Doctoral Program in Health Sciences

REDOX MODULATION BY SOD MIMICS IN RENAL CANCER: FROM ETIOLOGY TO PROGRESSION

Doctoral thesis presented by:

João Guilherme Feliciano da Costa

Alcalá de Henares, 2017

DEPARTAMENTO DE
CIENCIAS BIOMEDICAS

ESCOLA DE CIÊNCIAS E
TECNOLOGIAS DA SAÚDE

En virtud del acuerdo de colaboración com la Universidade Lusófona de Humanidades e Tecnologias

Programa de Doctorado en Ciencias de la Salud
Programa de Doutoramento em Ciências da Saúde

MODULACIÓN REDOX POR MIMÉTICOS DE LA SOD EN EL CÁNCER RENAL: DE LA ETIOLOGÍA A LA PROGRESSIÓN
MODULAÇÃO REDOX POR MIMÉTICOS DA SOD NO CANCRO RENAL: DA ETIOLOGIA À PROGRESSÃO

Doctoral thesis presented by:

João Guilherme Feliciano da Costa

Directores:

Dra. Ana Sofia Fernandes

Dr. Nuno Guerreiro Oliveira

Dr. Francisco Javier de Lucio Cazaña

Alcalá de Henares, 2017

PROGRAMA DE DOCTOADO EN CIENCIAS DE LA SALUD
Facultad de Medicina y Ciencias de la Salud
Escuela de Doctorado
Universidad de Alcalá
Ctra. Barcelona, Km. 33,600
28871 Alcalá de Henares (Madrid)

UNIVERSIDAD DE ALCALÁ, PATRIMONIO DE LA HUMANIDAD

DR. D. PEDRO DE LA VILLA POLO, COORDINADOR DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN CIENCIAS DE LA SALUD.

CERTIFICA que la Tesis Doctoral titulada **REDOX MODULATION BY SOD MIMICS IN RENAL CANCER: FROM ETIOLOGY TO PROGRESSION**, presentada por Don **JOÃO GUILHERME FELICIANO DA COSTA**, bajo la dirección de la Dr. Dña Ana Sofia Fernandes, del Dr. Don Nuno Guerreiro de Oliveira y del Dr. Don Francisco Javier de Lucio Cazaña reúne los requisitos científicos de originalidad y rigor metodológicos para ser defendida ante un tribunal. Esta Comisión ha tenido también en cuenta la evaluación del doctorando, habiendo obtenido las correspondientes competencias establecidas en el Programa.

Para que así conste y surta los efectos oportunos, se firma el presente certificado en Alcalá de Henares a 28 de julio de 2017.

Fdo.: Pedro de la Villa Polo

Universidad de Alcalá

DEPARTAMENTO DE CIENCIAS BIOMÉDICAS
Campus Universitario
Ctra. de Barcelona Km. 33,600
E-28871 Alcalá de Henares (Madrid)
Teléfono: 91 885 46 57

ANA SOFIA FERNANDES, PROFESSORA AUXILIAR Y PROFESSORA DE FARMACOLOGIA Y TOXICOLOGIA DE LA ESCOLA DE CIÊNCIAS E TECNOLOGIAS DA SAÚDE DE LA UNIVERSIDADE LUSÓFONA DE HUMANIDADES E TECNOLOGIAS DE LISBOA, NUNO GUERREIRO DE OLIVEIRA, PROFESSOR AUXILIAR Y PROFESSOR DE TOXICOLOGIA DE LA FACULDADE DE FARMACIA DE LA UNIVERSIDADE DE LISBOA, Y FRANCISCO JAVIER DE LUCIO CAZAÑA, PROFESSOR CATEDRÁTICO Y PROFESSOR DE FISIOLOGÍA DE LA FACULTAD DE MEDICINA Y CIENCIAS DE LA SALUD DE LA UNIVERSIDAD DE ALCALÁ

CERTIFICAN:

Que la memoria para optar al Grado de Doctor, elaborada por **João Guilherme Feliciano da Costa**, cuyo título es:

"REDOX MODULATION BY SOD MIMICS IN RENAL CANCER: FROM ETIOLOGY TO PROGRESSION"

ha sido realizada bajo su dirección y que reúne todos los requisitos necesarios para su juicio y calificación.

Y para que así conste, firman el presente certificado en Alcalá de Henares a 24 de julio de dos mil diecisiete.

Ana Sofia Fernandes	Nuno Guerreiro Oliveira	Francisco Javier de Lucio Cazaña
Prof. Doutor ULHT	Prof. Doutor FFUL	Prof. Doutor UAH

UNIVERSIDAD DE ALCALÁ, PATRIMONIO DE LA HUMANIDAD

Acknowledgments

I want to express my acknowledgments to my supervisors Professors Ana Sofia Fernandes and Nuno Oliveira. Thank you for your commitment, mentorship, precious scientific discussions and to allowing me to grow as a research scientist. Thank you for your constant encouragement, confidence and friendship. You have been incredible mentors for me, I am grateful to have been guided by you.

I want to express my acknowledgments to Professor Luís Monteiro Rodrigues for the opportunity to integrate the pedagogical and scientific activities at Escola de Ciências e Tecnologias da Saúde from Universidade Lusófona, which contributed to my personal, pedagogical and scientific development. Thank you for your confidence and support.

I want to thank my colleagues from CBIOS / Universidade Lusófona, particularly to Nuno Saraiva for his help in the laboratory. I also want to thank to Tânia Almeida and Catarina Rosado for their friendship and constant encouragement.

I want to thank my colleagues from the Chemical Biology and Toxicology (CBT) group from iMed.UL, particularly to Patrícia Guerreiro and Sérgio Camões for their help and availability. I also want to thank to Professor Matilde Castro for her encouragement and friendship.

I would like to thank to all the other researchers that were involved in this work. To Joana Miranda from CBT group (iMed.UL). My acknowledgment to Professor Ines Batinic-Haberle, from the Department of Radiation Oncology of Duke University Medical School. Thank you for the possibility to study your Manganese(III) porphyrins. I also want to thank Maria João Silva and Henriqueta Louro from Instituto Ricardo Jorge for their contribution to the experimental work presented herein.

To my family, especially to my parents, grandparents and sister. Thank you for your constant support and encouragement at every stage of my personal and academic life. Thank you for showing me how to keep motivated and always want to learn more. It has not been an easy road for you and I admire you for all you have done. Thank you for all.

To my wife, Sónia. Words cannot express how grateful I am. Thank you for all the sacrifices you have made in the last years. Thank you for your constant support, encouragement, understanding, friendship and love. You were always my support in the moments when there was no one to answer my queries. Thank you for being a great person with a big heart. I admire you. Thank you for being an exceptional friend, wife and mother. This PhD was only possible thanks to you.

To my son, Francisco. You were born during this work and despite all the difficulties, you filled the last three years with joy. You have given me all the courage that I need to face everything. Thank you.

Finally, I want to acknowledge ALIES, CBIOS and COST Action BM1203 / EU-ROS for the financial support.

Abstract

The incidence of renal cancer has been increasing over the last decades along with the interest in the research and development of novel anticancer drugs. Cancer is a multistep process and oxidative stress has been pointed out to have critical roles on its initiation, promotion and progression. This thesis addresses the redox modulation afforded by the superoxide dismutase mimic (SODm) MnTnHex-2-PyP (MnP), a prototype of the Manganese(III) porphyrins (MnPs), which are catalytical polyfunctional antioxidants with the ability to modulate different cellular redox pathways. Two different renal cell models were used to address initiation and progression cellular events. Firstly, the role of MnP on the toxicity of non-tumor renal cells exposed to the ochratoxin A (OTA) was evaluated. OTA is a well-known nephrotoxic agent and renal carcinogen and was used as a reference model to assess potential effects of the SODm in the initiation processes. Different endpoints of cytotoxicity and genotoxicity were evaluated in Vero cells exposed to OTA. The MnP protected cells from the OTA-induced cytotoxicity. In addition, it modulated the intracellular reactive oxygen species (ROS) levels and decreased the percentage of cells in apoptosis when compared with cells exposed only to OTA. The role of the MnP in renal cancer progression was subsequently studied in a human renal cancer cell model. MnP exposure resulted in a concentration and time-dependent decrease in cell viability of human 786-O cells and led to a significant increase in sub-G1 population. This treatment also induced a concentration-dependent increase in intracellular ROS. Notably, low concentrations of the MnP significantly decreased the chemotactic migration of the human renal cancer cells.

