro studies (29-31) and some experiments with tumor-bearing mice (32-34) suggested the inhibition of repair of radiation damage to DNA. Whereas from other investigations, it was postulated that owing to the binding of cisplatin to DNA, there is an increase in the immediate species created by the primary ionizing events in cells which causes the additional damage (35). Zheng et al. (15) measured DNA single-strand breaks (SSBs) and DNA double-strand breaks (DSBs) induced by the impact of 1, 10, 100 and 60,000 eV electrons on thin solid films of DNA with and without cisplatin bonded to two adjacent guanine bases. From a comparison of their results obtained at low energy (1-100 eV) with those obtained at 60 keV, they found that production of SSBs and DSBs induced by low-energy secondary electrons, created by the primary radiation, was substantially enhanced when cisplatin was covalently bonded to DNA. Such damage is known to promote cell death. The latter hypothesis implies that the effect of platinum drugs and radiation should be maximal when binding of platinum to DNA of cancer cells is maximized. This is precisely the result obtained in the present study. Determination of such fundamental mechanisms of the radiosensitizing action of platinum drugs may have implications in the design of new chemotherapeutic agents, as well as in the development of more efficient protocols for
chemoradiation therapy. In addition, since there was no correlation in the effect of combined therapies and cisplatin found either in blood or in normal tissues, this support the notion that the best combined effect can only be determined by quantifying the level of cisplatin–DNA adducts in tumor cells.

We reported previously that the intracellular platinum distribution in tumor from human HCT116 colorectal cancer cells for encapsulated cisplatin may be different than that of cisplatin alone (20). In this in vivo study, identical concentrations of platinum drugs (10 mg/kg) were administered to the animals. LipoplatinTM administration alone led to similar results in TGD to that of untreated animals; the liposomal formulation was therefore less effective than cisplatin administered alone. Indeed, most previous animal and human studies have administered 3-6 times higher doses of Lipoplatin™ than cisplatin (36). For example, Ravaioli and co-workers used Lipoplatin™ at 100 mg/m2 every two weeks as second-line chemotherapy in heavily pre-treated patients with advanced non-small cell lung cancer (NSCLC) and obtained 5% partial remission and 16% stable disease (36). On the other hand, Stathopoulos and co-workers used 400 mg/m2 every two weeks (200 mg/m2 D1 and 2 every 14 days) on a similar group of patients, and obtained 38% partial remission and 43% stable disease, with only minor toxicities of grade I (37). The dose of cisplatin in 14-day schedules was 75 mg/m² compared to 200 mg/m² for LipoplatinTM in randomized phase III study in combination with paclitaxel in nonsquamous NSCLC had shown a much lower toxicity profile in the Lipoplatin™ arm (38). Furthermore, the study of nonsquamous NSCLC demonstrated a statistically significantly higher response rate with the Lipoplatin™ treatment. Consequently, Lipoplatin™ should achieve a higher level of tumor accumulation of platinum and DNA-platinum adducts.
Although the radiosensitizing role of Lipoplatin™ has not yet been determined in clinical practice, the wider therapeutic index of this formulation suggests such potential (36-38). In addition, our previous study on the concurrent administration of Lipoplatin™ and radiation showed the high potential of concomitant effect in the HCT116 cells when radiation is applied at maximum formation of DNA-platinum adducts (16). In the present study, combined treatment of radiation and Lipoplatin™ led to the highest increase in antitumoral activity. An enhancement factor of 13.00 was obtained with Lipoplatin™, compared to only 4.09 for cisplatin, when tumor irradiation was performed 48 h after drug administration. The effect after treatment with Lipoplatin™ and radiation still correlated with maximum tumoral DNA-platinum levels (4 h and 48 h after drug administration). Administration of Lipoplatin™ also led to lower accumulation of platinum in the normal tissues tested, except for the liver.

Considering the lower toxicity of Lipoplatin™ compared to that of cisplatin reported in clinical trials (37-38), the therapeutic index should be larger for Lipoplatin™ than for cisplatin. It remains to be determined whether a larger dose of Lipoplatin™ administered in this preclinical model of cancer would result in an improvement of the antitumoral effect while maintaining an acceptable level of toxicity. Nevertheless, these data are valuable to highlight the potential of Lipoplatin™ as a radiosensitizer.

Although similar level of Lipoplatin™ and cisplatin bound to the DNA 24 h after drug administration was observed, superior treatment occurred with combined administration of Lipoplatin™ and radiation. Only a weak correlation between the cytotoxicity of liposomal platinum drug and the degree of DNA platination was found. This observation might imply that platinum-based drugs have important targets other than nuclear DNA (e.g. cytoplasmic compartments) (39). For example, it has been proposed that Lipoplatin™ can release cisplatin
molecules into the cytoplasm of the tumor cell, which then activate the mitochondrial apoptotic cascade (40). In addition, any released cisplatin molecules may i) undergo reaction with phospholipids; ii) inhibit amino acid transport, protein synthesis and ATPases; and iii) uncouple oxidative phosphorylation (41-43). However, the importance of targets other than DNA in relation to cytotoxicity is still unclear and remains a subject of future study.