This study contributed to clarify the role of oxidative stress in OTA-induced toxicity. ROS partially contributed to the cytotoxicity and genotoxicity of OTA in kidney cells, although other mechanisms may be relevant for OTA-induced deleterious effects. In addition, this work revealed the potential of MnTnHex-2-PyP in renal cancer treatment,

by decreasing cancer cells viability and migration. Overall, this MnP protected non-tumor cells from the toxic effects induced by OTA, while it had a beneficial effect against renal cancer cells. The results obtained herein reinforce the multiple potential applications of MnPs and warrant further studies towards their therapeutic use.

Keywords: renal cancer, oxidative stress, SOD mimics, MnTnHex-2-PyP

Resumen

La incidencia de cáncer renal ha aumentado en las últimas décadas, junto con el interés en la investigación y desarrollo de nuevos fármacos anticancerígenos. El cáncer es un proceso de múltiples pasos y el estrés oxidativo ha sido señalado por su papel crítico en la iniciación, promoción y progresión. Esta tesis aborda la modulación redox proporcionada por el mimético de la superóxido dismutasa (SODm) denominado MnTnHex-2-PyP (MnP), un prototipo de las porfirinas de manganeso(III) (MnPs), que son antioxidantes polifuncionales catalíticos capaces de modular diferentes vías redox celulares. Dos modelos diferentes de células renales se utilizaron para tratar la iniciación y la progressión de los eventos celulares. En primer lugar, se evaluó el papel del MnP en la toxicidad renal de células no tumorales expuestas a la ocratoxina A (OTA). OTA es un agente nefrotóxico bien conocido y carcinógeno renal que fue utilizado como un modelo de referencia para evaluar los efectos potenciales de SODm en los procesos de iniciación. Diferentes parámetros de citotoxicidad y genotoxicidad se evaluaron en células Vero expuestas a la OTA. El MnP protegió a las células de la citotoxicidad inducida por OTA. Además, hubo modulación de las especies reactivas de oxígeno (ROS) intracelulares, disminución de los niveles y el porcentaje de células en apoptosis en comparación con las células expuestas únicamente a OTA. Posteriormente, se estudió el papel del MnP en la progresión del cáncer usando un modelo de células de cáncer renal humano. La exposición al MnP resultó en una disminución dependiente de la concentración y del tiempo de la viabilidad de las células humanas 786-O y condujo a un aumento significativo en la población sub-G1. Asimismo, este tratamiento indujo a un aumento dependiente de la concentración de las ROS intracelulares. Cabe resaltar que concentraciones bajas del MnP disminuyeron significativamente la migración quimiotáctica de las células del cáncer renal humano.

Este estudio ha contribuido a clarificar el papel del estrés oxidativo en la toxicidad inducida por OTA. ROS contribuyó parcialmente a la citotoxicidad y a la genotoxicidad de OTA en células de riñón, aunque otros mecanismos pueden ser relevantes para los efectos deletéreos inducidos por OTA. Además, este trabajo reveló el potencial de MnTnHex-2-PyP en el tratamiento del cáncer de riñón mediante la disminución de la viabilidad y migración de las células cancerígenas. En general, este MnP protegió a las células no tumorales de los efectos tóxicos inducidos por OTA, mientras tuvo un efecto beneficioso contra las células de cáncer renal. Los resultados aquí obtenidos refuerzan las múltiples potenciales aplicaciones de las MnPs y sugieren más estudios para su uso terapéutico.

Palabras clave: cáncer renal, estrés oxidativo, miméticos de la SOD, MnTnHex-2-PyP

Scientific outputs

Articles

The data presented in this thesis led to two articles published in international refereed journals (references below). A third full paper is currently under preparation.

1**.** Ochratoxin A induced cytotoxicity, genotoxicity and reactive oxygen species in kidney cells: an integrative approach of complementary endpoints
Costa, João G.; Saraiva, Nuno; Guerreiro, Patrícia S.; Louro, Henriqueta; Silva, Maria J.; Miranda, Joana P.; Castro, Matilde; Batinic-Haberle, Ines; Fernandes, Ana S.; Oliveira, Nuno G. Food and Chemical Toxicology (2016), 87, 65-76

2. European Contribution to the study of ROS: A Summary of the Findings and Prospects for the Future from the COST Action BM1203 (EU-ROS)
Egea, Javier; Fabregat, Isabel; (…) **Costa, João G.** (…) Fernandes, Ana S. (…) Oliveira, Nuno G. (…) Di Lisa, Fabio; Daiber, Andreas. Redox Biology (2017), 13, 94-162

Beyond the scope of this thesis, I was involved in other research projects in which some of the methodologies used herein were implemented and optimized. The results from these works were published in the following papers:

1. The APE1 redox inhibitor E3330 reduces collective cell migration of human breast cancer cells and decreases chemoinvasion and colony formation when combined with docetaxel
Guerreiro, Patrícia S.; Corvacho, Eduardo; **Costa, João G.**; Saraiva, Nuno; Fernandes, Ana S.; Castro, Matilde; Miranda, Joana P.; Oliveira, Nuno G. Chemical Biology & Drug Design (2017), [In Press]

2. Structure-based virtual screening toward the discovery of novel inhibitors of the DNA repair activity of the human apurinic/apyrimidinic endonuclease 1
Guerreiro, Patrícia S.; Estácio, Sílvia G.; Antunes, F.; Fernandes; Ana S.; Pinheiro, Pedro F.; **Costa, João G.**; Castro, Matilde; Miranda, Joana P.; Guedes, Rita C.; Oliveira, Nuno G. Chemical Biology & Drug Design (2016), 88(6), 915-925

3. Xanthine Oxidase Inhibitory Activity of a Plectranthus saccatus aqueous extract
Caldeira, Francisco; **Costa, João G.**; Rijo, Patrícia; Saraiva, Nuno; Fernandes, Ana S. Biomedical and Biopharmaceutical Research (2016), 2(13), 259-269

4. Functionalized diterpene parvifloron D-loaded hybrid nanoparticles for targeted delivery in melanoma therapy
Silva, Catarina O.; Molpeceres, Jesús; Batanero, Belén; Fernandes, Ana S.; Saraiva, Nuno; **Costa**, **João G.**; Rijo, Patrícia; Figueiredo, Isabel V.; Faísca, Pedro; Reis, Catarina P. Therapeutic Delivery (2016), 7(8), 521–544

5. Differential effects of methoxyamine on doxorubicin cytotoxicity and genotoxicity in MDA-MB-231 human breast cancer cells
Guerreiro, Patrícia S.; Fernandes, Ana Sofia; **Costa, João G.**; Castro, Matilde; Miranda, Joana P.; Oliveira, Nuno G. Mutation Research/Genetic Toxicology and Environmental Mutagenesis (2013), 757(2), 140-147

Abstracts

In addition, the following abstracts were published in international peer-reviewed journals:

1. Effect of the SOD mimetic MnTnHex-2-PyP on the generation of ROS and cytotoxicity induced by ochratoxin A
Costa, J.; Saraiva, N.; Guerreiro, P.; Miranda, J.P.; Batinic-Haberle, I.; Castro, M.; Oliveira, N.G.; Fernandes, A.S. Toxicology Letters (2015), 238(2), S292-S292

2. Redox modulation by SOD mimics in renal cancer: from etiology to progression
Costa, J.. Biomed Biopharm Res. (2015), 1(12), 140-143

3. Targeting APE1 redox function with E3330: Effects on the migration of MDA-MB-231 cells
Guerreiro, P.; Corvacho, E.; Miranda, J.P.; **Costa, J.**; Fernandes, A.S.; Castro, M.; Oliveira, N.G. Toxicology Letters (2015), 238(2), S242-S242

4. Ionic liquids as solubility/permeation enhancers for topical formulations: Skin permeation and cytotoxicity characterization
Santos de Almeida, T.; Júlio, A.; Caparica, R.; Rosado, C.; Fernandes, A.S.; Saraiva, N.; Ribeiro, M.; Araújo, M.E.; Baby, A.R.; **Costa, J.**; Portugal Mota, J. Toxicology Letters (2015), 238(2), S293-S293

5. Hybrid nanoparticles for photodynamic and targeted cancer therapy: Cytotoxicity studies
Silva, C.O.; Coelho, J.P.; Vieira, P.; Lopes, J.; **Costa, J.**; Fernandes, A.S.; Gomes, R.; Gabriel, A.; Rijo, P.; Molpeceres, J.; Reis, C.P.. Toxicology Letters (2015), 238(2), S204-S204

6. Cytotoxicity screening of Plectranthus spp. extracts and individual components in MDAMB-231 cells

Matias, D.; Nicolai, M.; **Costa, J.**; Saraiva, N.; Fernandes, A.S.; Simões, M.F.; Diaz-Lanza, A.M.; Reis, C.P.; Rijo, P.. Toxicology Letters (2015), 238(2), S240-S240

7. Induction of micronuclei and cytotoxic effects of ochratoxin A in Vero cells

Costa, J.; Fernandes, A.S.; Guerreiro, P.S.; Filipe, E.; Miranda, J.P.; Castro, M.; Oliveira, N.G. Toxicology Letters (2013), 221, S121-S121

Book chapter

1. Nuno G. OLIVEIRA, Joana P. MIRANDA, **João G. COSTA**, António S. RODRIGUES, Matilde F. CASTRO, Ana S. FERNANDES. Toxicodinâmica. In Toxicologia Fundamental, eds Ricardo D. Oliveira, Félix D. Carvalho, Maria de Lourdes Bastos. Lidel, Portugal. [Accepted]

Oral communications

1. Influence of the SOD mimic MnTnHex-2-PyP on the viability and migration of human renal cancer cells

João G. Costa; Nuno Saraiva; Patrícia S. Guerreiro; Matilde Castro; Ines Batinic-Haberle; Nuno G. Oliveira; Ana S. Fernandes. XLVII Sociedade Portuguesa de Farmacologia Meeting, Coimbra, Portugal, 2017.

2. Case study: Ochratoxin A, ROS and Antioxidants

João G. Costa; Nuno Saraiva; Patrícia S. Guerreiro; Henriqueta Louro; Maria J. Silva; Joana P. Miranda; Matilde Castro; Ines Batinic-Haberle; Ana S. Fernandes; Nuno G. Oliveira. II Jornadas CBIOS – ECTS-ULHT, Lisbon, Portugal, 2016.

3. Reactive oxygen species generation by ochratoxin A: impact of the SOD mimic MnTnHex-2-PyP

João G. Costa. Training Course on Redox Biology in Health and Disease, Alicante, Spain, 2015.

4. Ochratoxin A-induced cytotoxicity and formation of ROS: effect of a manganese(III) porphyrin with superoxide dismutase mimetic activity

João G. Costa; N. Saraiva; Patrícia S. Guerreiro; Joana P. Miranda; Matilde Castro; Nuno G. Oliveira; Ana S. Fernandes. XLV Sociedade Portuguesa de Farmacologia Meeting, Lisbon, Portugal, 2015.

5. Cytotoxicity, genotoxicity and reactive oxygen species production by ochratoxin A in monkey kidney cells

João G. Costa; N. Saraiva; Patrícia S. Guerreiro; Joana P. Miranda; Matilde Castro; Nuno G. Oliveira; Ana S. Fernandes. II Jornadas Ibéricas de Toxicologia, Covilhã, Portugal, 2014.

6. Cytotoxicity, genotoxicity and ROS production by ochratoxin A in Vero cells

João G. Costa; Patrícia S. Guerreiro; Nuno Saraiva; Joana P. Miranda; Matilde Castro; Nuno G. Oliveira; Ana S. Fernandes. Spetses Summer School "Biochemical Basis of Healthy Aging", Spetses, Greece, 2014.

7. Redox modulation by SOD mimics in renal cancer: from etiology to progression

João G. Costa; Patrícia S. Guerreiro; Nuno Saraiva; Joana P. Miranda; Matilde Castro; Nuno G. Oliveira; Ana S. Fernandes. I Jornadas CBIOS – ECTS-ULHT, Lisbon, Portugal, 2014.

Poster communications

1. **J. Costa**, N. Saraiva, P. S. Guerreiro, M. Castro, I. Batinic-Haberle, N. G. Oliveira, A. S. Fernandes. Viability and migration of human renal cancer cells upon treatment with a superoxide dismutase mimic. [P-03-01-01]. 53rd Congress of the European Societies of Toxicology (EUROTOX), Bratislava, Slovakia, 2017.

2. **J. Costa**, N. Saraiva, P. S. Guerreiro, M. Castro, I. Batinic-Haberle, N. G. Oliveira, A. S. Fernandes. Influence of MnTnHex-2-PyP on the viability and migration of 786-O cells. [PC66]. 9th iMed.ULisboa Postgraduate Students Meeting, Lisbon, Portugal, 2017.

3. **J. Costa**, N. Saraiva, P. S. Guerreiro, J. P. Miranda, I. Batinic-Haberle, M. Castro, N. G. Oliveira, A. S. Fernandes. Ochratoxin A-induced cytotoxicity, genotoxicity and reactive oxygen species in kidney cells. Ciência 2017 - Encontro com a Ciência e Tecnologia em Portugal, Lisbon, Portugal, 2017.

4. **J. Costa**, N. Saraiva, P. S. Guerreiro, J. P. Miranda, I. Batinic-Haberle, M. Castro, N. G. Oliveira, A. S. Fernandes. Effect of the SOD mimetic MnTnHex-2-PyP on the generation of ROS and cytotoxicity induced by ochratoxin A. [P13-058]. 51st Congress of the European Societies of Toxicology (EUROTOX), Porto, Portugal, 2015.

5. P. Guerreiro, E. Corvacho, J. P. Miranda, **J. Costa**, A. S. Fernandes, M. Castro, N. G. Oliveira. Targeting APE1 redox function with E3330: effects on the migration of MDA-MB-231 cells. [P11-023]. 51st Congress of the European Societies of Toxicology (EUROTOX), Porto, Portugal, 2015.

6. D. Matias, M. Nicolai, **J. Costa**, N. Saraiva, A. S. Fernandes, M. F. Simões, A. M. Diaz-Lanza, C. P. Reis, P. Rijo. Cytotoxicity screening of Plectranthus spp. extracts and individual components in MDA-MB-231 cells. [P11-014]. 51st Congress of the European Societies of Toxicology (EUROTOX), Porto, Portugal, 2015.

7. C. O. Silva, J. P. Coelho, P. Vieira, J. Lopes, **J. Costa**, A. S. Fernandes, R. Gomes, A. Gabriel, P. Rijo, J. Molpeceres, C. P. Reis. Hybrid nanoparticles for photodynamic and targeted cancer therapy: Cytotoxicity studies. [P08-029]. 51st Congress of the European Societies of Toxicology (EUROTOX), Porto, Portugal, 2015.

8. T. S. Almeida, A. Júlio, R. Caparica, C. Rosado, A. S. Fernandes, N. Saraiva, M. Ribeiro, M. E. Araújo, A. R. Baby, **J. Costa**, J. P. Mota. Ionic liquids as solubility/permeation enhancers for topical formulations: Skin permeation and cytotoxicity characterization. [P13-060]. 51st Congress of the European Societies of Toxicology (EUROTOX), Porto, Portugal, 2015.

9. **J. Costa**, N. Saraiva, P. S. Guerreiro, J. P. Miranda, I. Batinic-Haberle, M. Castro, N. G. Oliveira, A. S. Fernandes. Reactive oxygen species generation by ochratoxin A: impact of the SOD mimic MnTnHex-2-PyP. [PC56]. 7th iMed.ULisboa Postgraduate Students Meeting, Lisbon, Portugal. 2015.

10. P. Guerreiro, E. Corvacho, **J. Costa**, A. S. Fernandes, M. Castro, N. Saraiva, J. P. Miranda, N. G. Oliveira. Evaluation of an inhibitor of APE1 redox function in docetaxel-treated MDA-MB-231 cells. [PC58]. 7th iMed.ULisboa Postgraduate Students Meeting, Lisbon, Portugal, 2015.

11. **J. Costa**, N. Saraiva, P. S. Guerreiro, J. P. Miranda, M. Castro, N. G. Oliveira, A. S. Fernandes. Cytotoxicity, genotoxicity and reactive oxygen species production by ochratoxin A in monkey kidney cells. [P.10]. II Jornadas Ibéricas de Toxicologia, Covilhã, Portugal, 2014.

12. **J. Costa**, P. S. Guerreiro, N. Saraiva, J. P. Miranda, M. Castro, N. G. Oliveira, A. S. Fernandes. Cytotoxicity, genotoxicity and ROS production by ochratoxin A in Vero cells. [P7]. Spetses Summer School "Biochemical Basis of Healthy Aging", Spetses, Greece, 2014.