It has been proposed that the binding affinity of drugs to the mitochondria is regulated by lipid solubility (44). Hori and co-workers reported that highly lipophilic drugs are more rapidly accumulated in the mitochondria than are non- or poorly lipophilic drugs (45). The improvement in terms of lipophilicity for Lipoplatin™ compared to free-platinum forms could indicate a higher efficacy for binding to the mitochondrial membrane that would lead to a greater accumulation of drug within the mitochondria. Thus, promotion of liposomal platinum drug accumulation in the mitochondria may contribute to the enhancement of apoptotic cancer cell death. Although this mechanism may explain in part the anticancer effect of Lipoplatin™ for some cancer types, we observed similar induction of apoptosis with cisplatin and Lipoplatin™ in the HCT116 colorectal carcinoma model used in this study. However, the fact remains that Lipoplatin™ was also associated with a large improvement of the radiosensitizing effect.

In conclusion, the narrow therapeutic index of cisplatin excludes dose escalation to improve its radiosensitizing action in the treatment of various types of cancer. There exists a general impression that the maximum potential of this drug may have been reached. However, our current report shows that the radiosensitizing effect of platinum drugs varies considerably according to the time after exposure to the drugs and to the platinum level in different tumor cellular compartments. The efficacy of combined treatments essentially correlates with the
amount of DNA-bound platinum in tumor cells: it reaches a maximum at the times corresponding to the highest DNA-platinum concentration in the nucleus (4 h and 48 h); furthermore, efficacy reaches a minimum at low DNA-platinum levels (24 h). The best combined effect was observed with Lipoplatin™ and was also correlated with the maximum DNA-platinum levels in the tumor cells.

Acknowledgements

This research was financed by the Canadian Institute of Health Research (grant #81356). Léon Sanche, Benoit Paquette and Rami Kotb are membres of the Centre de recherche Clinique-Étienne Lebel supported by the Fonds de la Recherche en Santé du Québec. Sanofi-Aventis Canada also provided a partial unrestricted grant to support this project.

References


CHAPTER VI
DISCUSSION

The scope of this thesis

Concomitant chemoradiotherapy consists of administering chemotherapeutic agents, such as radiosensitizers, during the course of radiotherapy. Concomitant chemoradiotherapy is proposed to locally improve primary tumor control and eliminate metastatic foci. However, some difficulties in managing the side effects from this combined treatment are still commonly encountered in clinical practice. Therefore, the scheduling of radiosensitizing drugs in relation to radiotherapy is crucial to gain the benefit of drug-radiation interactions for maximizing tumor response. The aim of this study was to develop an optimal schedule for chemoradiotherapy to assess the best concomitant effect while minimizing the level of toxicities.

The articles presented in this thesis emphasize preclinical in vitro and in vivo studies including; studies on the cytotoxicity, pharmacokinetic characteristics of platinum-based drugs (e.g. intracellular accumulation and platinum-DNA adducts) and key information on cell proliferation, cell cycle progression and cell death in the response to chemoradiotherapy. We also investigated the optimal chemoradiation schedule regarding the hypothesis of the “true” concomitant chemoradiotherapy that the maximum therapeutic effect of concomitance is obtained if radiation is administered at the time at which the maximum quantity of platinum is bound to the DNA in tumor cells. We first performed experiments in vitro to verify this hypothesis. Since vascularization is not present in experiments with the cells, an animal model was additionally investigated to model human tumors and be more predictive of the therapeutic effects that might be observed in humans.

147
Cytotoxicity potential of cisplatin and oxaliplatin

DNA damage is mainly responsible for the cytotoxic properties of platinum drugs (Saris et al., 1996). Thus differences in the cytotoxicity of oxaliplatin and cisplatin likely derive from differences in their interactions at the DNA level. Our results and existing evidences (Saris et al., 1996; Woynarowski et al., 2000), showed that oxaliplatin forms fewer platinum-DNA adducts, but inhibits DNA synthesis more efficiently than equimolar concentrations of cisplatin.

That cells were more sensitive to oxaliplatin than to cisplatin may be because oxaliplatin affects DNA with different and more efficient pathways than does cisplatin. This was attributed to the fact that oxaliplatin-DNA adducts have different biological properties, specifically they are bulkier and more hydrophobic than are cisplatin-DNA adducts, and can therefore cause different damage to DNA by altering the conformation of the molecule (Floltinova et al., 2008). Moreover, the formation of a bifunctional adduct with oxaliplatin was reported to be greater, and this could also represent a mechanism for the increased activity of oxaliplatin (Jennerwein et al., 1989; Kim et al., 2010). The formation of bifunctional adducts of oxaliplatin may associated with more lethal DNA damage than cisplatin-DNA adduct (Vaisman et al., 1998; Ferry et al., 1999).

The difference of cell sensitivity to the two platinum drugs may also be explained by differences in the cell’s ability to repair platinum-induced DNA damage lesions after cisplatin and oxaliplatin treatment. Scheeff and coworkers have shown that oxaliplatin-induced damage was more difficult to repair than cisplatin-induced damage (Scheeff et al., 1999). Nehme and co-workers have suggested that cisplatin activates particular components of damage-response pathways, such as JNK and c-Abl kinases (Nehme et al., 1999). Consequently, cisplatin
depends on an intact mismatch repair (MMR) system for its minimal cytotoxicity for signaling apoptosis via the JNK-mediated pathway. In contrast, the DACH ligand which is present in oxaliplatin, but not in cisplatin, inhibit recognition by the MMR protein complex and so not activate JNK and c-Abl (Vaisman et al., 1999), thus providing oxaliplatin with a means to maintain its cytotoxicity in cells.

Pharmacokinetics of platinum drugs

Our studies on the pharmacokinetic characteristics of platinum drugs were performed to obtain a better understanding of their cytotoxicity and to better identify differences between the drugs.

Previously, Boulikas has reported that the cisplatin concentration can reach 0.4 cisplatin molecules/1000 b.p. in DNA (Boulikas et al., 2005). Zheng and co-workers demonstrated the considerable sensitivity of DNA/cisplatin complexes to low energy electrons that resulted in an increase of SSB and DSB with an estimated 0.625 cisplatin molecules/1000 b.p. covalently bound to DNA, when samples were irradiated by a 60 keV primary electron beam (Zheng et al., 2008). By conducting in vitro studies using an inductively coupled plasma mass spectrometer (ICP-MS), we found the fraction of platinum drugs bound to DNA. The pharmacokinetic characteristics of the various platinum drugs at the DNA level are shown in Table 1. The cisplatin and oxaliplatin concentration reached values of respectively 0.795 and 0.726 platinum molecules/1000 base pair (b.p.) in DNA, which is consistent with previous studies. These observations suggest an adequate distribution of these platinum drugs into the DNA of the cancer cells, which could lead to improvements in efficiency in combination with radiation treatment.
Table 1 shows that while the quantities of cisplatin- or oxaliplatin-DNA adducts decreases by a factor 3 as incubation periods increase from 4 h to 24 h, there is an augmentation by a factor 10 in the concentration of platinum-DNA adducts from treatment with liposomal platinum formulations. This suggests that the comparatively slow release of platinum compounds into the cell by liposomal-platinum drug administration may prolong exposure of tumor cell to the cytotoxic drugs and consequently promote their binding to DNA.

In our study (Table 1) and as discussed in Chapter 2, the cellular uptake and cytoplasmic/DNA distribution of platinum-based drugs in HCT116 cells showed similar levels for cisplatin and oxaliplatin accumulation after 4 h of incubation, subsequently, the level for oxaliplatin attained a plateau; whereas cisplatin continued to accumulate. These results are consistent with a recent study by Virag and co-workers (Virag et al., 2012). They observed that the cellular uptake of oxaliplatin gradually increased with time, while cisplatin entered rapidly into the cells. Thus, the cellular accumulation of cisplatin was several times that of oxaliplatin. In our study, the concentration of cisplatin in DNA has to be eight times higher than that of oxaliplatin to reach the same level of both platinum-DNA adducts and cancer cell toxicity. A higher concentration of cisplatin was accumulated in cells compared to the oxaliplatin, probably because of the less limiting efflux transporters (e.g. some copper transporters) for cisplatin versus oxaliplatin. The plateau level of oxaliplatin in the cells after 24 h of drug incubation may be related to the reaction of oxaliplatin with other targets in the cytoplasm, such as proteins, with higher frequency than cisplatin. Cisplatin-DNA or cisplatin-protein adducts may be removed less efficiently than oxaliplatin-DNA adducts, resulting in a higher accumulation of cisplatin in the cells (Virag et al., 2012).
Table 1. The platinum-DNA binding after HCT116 cells exposed to cisplatin, oxaliplatin, and their liposomal formulation for 4 h and 24 h of drug incubation

<table>
<thead>
<tr>
<th>Platinum derivatives</th>
<th>DNA-Pt (Molecule Pt/1000 b.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h drug incubation</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.795 ± 0.09</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>0.726 ± 0.04</td>
</tr>
<tr>
<td>Lipoplatin</td>
<td>0.069 ± 0.02</td>
</tr>
<tr>
<td>Lipoxal</td>
<td>0.007 ± 0.002</td>
</tr>
</tbody>
</table>

In the *in vivo* studies following the administration of a fixed intravenous dose (10 mg/kg) of cisplatin, oxaliplatin or their liposomal formulations, only a small fraction of platinum found in the serum, suggesting that the most of the platinum drug molecules are free to diffuse out from blood vessels into tumor and/or normal tissues (Hall et al., 2008; Wang and Lippard, 2005). While there is a gradual decrease of platinum drug concentration in the serum over time, the total concentration of platinum drugs in the blood tended to stabilize or to increase, probably via redistribution from other tissues. While the design of this study consists of a single i.v. injection of each of the tested drugs, these particular results underline the possible long term blood accumulation of platinum drugs especially with repetitive administrations, which may have an impact on drug pharmacokinetics and tolerability. This result also suggests that the haemoglobin level may have an effect on the efficacy and toxicity of the platinum drugs (Mandal et al., 2004; Mantovani et al., 1999).