13. **J. Costa**, P. S. Guerreiro, N. Saraiva, J. P. Miranda, M. Castro, N. G. Oliveira, A. S. Fernandes. Cytotoxicity, genotoxicity and ROS production by ochratoxin A in Vero cells. [PC55]. 6th iMed.ULisboa Postgraduate Students Meeting, Lisbon, Portugal, 2014.

14. P. Guerreiro, E. Corvacho, J. P. Miranda, **J. Costa**, A. S. Fernandes, M. Castro, N. G. Oliveira. Targeting APE1 redox activity to modulate the migration of MDA-MB-231 breast cancer cells. [PC60]. 6th iMed.ULisboa Postgraduate Students Meeting, Lisbon, Portugal, 2014.

15. **J. Costa**, A. S. Fernandes, P. Guerreiro, E. Filipe, J. P. Miranda, M. Castro, N. G. Oliveira. Induction of micronuclei and cytotoxic effects of ochratoxin A in Vero cells. [P09-15]. 49th Congress of the European Societies of Toxicology (EUROTOX), Interlaken, Switzerland, 2013.

Awards

1. Honorable mention at II Jornadas CBIOS, ECTS-ULHT, Lisbon, Portugal (2016), for the oral communication "Case study: ochratoxin A, ROS and antioxidants".

2. Best poster award at II Jornadas Ibéricas de Toxicologia, Covilhã, Portugal (2014), attributed for the work "Cytotoxicity, genotoxicity and reactive oxygen species production by ochratoxin A in monkey kidney cells".

Grants

1. PADDIC grants 2016 and 2017 from ALIES-COFAC as part of the PhD program in Health Sciences from Univ. Alcalá and Univ. Lusófona.

2. EU-ROS COST Action BM1203 grants to attend the Spetses and Alicante training schools in 2014 and 2015.

Table of contents

List of Figures

List of Tables

List of abbreviations

$^1O_2$	Singlet oxygen
4-HNE	4-Hydroxynonenal
AA	Aristolochic acid
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
ANOVA	One-way analysis of variance
AP-1	Activator protein-1
BAP1	Breast cancer 1 associated protein-1
BBB	Blood–brain barrier
BMI	Body mass index
BN	Binucleated
BRCA1	Breast cancer 1
CAT	Catalase
CBMN	Cytokinesis-block micronucleus
ccRCC	Clear cell renal cell carcinoma
CV	Crystal violet
DDAH	Dimethylarginine dimethylaminohydrolase
DHE	Dihydroethidium
DHR	Dihydrorhodamine 123
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
$E_{1/2}$	Half-wave metal-centered reduction potential
EMS	Ethyl methanesulfonate
EPO	Erythropoietin
ESMO	Europe's leading medical oncology society
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FPG	Formamidopyrimidine DNA glycosylase
FR	Free radicals

GLUT-1	Glucose transporter protein-1
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HIF	Hypoxia inducible factors
HIF-1α	Hypoxia inducible factor-1α
HO-1	Heme-oxygenase 1
$HOO^{\bullet}$	Hydroperoxyl
HPRCC	Hereditary papillary RCC
IARC	International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration
iNOS	Nitric oxide synthase
IR	Ionizing radiation
JARID1A	Jumonji AT-rich interactive domain 1A
k_{cat}	catalytic constant
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
MOA	Mode of action
MN	Micronuclei
MNBN	Micronucleated binucleated
MnPs	Mn(III) porphyrins
mRCC	Metastatic Renal-Cell Carcinoma
mTOR	Mammalian target of rapamycin
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4--sulphophenyl)-2H-Tetrazolium]
NADPH	Nicotinamide adenine dinucleotide phosphate
NDI	Nuclear division index
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NOX	NADPH oxidases
NR	Neutral red

Nrf2	Nuclear factor erythroid 2–related factor 2
NSS	Nephron-sparing surgery
$O_2^{\bullet -}$	Superoxide anion radical
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
OTA	Ochratoxin A
PBRM1	Polybromo 1
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
Pen/strep	Penicillin/streptomycin
PI	Propidium iodide
PI3K	Phosphatidylinositide 3-kinase
PRDX4	Peroxiredoxin-4
RCC	Renal cell carcinoma
RNS	Reactive nitrogen species
$RO^{\bullet}$	Alkoxyl radicals
$ROO^{\bullet}$	Peroxyl radicals
ROS	Reactive oxygen species
RS	Reactive species
SETD2	SET domain-containing protein 2
SOD	Superoxide dismutase
SODm	Superoxide dismutase mimics
SP-1	Specificity protein-1
TBARS	Thiobarbituric acid reactive substances
TCE	Trichloroethylene
TGF-α	Transforming growth factor alpha
Trolox	(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
USA	United States of America
VDAC1	Voltage-dependent anion channel 1
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
XO	Xanthine oxidase

Chapter 1

General Introduction

1.1. Oxidative stress

The concept of oxidative stress was introduced by Helmut Sies in 1985 and was defined as an imbalance between oxidants and antioxidants in favor of the oxidants (Sies, 1985). Due to the increasing knowledge in the redox biology area, this concept has been slightly changing. The current definition of oxidative stress include "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" (Sies, 2015; Sies and Jones, 2007). The levels of cellular oxidants are well controlled, not only via their production, but also via elimination by antioxidant systems. If this control fails, oxidative damage may occur. Oxidative stress is therefore associated with several diseases (Fig. 1.1), such as cardiovascular disorders, neurodegenerative, metabolic and autoimmune diseases (Reuter et al., 2010). Furthermore, oxidative stress is associated with inflammatory disorders and cancer, among other diseases (Armstrong and Stratton, 2016; Kehrer and Klotz, 2015; Rahal et al., 2014; Reuter et al., 2010).

Fig. 1.1 – Examples of pathological conditions associated with oxidative stress.

The term reactive species (RS) includes oxygen, nitrogen, carbon, sulfur, chlorine, bromine or halogens species with high reactivity (Kehrer and Klotz, 2015; Rahal et al., 2014). The RS can be free radicals or non-radical derivatives. Reactive oxygen species (ROS) include different chemical species, such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), alkoxyl radicals ($RO^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$). The $O_2^{\bullet-}$, mainly produced in mitochondrial electron transport reactions, is dismutated by superoxide dismutase (SOD) enzymes to H_2O_2 and O_2. In the presence of reduced free iron (Fe^{2+}) and copper (Cu^{+}) ions, H_2O_2 participates in Fenton reactions to produce $HO^{\bullet}$. The oxidized forms Fe^{3+} and Cu^{2+} can then oxidize H_2O_2 to form hydroperoxyl ($HOO^{\bullet}$; Armstrong and Stratton, 2016; Birben et al., 2012). The reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^{-}$), are also important in various pathophysiological processes (Dedon and Tannenbaum, 2004; Weidinger and Kozlov, 2015).

The major endogenous sources of ROS include cellular metabolism and respiration processes. During cell respiration, oxygen is reduced by sequential transfer of electrons and some intermediates with odd electrons can escape from mitochondrial electron-transport chain (Brieger et al., 2012). These electrons interact with O_2 to originate $O_2^{\bullet-}$, which spontaneously or enzymatically is converted to H_2O_2 and $HO^{\bullet}$ (Lushchak, 2014). In the metabolic processes that occur in different cellular organelles, such as endoplasmic reticulum (ER) or peroxisome, ROS are also generated. Various enzymes, for example xanthine oxidase (XO), NADPH oxidases (Nox) or P450 cytochromes, are also relevant ROS producers (Brieger et al., 2012; Lushchak, 2014). ROS may also be generated from exogenous sources, including cigarette smoke, ozone exposure, hyperoxia, ionizing radiation, exposure to heavy metals and food toxicants (Birben et al., 2012; Shibamoto and Bjeldanes, 2009).

The principal biological targets of RS include lipids, proteins, carbohydrates, DNA and RNA (Kehrer and Klotz, 2015). The RS can oxidize directly many substrates but are also able to initiate a number of addition reactions, which result in a covalent

binding to biomolecules, including lipids (Birben et al., 2012). Oxidative stress can lead to lipid peroxidation, protein oxidation and DNA oxidation (Birben et al., 2012; Valko et al., 2006). Moreover, RS can interact with cellular signaling pathways that can alter gene activation/inactivation with subsequent cellular responses such as induction of antioxidant enzymes, cell death or cancer initiation (Kehrer and Klotz, 2015).