Our results agree with previous observations that the binding of oxaliplatin to red blood cells (RBCs) is rapid ($t_{1/2}$ RBCs = 0.58 h, reaching equilibrium by 4 hours) (Luo et al., 1999).
and its clearance was subsequently slow (Kim and Erlichman, 2007). These data indicate the ratio of haemoglobin (Hb) level / packed cell volume (PCV) might affect oxaliplatin metabolism. Since many patients treated with oxaliplatin are anemic from digestive bleeding and/or bone marrow toxicity (Gundling et al., 2008; Noronha et al., 2005), the effect of the variation in Hb level on the efficacy and toxicity of oxaliplatin should be clarified. Fortunately, the relative contribution of Hb level on the anticancer efficacy in the clinical practice may be small since the drugs are captured mainly in DNA in tissues throughout the body (Culy et al., 2000).

Cisplatin and oxaliplatin exhibited levels of platinum drug accumulation in liver tissues similar to those of their liposomal formulations. The free platinum forms however showed a higher peak levels in kidney tissues than those observed with their liposomal formulations. Indeed, the maximum level of platinum drug accumulation in kidney tissues was 3-5 times higher with cisplatin and oxaliplatin than with to Lipoplatin™ or Lipoxal™. This observation is in agreement with previous studies (Boulikas, 2004; Devarajan et al., 2004b). These pharmacokinetic data may account for the low renal toxicity of liposomal platinum formulations. A low concentration of platinum was accumulated in muscle tissues after treatment with Lipoplatin™ and Lipoxal™ as it was with the free formulations, suggesting slow clearance of all drugs.

The accumulation of platinum drugs in the tumor tissue after cisplatin and oxaliplatin administration was higher relative to their liposomal forms in the animal model. However, this is the opposite of what was observed in the in vitro study with HCT116 cells, where higher levels of platinum accumulation were observed after treatment with Lipoplatin™ and Lipoxal™ relative to the free-platinum forms. This difference in accumulation between in vitro
and in vivo studies is likely explained by our experimental setup. To determine the platinum accumulation in the HCT116 cells, we incubated all platinum drugs at the same equitoxic dose (or their IC\textsubscript{50} concentration). With in vivo studies, the identical concentrations of platinum drugs at 10 mg/kg were administered to the animals because of the difficulty to determine the exact equitoxic dose among these platinum drugs. Indeed, most animal and human studies have used 3-6 times higher doses of Lipoplatin\textsuperscript{TM} compared to cisplatin. For example, Ravaioli and coworkers (Ravaioli et al., 2009) used Lipoplatin\textsuperscript{TM} 100 mg/m\textsuperscript{2} every two weeks, as second line chemotherapy in heavily pre-treated patients with advanced non-small-cell lung cancer and obtained 5% partial remission and 16% stable disease. On the other hand, Stathopoulos and coworkers (Stathopoulos, 2010) used 400 mg/m\textsuperscript{2} every two weeks (actually 200 mg/m\textsuperscript{2} D1,2 every 14 days) on a similar group of patients, and obtained 38% partial remission and 43% stable disease with only minor toxicities of Grade I. The dose of cisplatin in 14-day schedules is 75 mg/m\textsuperscript{2} compared to 200 mg/m\textsuperscript{2} for Lipoplatin\textsuperscript{TM} in two randomized Phase III studies (Stathopoulos et al., 2011) in combination with paclitaxel one in NSCLC and the other in non-squamous-NSCLC patients; both studies have shown a much lower toxicity profile in the Lipoplatin\textsuperscript{TM} arm. Furthermore, the non-squamous-NSCLC study demonstrated a statistically-significant higher response rate in the Lipoplatin\textsuperscript{TM} treatment. Consequently, Lipoplatin\textsuperscript{TM} should achieve a higher level of tumor accumulation and also in platinum-DNA adducts.

Despite the fact that the amount of platinum accumulated in the tumoral tissue gradually decreased with the time (Chapter 4 and 5), the concentration of platinum-DNA adducts showed a more complicated behaviour and two maxima were observed at 4 h and 48 h after drug administration. The rational for measuring the number of platinum-DNA adducts as a predictive assay is based on the assumption that higher levels of platinum adducts predicts
favourable treatment outcomes (van de Vaart et al., 2000). Furthermore, it seems plausible that the maximum combination effects with radiation treatment might be observed if radiation were administered when platinum-DNA adducts levels are highest. Indeed, we observed a high concomitant effect under such conditions (Chapter 4 and 5).

The poor accessibility of tumor tissues is a major obstacle for the routine measurement of platinum-DNA adducts formation, but it is known that platinum-DNA adducts form in both tumors and in normal tissues (Hoebers et al., 2008). Might levels in normal tissue be useful markers to levels in tumors? The measurement of platinum-DNA adducts in normal tissues and tumor response was performed previously (Blommaert et al., 1993; Schellens et al., 1996; Van der Vaart et al., 2000). The data on correlations between the amounts of platinum-DNA adducts and tumor responses are contradictory. In one experimental study, no significant correlations were found between adduct levels in normal tissues and primary tumor biopsies, nor between white blood cells and buccal cells (Hoebers et al., 2008). However, various other studies (Blommaert et al., 1993; Redd et al., 1987, Schellens et al., 1996; Van der Vaart et al., 2000) showed a positive correlation between the numbers of platinum-DNA adducts in normal tissues and the response to therapy in patients. They suggested that the higher levels of platinum-DNA adducts in normal tissue could predict a favourable treatment outcome. In future study, the evaluation of the concentration of platinum-DNA adducts in normal tissue as well as in leucocytes could be performed as a potential predictive assay, with the assumption that normal tissues may be used as a surrogate marker for the tumor. Additionally, such a study might allow for the assessment of the concomitant effects in a clinical setting, where repetitively obtaining cancerous tissue samples to determine platinum-adduct concentration would be problematic.
Such a study would look to identify correlations between in platinum-DNA adduct levels in normal tissue and patient response to chemoradiotherapy in clinical trials.

**Platinum-based radiosensitizers and cell cycle progression to chemoradiotherapy in human colorectal cancer HCT116 cell**

To determine the radiosensitivity of cisplatin and oxaliplatin in colorectal cancer *in vitro* and *in vivo* studies were performed. For *in vitro* study (Chapter 3), the colony formation assay demonstrated that cisplatin and oxaliplatin act as radiosensitizers in human colorectal cancer HCT116 cells. It should be noted that the concentration range used in this study (cisplatin = 1 - 10 μM; oxaliplatin = 0.5 - 3.5 μM) is close to clinically obtainable concentrations, e.g. oxaliplatin at 2 h i.v. injection of a normal patient dose (85 mg/m²) reaches a peak plasma concentration of 3.6 μM (Boulikas and Vougiouka, 2003).

While cisplatin and oxaliplatin belong to the alkylating agent group of chemotherapies (Kweekel et al., 2005), they are not cell cycle specific. However, their radiosensitizing activity is a function of the cell cycle (Hall and Giaccia, 2005). The diagram in Figure 1 illustrates the cell cycle phases and their relative radiosensitivity or radioresistance. Cells appear to be maximally sensitive to platinum drugs and radiation in G₀/G₁ and G₂/M phases and minimally sensitive at peak DNA synthesis in the S phase. Unlike G₂/M arrest, the S phase delay lacks the sustained maintenance phase of the cell cycle arrest (Shiloh, 2003). Moreover, DNA damage induction by platinum drug and radiation has been shown to cause G₂/M arrest prior to inducing apoptosis (Sorenson et al., 1990).

The cell cycle progression after chemoradiotherapy was investigated in HCT116 human colorectal cancer cells to better understand of how chemotherapeutic agents cisplatin, oxaliplatin and their liposomal formulations (Lipoplatin™ and Lipoxal™) can act as
radiosensitizers *in vitro*, and to determine the effect of platinum drugs on cell cycle progression. To examine cell distribution in the different phases of the cell cycle, we used flow cytometry at 8 h and 48 h after drug exposure, with and without radiation treatment.

![Diagram](image)

**Figure 1.** Diagram illustrated phases of cell cycle and their relative radiosensitivity or radioresistance (Adapted with permission from Hill) (Hill et al., 2012).

A similar characteristic of cell progression at both 8 h and 48 h after drug incubation, with and without radiation treatment, in that the majority of cells were accumulated in the G₀/G₁ phases. The combined treatment (i.e., with radiation) yielded a higher level of cell accumulated in the G₂/M phase than with platinum drug treatment alone. However, we observed a difference in cell accumulation in the G₂/M phase, (which is the most radiosensitive phase) between 8 h and 48 h of drug incubation. Samples incubated for 8 hours and then irradiated showed a higher level of cell blocking at G₂/M phase than the untreated controls,
while a lower level of cell accumulation at $G_2/M$ phase was measured in sample irradiated after 48 h of drug incubation. Moreover, a higher level of apoptotic cell death was observed with the combined radiation treatment at 8 h than was the case when radiation was administered after 48 h of drug incubation. Consideration of time-dependence in cell cycle progressions may contribute to the variation of the concomitant effect of combined treatment, and consequently have implications for the enhancement for cancer cell death in chemoradiotherapy involving platinum-based drugs.

As described in chapter 3, the concentration of platinum drugs used to incubate the HCT116 human colorectal cancer cells (e.g. at concentrations lower, equal and higher than their $IC_{50}$ values) may affect the cell progression through the cell cycle, affecting the potential for a concomitant effect with combined treatment. It has been suggested that a high level of cell accumulation at $G_2/M$ phase, combined with a delay in the S phase, resulting from a reduction in DNA synthesis, is correlated with the augmentation of drug concentrations (William-Faltaos et al., 2006). Such an increase of cell accumulation at $G_2/M$ phase could lead to an enhancement in apoptotic cell death (Zheng et al., 2007). We clearly observed a synergetic effect as well as an additive effect when assessing radiation treatment at high or median concentration of the drug ($> IC_{50}$ or $= IC_{50}$ values) for both 8 h and 48 h of drug incubation, except in the case of oxaliplatin after 48 h, where a reduction of cell accumulation at $G_2/M$ phase of more than 60% was measured. Similarly previous studies have demonstrated a decrease in $G_2/M$ accumulation when HCT116 cells were exposed to oxaliplatin for a long incubation period (Arnould et al., 2002). Such a decrease suggests that a reduction in the radiosensitizing activity of combined treatment at long incubation times with this drug. The treatment with all drugs, excluding Lipoxal™, at low concentration (i.e., below the $IC_{50}$ values)
for 48 h, showed a significantly antagonistic effect, indicating no pronounced G₂/M arrest in these cases.