Beyond the negative role associated to the RS over the years, nowadays it is also clear that these species have indeed beneficial functions, participating in diverse processes essential to live (Lushchak, 2014). RS, namely ROS are vital as mediators of multiple cellular processes, including cell growth and differentiation, cell signaling, the immune response, biosynthesis, cell adhesion and apoptosis (Brieger et al., 2012; Dröge, 2002; Ray et al., 2012; Verbon et al., 2012). Therefore, the intracellular levels of ROS need to be well controlled to avoid cellular damage (Fig. 1.2).

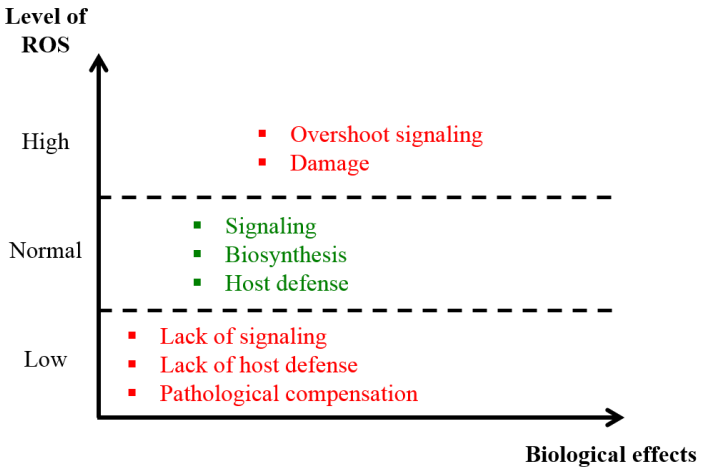

Fig. 1.2 – The role of ROS in biological systems (adapted from Brieger et al., 2012).

The cellular antioxidant system is vital to eliminate excess ROS or to minimize their negative effects. This antioxidant system include non-enzymatic antioxidants, such as ascorbic acid, tocopherols, carotenoids, anthocyanins, polyphenols and uric acid (Birben et al., 2012; Lushchak, 2014). Glutathione (GSH) is a major antioxidant that can interact directly with ROS and serve as a cofactor to antioxidant enzymes (Aquilano

et al., 2014). Nevertheless, the main cellular antioxidants group includes the enzymes superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidases (GPx). The SOD catalyzes the dismutation of the $O_2^{\bullet -}$ with formation of O_2 and H_2O_2, which can be further dismutated by CAT or reduced by GPx (Fig. 1.3).

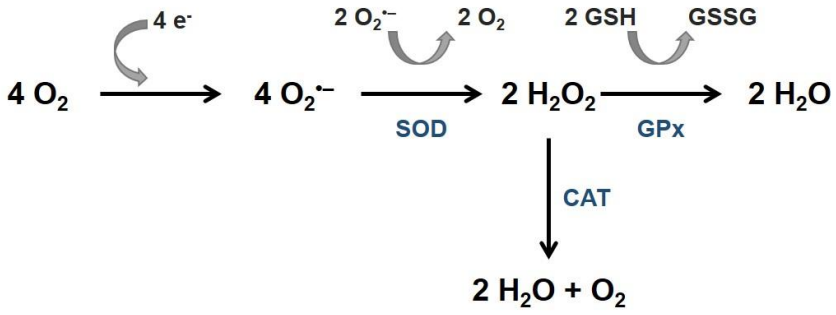

Fig. 1.3 – Major cellular enzymatic antioxidant defenses.

1.1.1. Oxidative stress and Cancer

The development of cancer is a complex and multistep process. The cancer progression can occur through various defects in signaling cascades regulating the processes of cell differentiation, proliferation, migration, and apoptosis (Hanahan and Weinberg, 2011; Kruck et al., 2014).

In 2000, Hanahan and Weinberg published a landmark review that pointed out to six classical cancer hallmarks: induction of angiogenesis, resistance to cell death, capability to sustain proliferative signaling, evasion of growth suppressors and tissue invasion and metastasis (Hanahan and Weinberg, 2000). One decade later, due to the scientific progress, additional cancer hallmarks were proposed: genome instability and mutation, deregulation of cellular energetics, the ability to avoid immune destruction and the presence of an inflammatory response (Hanahan and Weinberg, 2011). The

hallmarks play a collective and interlinked role promoting tumorigenesis (Fig. 1.4). Oxidative stress is a key element in cancer growth. ROS can be involved in all tumorigenesis steps: initiation, promotion and progression. ROS also participate in many aspects of tumor development, such as cellular proliferation, evasion of apoptosis, tissue invasion and metastasis and angiogenesis (Fulda, 2010; Sosa et al., 2013; Valko et al., 2006).

Fig. 1.4 – The Hallmarks of Cancer (adapted from Hanahan and Weinberg, 2011).

The levels of ROS change throughout the tumorigenesis stages (Fig. 1.5). While in normal cells an equilibrium between cellular antioxidants and ROS generation is present, increased ROS levels constitute hallmarks of tumor cells. The higher levels of ROS lead to genomic instability, pro-survival signaling and increased cell proliferation and motility (Storz, 2013). At this stage, antioxidant therapeutic approaches could be effective against tumor progression. On the other hand, some chemotherapeutic drugs can increase ROS to a much higher levels, which induce irreversible cell damage and tumor regression. Conversely, some tumor cells (i.e. cancer stem cells) have the ability

to resist to cell death by upregulating the antioxidant systems, which leads to tumor resistance (Storz, 2013).

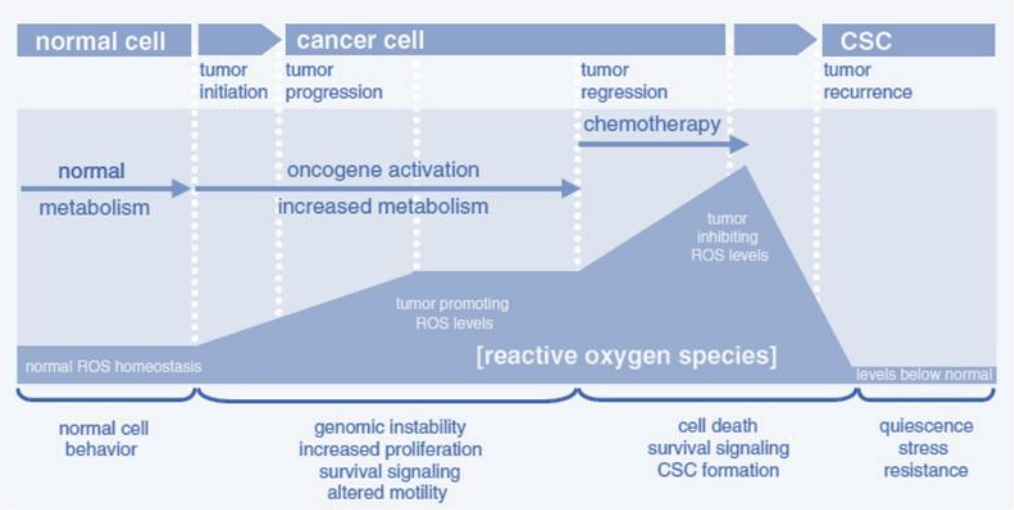

Fig. 1.5 – ROS levels in the different tumor stages (adapted from Storz, 2013).

1.1.2. Superoxide dismutase (SOD)

The SOD enzymes are the major cellular defense against $O_2^{\bullet-}$. These enzymes are oxidoreductases that catalyze the dismutation of $O_2^{\bullet-}$ (Fukai and Ushio-Fukai, 2011). In this reaction, two molecules of $O_2^{\bullet-}$ react with SOD to form O_2 and H_2O_2 (Eq 1/ Eq2).

$$O_2^{\bullet-} + M^{(n+1)+} \rightarrow O_2 + M^{n+} \qquad \text{(Eq 1)}$$

$$O_2^{\bullet-} + M^{n+} + 2H^+ \rightarrow H_2O_2 + M^{(n+1)+} \qquad \text{(Eq 2)}$$

In the first equation, the oxidized form of the metalloenzyme SOD ($M^{(n+1)+}$) reacts with $O_2^{\bullet-}$ to form O_2 and generates the reduced form of the enzyme (M^{n+}). In the second step, the reduced form of the metalloenzyme reacts with another $O_2^{\bullet-}$ and two protons to form H_2O_2, regenerating the oxidized form of the enzyme (Miller, 2012).

In mammals, there are three isoforms of SOD: SOD1 (CuZnSOD), SOD2 (MnSOD) and SOD3 (ecSOD) that differ mainly in their metal center and distribution in the different cellular compartments (Table I.1).

Table I.1 – Types of superoxide dismutase enzymes in mammals (SOD).

Types of SOD	Active center	Characteristics	Location
SOD1	Cu(II)/(I) and Zn(II)	32 kDa, homodimer	Cytoplasm, mitochondrial intermembrane space and others (nucleus, lysosomes, peroxisomes)
SOD2	Mn(III)/(II)	96 kDa, homotetramer	Mitochondrial matrix
SOD3 (ecSOD)	Cu(II)/(I) and Zn(II)	135 kDa, homotetrameric glycoprotein	Extracellular matrix, cell surface, extracellular fluids

Adapted from Fukai and Ushio-Fukai, 2011.

Although each SOD isoform is coded by different genes and present distinct subcellular localization, they all catalyze the same reaction (Fukai and Ushio-Fukai, 2011; Miller, 2012; Zelko et al., 2002). The SOD1 is mainly localized in the cytosol, although it is also present in a variety of cellular organelles. The SOD2 is a mitochondrial enzyme essential for the dismutation of $O_2^{\bullet -}$ generated by the respiratory chain of enzymes. Finally, the SOD3 is a secretory extracellular enzyme (Fukai and Ushio-Fukai, 2011).

Due to the vital functions of SOD, alterations in these enzymes are frequently associated with oxidative stress-induced pathophysiological conditions. Moreover, metal deficiencies related with diet or diseases associated with increased levels of metals, such as copper in Wilson's disease, affect the SOD function (Johnson and Giulivi, 2005). There is a strong connection between the activity of SOD and Alzheimer's disease (Marcus et al., 1998). In addition, several studies have addressed

the association of the SOD1 with the neurodegenerative disease amyotrophic lateral sclerosis (ALS; Valentine et al., 2005; Wei et al., 2017). An inverse relationship between SOD activity and human diseases, such as in cardiovascular diseases, is also usually observed (Fukai and Ushio-Fukai, 2011). The MnSOD is an isoform that is essential for life in aerobic environment, probably due to its crucial location. Complete knockout of this enzyme results in death shortly after birth in different animal models (Holley and St. Clair, 2016). In addition, several studies have showed that MnSOD plays an important role in the development and progression of cancer (Dhar and St. Clair, 2012). Particularly in renal cancer there is an association between the low levels or the low activity of SOD and its development, which will be further discussed in this thesis.

1.1.3. Superoxide dismutase mimics (SODm)

The importance of SOD to all aerobic life and its beneficial effects in oxidative stress-related pathologies led to the development of SOD mimics (SODm), which have the ability to mimic the reaction of native SOD - the dismutation of $O_2^{\bullet-}$. There are diverse classes of SODm, with different properties and characteristics as summarized in Table I.2.

The Mn(III) porphyrins (MnPs) are among the most potent SODm developed. MnPs have a macrocyclic structure similar to the structure of natural porphyrin-containing proteins (Fig. 1.6), such as hemoglobin, nitric oxide synthases and cytochrome P450 family of enzymes (Batinic-Haberle et al., 2015).

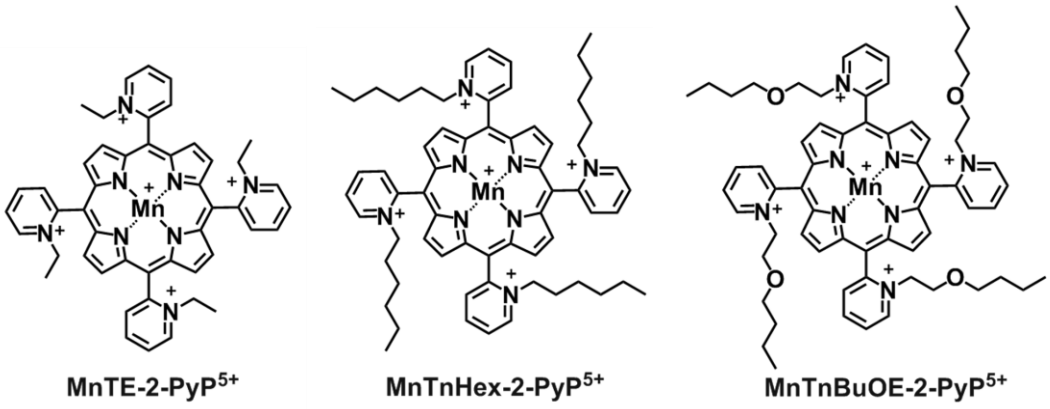

Fig. 1.6 – Chemical structures of some representative MnPs.

Most studies have focused on cationic Mn(III)-substituted *N*-pyridyl- and *N,N'*-imidazolyl-porphyrins, which were optimized to reach high SOD-like activities with a rate constant for the catalysis of $O_2^{\bullet-}$ dismutation ($k_{cat}(O_2^{\bullet-})$) very similar to that of SOD enzymes (Batinić-Haberle et al., 2016; Gauter-Fleckenstein et al., 2014). The dismutation reaction of MnPs includes two steps (Fig. 1.7). In the first step, MnPs act as an oxidant and in the second step as an antioxidant, closing the catalytic cycle (Batinic-Haberle et al., 2015). It is important to note that H_2O_2 is produced during the dismutation process, an oxidant which is detoxified by enzymatic systems, such as CAT or GPx, as previous described (Fig. 1.3).

Table I.2 – Major classes of SOD mimics (SODm).

Classes of SODm	Characteristics	Examples
Mn(III) porphyrins	▪ Scavengers of ROS ($O_2^{\bullet-}$) and RNS (e.g. $ONOO^-$) ▪ Modulators of redox transcription factors (e.g. NF-κB)	MnTM-2-PyP^{5+} MnTE-2-PyP^{5+} MnTnHex-2-PyP^{5+} MnTnBuOE-2-PyP^{5+}
Mn(III) biliverdins	▪ Scavengers of ROS ($O_2^{\bullet-}$)	$(MnBVD)_2$
Mn(III) corroles	▪ Scavengers of ROS (e.g. $O_2^{\bullet-}$ and H_2O_2) and RNS (e.g. $ONOO^-$)	MnDiM-4-PyMAn-$Corrole^{2+}$ $MnTrF_5Ph$-β$(SO_3)_2$-$Corrole^{2-}$
Mn(III) salen complexes	▪ Mn enclosed in a salen = = *N*,*N*'-bis-(salicylideneamido)ethane complex ▪ Scavengers of ROS ($O_2^{\bullet-}$ and H_2O_2) and RNS ($ONOO^-$)	EUK-8 EUK-134 EUK-207
Mn(II) cyclic polyamines	▪ Mn(II)-pentaazamacrocyclic complexes ▪ Transfer only one electron ▪ Scavengers of $O_2^{\bullet-}$	M40401 SC55858 M40403 GC4419
Cu(II) complexes	▪ Mono- and binuclear complexes of Cu(II) with different classes of ligands (e.g. amines, quinolone, macrocycles) ▪ Scavengers of ROS ($O_2^{\bullet-}$)	Cu(II) 3',5'-diisopropylsalicylate (CuDIPs) $CuBr_8$TM-4-PyP $Cu[15]pyN_5$
Fe(III) complexes	▪ Fe(III) complexes with macrocycle (e.g. porphyrins) and non-macrocycle ligands (e.g. aminopolycarboxylates) ▪ Scavengers of ROS ($O_2^{\bullet-}$) and RNS ($ONOO^-$)	(OH)FeTM-4-PyP^{4+} (OH)FeTE-2-PyP^{4+} FeTM-4-PyP^{5+} FeTnOct-3-PyP^{5+}
Fe(III) corroles	▪ Scavengers of ROS ($O_2^{\bullet-}$)	$FeTrF_5Ph$-β$(SO_3)_2$-$Corrole^{2-}$
Other metal based compounds	▪ Scavengers of ROS ($O_2^{\bullet-}$)	CeO_2 OsO_4
Nitroxides and Nitrones	▪ Scavengers of ROS ($O_2^{\bullet-}$)	Tempol Tempone Mito-tempol NXY-059

Table elaborated with data from references Batinić-Haberle et al., 2016, 2014, 2010; Fernandes et al., 2015.