**Radiosensitizing activity of Lipoplatin™ and Lipoxal™**

The radiosensitizing activity of liposomal platinum formulations, Lipoplatin™ and Lipoxal™, has been evaluated for cellular response, for systemic cytotoxicity and for the potential for synergetic effects with radiation treatment. In previous a phase I/II study concerning concurrent liposomal cisplatin (Lipoplatin), 5-fluorouracil and radiotherapy for the treatment of locally advanced gastric cancer (Koukourakis et al., 2010), Lipoplatin™ was given at a dose of 120 mg/m²/week, 5-fluorouracil at 400mg/m²/week (Day 1), and radiotherapy was given as 3.5-Gy fractions on Days 2, 3, and 4. Two groups of 6 patients received four and five consecutive cycles, respectively. The concurrent administration of Lipoplatin™ and radiation showed a high potential for improving complete response rates to chemoradiation treatment in locally advanced gastric cancer with acceptable toxicities. Such results appear consistent with those of this study (e.g., chapters 4 and 5), where we observed a radiosensitizing enhancement after combined radiation treatment with Lipoplatin™ and Lipoxal™ in both cell cultures and mouse xenografts.

While in chapter 4 and 5, Lipoplatin™ and Lipoxal™ were found to produce fewer platinum-DNA adducts than did their free platinum formulations, a superior concomitant effect was observed with concurrent radiation treatment. Only a weak correlation between the cytotoxicity of liposomal platinum drugs and the degree of DNA platination could be found. This might imply that the platinum-based drugs have important targets other than from nuclear DNA (e.g. the cytoplasmic compartments) (Yang et al., 2000). For example it has been proposed that Lipoplatin™ and Lipoxal™ can release cisplatin and oxaliplatin molecules into
the cytoplasm of the tumor cell which then activate the mitochondrial apoptotic cascade (Stathopoulos and Boulikas, 2012). In addition, any released cisplatin and oxaliplatin molecules may i) undergo reaction with phospholipids; ii) inhibit amino acid transport, protein synthesis and ATPases; and iii) uncouple oxidative phosphorylation (Gourdier et al., 2004; Mandic et al., 2003; Wang et al., 1996). However, the importance of targets other than DNA in relation to cytotoxicity is still unclear and remains the subject of future study.

Due to the abundance of mitochondria in almost all cells, platinum drugs may interact with components of the mitochondrial membrane (Weissig et al., 2006). It has been suggested that platinum drugs can induce high levels of mitochondrial reactive oxygen species (ROS) such as the superoxide radical anion (O$_{2}^{-}$), hydroxyl radical (OH) and hydrogen peroxide (H$_{2}$O$_{2}$) (Mukhopadhyay et al., 2012). ROS can interact with cellular macromolecules to interfere with vital cellular functions and so lead to cell death (Conklin, 2004). Yakes and Van Houten have been demonstrated oxidative stress-induced mitochondrial DNA damage (Yakes and Van Houten, 1997). Figure 2 shows a schematic illustrating the induction of the mitochondrial apoptotic pathway by cisplatin. Cisplatin-induced mitochondrial ROS trigger the release of cytochrome c from the mitochondrial intermembrane space onto the cytosol, which induces the formation of the apoptosome. The apoptosome is an Apaf-1 cytochrome c complex which activates procaspase-9. Procaspase-9 molecules can bind to the inner "hub" region of the apoptosome. This complex promotes the efficient activation of procaspase-3. Therefore, the cleavage of procaspase-9 is not required to form an active cell death complex (Boulikas, 2007). Cisplatin can activate the proapoptotic protein Bax, resulting in cytochrome c release, caspase activation, and apoptosis (Wei et al., 2007). Bcl-2 also plays an important role in the mitochondrial apoptotic pathway.
It has been proposed that the binding affinity of drugs to the mitochondria is regulated by its lipid solubility (Boddapati et al., 2005). Hori and co-workers have reported that high lipophilicity drugs are more rapidly accumulated in the mitochondria than are non- or low-lipophilic drugs (Hori et al., 1987). The improvement in terms of lipophilicity for Lipoplatin™ and Lipoxal™ above that of their free-platinum forms could indicate a higher efficacy for binding to the mitochondria membrane, that would lead to a greater accumulation of the drugs within the mitochondria. Thus, induction of the liposomal platinum drug accumulation in the mitochondria may contribute to the enhancement of apoptotic cancer cell death. This characteristic of Lipoplatin™ and Lipoxal™ may be further associated with the improvement of the radiosensitizing effect after the chemoradiation treatment.
Figure 2. Induction of the mitochondrial apoptotic pathway by cisplatin (Adapted with permission from Boulakis) (Boulakis, 2007).

Histopathological analysis

Part of the work of this thesis was to evaluate the efficiency of various combinations of radiation with platinum-based drugs on the basis of their effect on tumor growth delay (Chapter 4 and 5). Additionally, histopathologic changes were also studied to determine the response of
HCT116 colorectal tumor xenografts to chemoradiation treatments. Examples are shown in the Figure 3A. Mitotic cells were not counted in necrotic areas. Figure 3B shows a mitotic cell count after chemoradiotherapy. The tumor sections treated with chemotherapy alone remained mitotically active compared to the control group. In contrast, HCT116 tumor sections with combined platinum-based drugs and radiotherapy, display a significant decrease in the number of mitotic cells. In addition, the combined treatments demonstrate a shrinking nuclear pleomorphism in relation to form and size; one or two nucleoli observed in the nuclei. Islands of geographic necrosis are frequent between the sheets of tumor cells. Vascularisation is poor. The tumoral mass seems contained in a very thin collagen capsule-like structure.