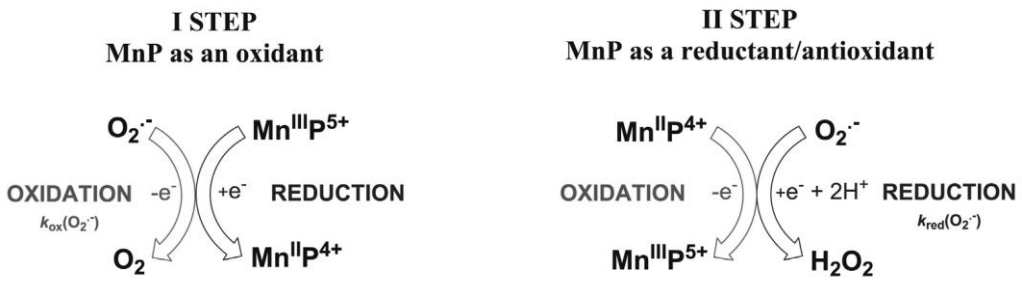

Fig. 1.7 – The $O_2^{\bullet-}$ dismutation reaction catalyzed by Mn porphyrins (MnPs), reproduced from Batinic-Haberle et al., 2015.

MnPs have a high capability to exchange electrons with cellular reductants, RS and redox-active signaling proteins (Gauter-Fleckenstein et al., 2014). Those facile exchange of electrons with bio-targets is controlled by the reduction potential of Mn^{III}/Mn^{II} redox couple and the $k_{cat}(O_2^{\bullet-}$; Gauter-Fleckenstein et al., 2014). MnPs are scavengers of a plethora of different ROS and RNS (Batinić-Haberle et al., 2016).

MnPs have also impact in cellular redoxome, the redox-based signaling pathways (Batinić-Haberle et al., 2014). Moreover, MnPs affect the cellular transcription, modulating transcription factors, such as the hypoxia inducible factor-1α (HIF-1α), the nuclear factor-kappaB (NF-κB), the activator protein-1 (AP-1) and the specificity protein-1 (SP-1; Batinić-Haberle et al., 2012). The MnPs react directly with thiol groups of those transcription factors, preventing its activation, and have also an indirect action on those signaling pathways via RS scavenging (Weitzel et al., 2015). MnPs have been also associated with an activation of Nrf2 signaling pathway (Batinić-Haberle et al., 2016).

The MnTE-2-PyP^{5+} was the first developed and consequently the most studied cationic MnP. Afterwards, other MnPs were developed and characterized, with higher lipophilicity, with the ability to accumulate in mitochondria and to cross the blood–brain barrier (BBB), and less toxic, such as MnTnHex-2-PyP^{5+} and MnTnBuOE-2-PyP^{5+} (Batinić-Haberle et al., 2016). A different MnP, $MnTBAP^{3-}$ was thoroughly

studied and has shown remarkable protective actions in various experimental disorders models. However, this anionic porphyrin was recently classified as a non-SODm and the protective effects reported should be ascribed to other mechanisms of action, rather than to a SOD-like activity (Celic et al., 2014; Tovmasyan et al., 2015a).

Besides MnPs, several other groups of compounds have been explored over the last decades. Those include: Mn salen derivatives (e.g. EUK-207), Mn cyclic polyamines (e.g. M40403), Mn and Fe corroles, Fe(III) polyethyleneglycolated porphyrin (e.g. FP-15), Fe(III) porphyrin with carboxylate substituents on pyridyl nitrogens (e.g. INO-4885) and metal oxides (e.g. CeO_2). In addition, there are non-metal based nitroxides (e.g. tempol) and nitrones (e.g. NXY-059) with superoxide scavenger activity (Batinić-Haberle et al., 2016). However, it is controversial whether such compounds are real SODm, since under physiological pH they may lose the SOD-like activity (Batinić-Haberle et al., 2016).

In addition to their higher potency, the Mn-containing compounds have advantages in terms of biological stability and toxicity (Batinić-Haberle et al., 2014). Many of the Cu(II) and Fe(III) complexes developed so far could dissociate *in vivo* and release the metals ions, which can contribute to their toxicity by producing $HO^{\bullet}$ radical via Fenton reaction (Batinić-Haberle et al., 2010).

1.1.4. SODm in different pathological conditions

Several diseases and pathological disorders are related with oxidative stress. SODm can be very useful as a therapeutic option, due to their capability to reduce the oxidative environment and cellular damage, but also through its ability to modulate different redox pathways.

The therapeutic potential of SODm has been reported in the last years, in different experimental models (Table I.3). The neurologic disorders are one of the major areas of potential use of SODm, particularly of MnPs. The MnTE-2-PyP^{5+} was the first MnP to show neuroprotection in ischemic brain injuries (Mackensen et al., 2001), followed by others MnPs, namely the MnTDE-2-ImP^{5+} and the MnTnHex-2-PyP^{5+} (Sheng et al., 2002; Sheng and Warner, 2016). The MnTDE-2-ImP^{5+} and MnTnHex-2-PyP^{5+} also showed remarkable effects in rat spinal cord injury models (Celic et al., 2014; Sheng et al., 2004). Both MnTE-2-PyP^{5+} and MnTnHex-2-PyP^{5+} inhibited the chronic morphine tolerance and reduced the neuropathic pain in a significant way, in *in vivo* models (Batinić-Haberle et al., 2009).

In the last decades, due the discovery of the central role of SOD in ALS, the most common fatal neurodegenerative disease, SODm were suggested as a therapeutic option for this illness (Hubens and Okado-Matsumoto, 2016; Ramdial et al., 2016). The MnTDE-2-ImP^{5+} alleviated ALS symptoms and extended survival in animal experimental models (Crow, 2006; Crow et al., 2005). Furthermore, a phase I clinical trial with ALS patients with MnTDE-2-ImP^{5+} was performed (Benatar, 2007).

SODm have been also showing beneficial effects in cardiovascular and metabolic diseases. Different MnPs showed to suppress stroke injury, particularly the MnTnHex-2-PyP^{5+} (Sheng et al., 2011). Both MnTDE-2-ImP^{5+} and MnTE-2-PyP^{5+} revealed positive results in diabetes. Those MnPs increased the survival of cultured human pancreatic islet cells and reduced the levels of IL-6 and IL-8, pro-inflammatory cytokines (Bottino et al., 2002). The MnTE-2-PyP^{5+} also showed beneficial effects in diabetes progression in mice (Batinić-Haberle et al., 2014).

SODm showed also protective effects in some lung injuries. The MnTDE-2-ImP^{5+} decreased significantly the adverse effects in animals exposed to tobacco smoke (Smith et al., 2002). SODm can also protect the liver and the kidney in some disorders. For example, MnTDE-2-ImP^{5+} protected the liver from ischemic injury in a rat model (Hines et al., 2003). Furthermore, EUK-134, Tempol and MnTnHex-2-PyP^{5+} protected

the kidney in different experimental rat models. EUK-134 significantly reduced the renal dysfunction and injury in a bilateral renal ischemia rat model, by decreasing the serum creatinine levels, an indicator of renal dysfunction, as well as the urinary N-acetyl-ß-D-glucosaminidase activity, an indicator of tubular damage. In addition, EUK-134 reduced the renal injury related with oxidative stress (Chatterjee et al., 2004). Moreover, Tempol attenuated dose-dependently the ischemia/reperfusion-induced renal dysfunction, as well as the development of kidney histopathological lesions in a rat model (Fujii et al., 2005). The pretreatment with MnTnHex-2-PyP^{5+} was shown to be beneficial in an oxidative stress injury rat model, protecting from ATP depletion, MnSOD inactivation, nitrotyrosine formation and renal dysfunction (Saba et al., 2007). The impact of MnPs in other renal experimental models, including the evaluation of additional endpoints will be further discussed in *Chapter 3* and *Chapter 4*.

Some of those valuable effects of MnPs previous described were possibly related with the inhibition of the NF-κB, a central transcription factor that controls several inflammatory and immune pathways (Batinić-Haberle et al., 2014).

Table I.3 – Examples of beneficial effects of SODm in different disorders.