The relationship between mitotic activity and apoptotic cell death has been reported (Brustmann, 2004). In the present study, an intense reduction in mitotic activity was observed when radiation was applied 48 h after drug administration. In addition, we also observed an increased number of cells dying following the combined treatment when compared to the control group. After apoptotic analysis using TUNEL assay, we observed a higher percentage of apoptotic cells at 48 h of drug administration combined with radiation treatment, compared to the control group or to both chemotherapy and radiotherapy alone. These results on mitotic activity and the fraction of apoptotic cells are remarkably consistent with idea that a strong concomitant effect is obtained when radiotherapy is performed 48 h after drug administration (i.e., when there is a high level of platinum-DNA adducts).
A)

i) Control

ii) Irradiation alone

iii) Cisplatin

iv) Lipoplatin™
v) Oxaliplatin

vi) Lipoxal™

vii) Cisplatin (24h)-IR

viii) Lipoplatin™ (24h)-IR

ix) Oxaliplatin (24h)-IR

x) Lipoxal™ (24h)-IR

164
xi) Cisplatin (48h)-IR  

diagram of Cisplatin (48h)-IR

xii) Lipoplastin™ (48h)-IR  

diagram of Lipoplastin™ (48h)-IR

xiii) Oxaliplatin (48h)-IR  

diagram of Oxaliplatin (48h)-IR

xiv) Lipoxal™ (48h)-IR  

diagram of Lipoxal™ (48h)-IR
Figure 3. Histophatologic changes of HCT116 human colorectal cancer tumors treated with platinum drugs and radiation. A) H&E staining after chemoradiotherapy. Animals were irradiated at 24 and 48 h of drug administration. Treatments were administered with a single dose of cisplatin, oxaliplatin, Lipoplatin™, Lipoxal™ of 10 mg/kg; and a single dose of radiation of 15 Gy. Arrow indicated mitotic figures and arrow head indicated cells undergoing death. Mitotic cells were counted in 10 random fields per tumor (n=3) (Mean ± S.D.). Magnification, X40. B) Mitotic cells count after chemoradiotherapy.

Emerging strategies for improvement in chemoradiotherapy: Clinical aspects

The efficacy of radiation treatment is often increased by using chemotherapeutic agents such as radiosensitizers. Concurrent chemoradiotherapy has become the standard treatment of colorectal cancer (Prestwich et al., 2007; Glimelius et al., 2003; Minsky et al., 1992). Bosset and co-workers reported downsizing and down-staging of tumors, as well as changes in histologic characteristics of tumors after chemoradiotherapy. These effects were associated
with a significant increase in local control, but no improvement in progression-free or overall survival (Bosset et al., 2005). The combined therapy is still much restricted by its narrow therapeutic index. The chemotherapeutic drugs scheduling in relation to radiation fractions is one of the important. Optimal scheduling is essential in concomitant chemoradiotherapy, not only to maximize tumor radioresponse, but also minimize toxicity to critical normal tissues.

Before novel clinical treatment strategies are attempted in the field of chemoradiation, there is a need for adequate preclinical data that establishes whether the addition of drugs to radiation is likely to be of clinical benefit. The selection of optimal timing of drug administration must be based on multiple factors including; mechanisms of tumor radioenhancement by a given drug, the drug’s normal tissues toxicity, and conditions under which the highest enhancement is achieved. The successes in our preclinical studies offer the possibility to develop an optimal concomitant chemoradiotherapy schedule, and this should encourage further clinical implementation. The hypothesis for further development of an optimal clinical treatment schedule that follows the observation in vitro and in vivo studies is shown in the Figure 4.

For most current chemoradiotherapy protocols, the radiation is given at equal doses on a daily basis (five sessions per week, for about five weeks) while the addition of cisplatin or oxaliplatin is given intermittently (Bosset et al., 2006; Marcu, van Doorn and Olver, 2003). Assessment of radiotherapy at the time at which the amount of platinum accumulated in the tumor or bound to DNA is low, likely to be clinically relevant. This may reduce the anticancer effectiveness of the combined treatment.
Figure 4. Possible improvement for clinical chemoradiation treatment based on our preclinical studies (\(\bullet\)) for radiation treatment.

The improvement of antitumor effectiveness after fixed sessions of radiotherapy at which the maximum level of platinum bound to DNA, so far not tested yet in the clinical practice. Cividalli and co-workers reported the improvement of tumor growth delay in mouse adenocarcinoma after combined treatment of oxaliplatin (10 mg/kg) with a single dose of 10 Gy X-rays better relative to a treatment with 10 daily X-rays sessions (Choi and Deasy, 2002). The modifying of the conventional chemoradiation treatment schedule from daily 5 x 2 Gy per week to a few sessions radiotherapy which correspond to the amount of platinum-DNA adducts (e.g. 2 x 5 Gy per week) should be further explored.

Furthermore, considering that the majority of platinum radiosensitizers are not administered alone, but combined with other drugs. Table 2 shows the lists of other
chemotherapeutic agents that are commonly combined with platinum drugs (Nieder and Lordick, 2006). Here, we used platinum-based radiosensitizers as a monotherapy, but concurrent administration of 5-FU or other drugs could impact the distribution of those drugs, and vice versa. Kjellstrom and co-workers reported the importance of the period of incubation with oxaliplatin in a colorectal cancer cell line exposed to ionizing radiation and 5-FU [33]. It has shown also the integration of drugs such as 5-fluorouracil, folinic acid, capecitabine and irinotecan to oxaliplatin chemoradiotherapy protocols (Andre and Schmiegel, 2005; William-Faltaos et al., 2006). For cisplatin combined with 5-fluorouracil, folinic acid and gemcitabine has been purposed (Chang et al., 2007). Thus, the use of cisplatin, oxaliplatin and their liposomal formulations in combination with other drugs plus radiation could be further explored.
Table 2 Lists of other chemotherapeutic agents that are commonly combined with platinum drugs (Nieder and Lordick, 2006).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irinotecan</td>
<td>Topoisomerase-I inhibitor</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Topoisomerase-II inhibitor</td>
</tr>
<tr>
<td>Taxanes (Paclitaxel, Docetaxel)</td>
<td>Microtubule inhibitors, disruption of the centrosome network, inhibition of mitotic spindle formation</td>
</tr>
<tr>
<td>Vinblastine, Vincristine, Vinorelbine</td>
<td>Tubuling-binding vinca alkaloids, inhibition of mitosis</td>
</tr>
<tr>
<td>5-Fluorouracil, Capecitabine, Gemcitabine</td>
<td>Affect DNA synthesis, nucleoside, and nucleotide metabolism deplete the deoxynucleoside triphosphate pool (5-FU: inhibition of thymidilate synthase; Capecitabine: oral prodrug, converted to FU by thymidine phosphorylase; Gemcitabine diphosphate inhibits ribonucleotide reductase inducing a depletion of cellular deoxynucleotides (dNTP)</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Affects DNA synthesis in hypoxic cells through formation of adducts and crosslinks</td>
</tr>
<tr>
<td>Tirapazamine</td>
<td>Affects hypoxic cells through intracellular reduction resulting in a highly reactive radical capable of causing DNA strand breaks</td>
</tr>
</tbody>
</table>
CHAPTER VII
CONCLUSIONS

Aiming to enhance the synergism and increase the efficiency of concomitant chemoradiotherapy in patients with rectal cancers, we tried to identify the optimal conditions associated with higher synergistic anticancer effect with no or minimal added toxicity.

The in vitro experiments confirm the sensitization of the colorectal cell line HCT116 to radiation therapy after exposure to cisplatin, oxaliplatin and their liposomal formulations Lipoplatin™ and Lipoxal™. Such radiosensitization was shown to depend on the drug concentration (that should exceed IC$_{50}$), and the duration of exposure. The timing of radiation in relation to drug exposure, which implies variation of drug concentration in tumor cells and its different intra-cellular compartments, was shown to be a major element impacting synergism. A maximal synergism appears to correlate to the time of highest platinum-DNA adducts.

These data was confirmed in animal model, where the outcome of combined chemoradiotherapy varied in relation to the timing of drug exposure and radiotherapy. The maximal synergism and antitumor effect was observed when platinum drug was administered 48 h prior to radiation, which mainly correlated to the highest level of platinum bound to the DNA.

These observations goes with the hypothesis of “true” or “physical” synergism between radiation and platinum molecules bound to the DNA, rather than a concomitant or complimentary biologic effects.
These studies show that the time-dependent accumulation of platinum in tumor cells and in its different cellular compartments is not linear, and is not completely similar to other healthy tissue cells. This opened the way to define a time-window where radiation has the highest synergism with platinum.

Given that most clinical platinum based chemoradiotherapy protocols in rectal cancers or other neoplasia consist of daily hyperfractionated radiation and intermittent chemotherapy, it appears clear that this window of maximal synergism correlates only to a small part of the radiation treatment, and most of radiation is given at times of sub-optimal or minimal synergism. There is clearly a wide room to improve these clinical protocols for better patient outcomes.

The liposomal platinum formulations (Lipoplatin™ and Lipoxal™) have been investigated to overcome side effects of cisplatin and oxaliplatin. Our in vitro and in vivo data confirm their potential roles as radiosensitizers. While the general concepts of maximal synergism correlating with highest DNA platinum still applies, there were some kinetic different between each of these drugs, as described, and these should be taken in consideration in defining the “platinum window” of each drug.

Collectively, these studies allow a better understanding of the pharmacokinetics and the synergistic mechanisms at the base of platinum-based chemoradiotherapy, and open the door to a better patient outcome with minimal or no added toxicity. This should be confirmed in clinical trials.


175

Canadian Cancer Society, www.cancer.ca


Rodel, C., Liersch, T., Becker, H., Fietkau, R., Hohenberger, W., Hothorn, T. et al. (2012). Preoperative chemoradiotherapy and postoperative chemotherapy with fluorouracil and


Oxaliplatin-induced damage of cellular DNA. Molecular Pharmacology, vol. 58, n° 5, p. 920-927.