Disorder	**SODm**	**Model**	**References**
Nervous system			
Ischemic brain injury	MnTE-2-PyP^{5+}	Rat	(Mackensen et al., 2001)
	MnTDE-2-ImP^{5+}	Mouse	(Sheng et al., 2002)
	MnTnHex-2-PyP^{5+}	Rat	(Celic et al., 2014)
Spinal cord injury	MnTDE-2-ImP^{5+}	Mouse	(Sheng et al., 2004)
	MnTnHex-2-PyP^{5+}	Rat	(Celic et al., 2014)
Hyperalgesia	M40403	Rat	(Wang et al., 2004)
	MnTE-2-PyP^{5+}	Mouse	(Batinić-Haberle et al.,
	MnTnHex-2-PyP^{5+}		2009)
Amyotrophic lateral sclerosis (ALS)	MnTDE-2-ImP^{5+}	Mouse	(Crow et al., 2005)

Table I.3 *(cont.)*

Cardiovascular system			
Stroke injury	MnTnHex-2-PyP^{5+}	Rat	(Sheng et al., 2011)
Heart ischemia–reperfusion	M40403	Rat	(Masini et al., 2002)
	EUK-8	Rat	(Xu et al., 2004)
Hemorrhagic shock	EUK-8	Rat	(Izumi et al., 2002)
	EUK-134		
Endocrine system			
Diabetes	MnTE-2-PyP^{5+}	Human islets cells	(Bottino et al., 2002)
	MnTDE-2-ImP^{5+}		
Lung			
Cigarrete smoke injury	MnTDE-2-ImP^{5+}	Rat	(Smith et al., 2002)
Liver			
Isquemic injury	MnTDE-2-ImP^{5+}	Rat	(Hines et al., 2003)
Kidney			
Isquemic injury	EUK-134	Rat	(Chatterjee et al., 2004)
	Tempol	Rat	(Fujii et al., 2005)
	MnTnHex-2-PyP^{5+}	Rat	(Saba et al., 2007)
Gentamicin injury	M40403	Rat	(Cuzzocrea et al., 2002)

1.1.5. SODm and cancer

SODm have also been used with success in cancer models. These compounds have the ability to protect normal tissues from oxidative stress damage induced by chemotherapy and radiotherapy and, conversely, to increase the effect of these anticancer treatments in tumor tissues. SODm can be selective to tumor tissues and usually presented low toxicity to non-tumor tissues (Tovmasyan et al., 2015b). In addition, SODm have been pointed out as anticancer agents through its antiproliferative effects in tumoral cells, as assessed in different experimental models. Those effects can be justified by their differential redox environments (Tovmasyan et al., 2015b). The SODm can increase some RS levels, namely H_2O_2 by its own mechanism, and both SOD and SODm under such conditions cannot be considered as antioxidative defenses (Batinic-Haberle et al., 2015). Furthermore, in the most tumor cells the expression or the activity of the antioxidant defenses, such as CAT or GPx enzymes with capability to detoxify H_2O_2, are lower (Fernandes et al., 2016; Gauter-Fleckenstein et al., 2014; Tovmasyan et al., 2015b; Fig. 1.8). The differences observed may also be justified due to the different transcription factor profile in tumor *versus* non-tumor cells (Keir et al., 2011). The H_2O_2 can contribute to the oxidation or the glutathionylation of redox-sensitive subunits of NF-κB, a critical cellular transcription factor (Batinić-Haberle et al., 2014). Additionally, MnPs can inhibit mitochondrial respiration, with reduction in ATP production and subsequently induction of cell death (Jaramillo et al., 2015). Consequently, SODm can act both as anti- and pro-oxidant, depending on cell type, the basal levels of RS and antioxidant enzymes, the cellular redox balance and the cellular distribution of SODm.

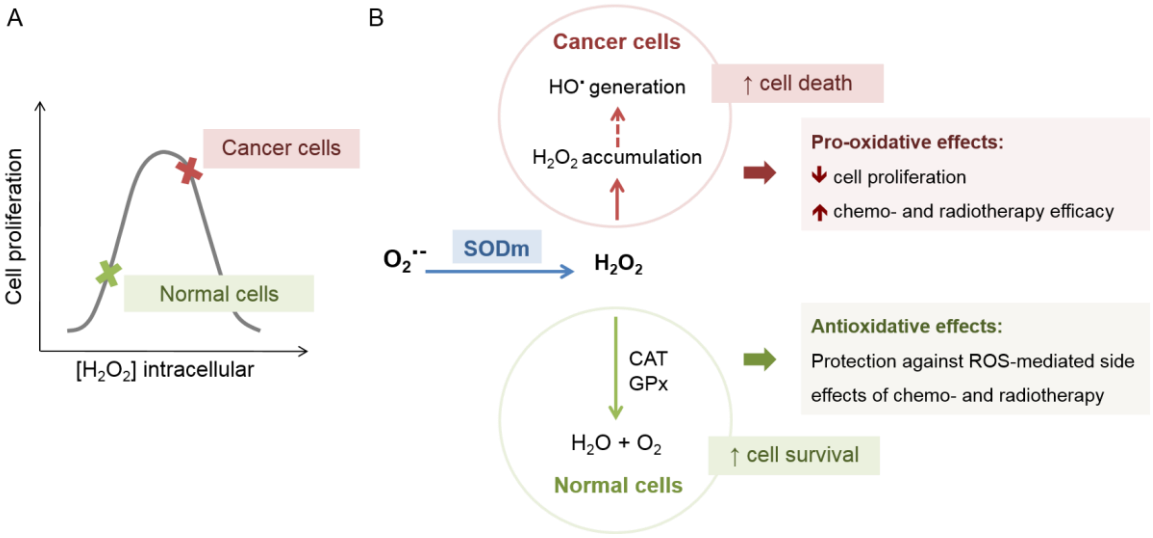

Fig. 1.8 – Differential role of SOD mimics in cancer and normal cells. (A) The opposing effects of intracellular H_2O_2 concentrations on the proliferation of cancer and normal cells. (B) The redox status of cancer and normal cells differs significantly, controlling their differential sensitivity to additional increase in H_2O_2. In cancer cells, SODm may decrease cell proliferation and boost chemo- and radiotherapy treatments. Conversely, SODm may increase the survival of normal cells, protecting against RS-mediated adverse effects of chemo- and radiotherapy (reproduced from Fernandes et al., 2016).

Most cancer treatments, including ionizing radiation (IR), chemotherapy and photodynamic therapy, have a mode of action (MOA) associated with the generation of ROS, which are able to damage DNA and to kill cancer cells. Nonetheless, these treatments can also induce adverse effects in non-tumor tissues (Liou and Storz, 2010; Wang and Yi, 2008). In this context, several studies have been performed towards the application of SODm to protect non-tumor tissues against the toxicity of cancer treatments as exemplified in Table I.4.

Table I.4 – Examples of the application of SODm in cancer therapy.

Action	SODm	Model	References
Radioprotection			
Lung	MnTDE-2-ImP^{5+}	Mouse	(Jackson et al., 2012)
		Mouse	(Zhang et al., 2012)
	MnTE-2-PyP^{5+}	Rat	(Gauter-Fleckenstein et al., 2010, 2008)
	MnTnHex-2-PyP^{5+}	Rat	(Gauter-Fleckenstein et al., 2014, 2008)
	EUK-207	Rat	(Mahmood et al., 2011)
Prostate	MnTE-2-PyP^{5+}	Rat	(Oberley-Deegan et al., 2012)
Brain	MnTDE-2-ImP^{5+}	Rat	(Pearlstein et al., 2010)
Salivary gland	Tempol	Mouse	(Cotrim et al., 2007)
	MnTnBuOE-2-PyP^{5+}	Mouse	(Ashcraft et al., 2015)
Chemoprotection			
Cisplatin-induced nephrotoxicity	$Cu(II)_2(3,5\text{-}DTBS)_4(Eth)_4$	LLC-PK cells	(Wangila et al., 2006)
Oxaliplatin toxicity to normal breast cells	$Cu[15]pyN_5$	MCF-10A cells	(Fernandes et al., 2012)
Paclitaxel-induced neuropathic pain	MnTE-2-PyP^{5+}	Rat	(Doyle et al., 2012)
	Tempol	Rat	(Fernandes et al., 2012)

Both MnTE-2-PyP^{5+} and MnTnHex-2-PyP^{5+} showed remarkable pulmonary radioprotective effects in rat models exposed to IR (Gauter-Fleckenstein et al., 2014, 2010, 2008). The MnTDE-2-ImP^{5+} also showed pulmonary radioprotection with lower expression of several hypoxia-associated genes (Jackson et al., 2012), as well as lower percentage of apoptotic cells (Zhang et al., 2012). Furthermore, SODm protected other

tissues, organs or glands, such as prostate, eyes, gastrointestinal tract, brain, head and neck, salivary gland and hematopoietic stem cells (Batinić-Haberle et al., 2014).

In addition, SODm are capable to counteract the adverse effects of chemotherapy, such as the *in vitro* protection from cisplatin-induced nephrotoxicity by $Cu(II)_2(3,5\text{-}DTBS)_4(Eth)_4$ (Wangila et al., 2006) or from oxaliplatin toxicity to normal breast cells by $Cu[15]pyN_5$ (Fernandes et al., 2012).

SODm have been also associated with antitumor effects as assessed in several experimental models. SODm showed antiproliferative effects, decreased the tumor cell viability or tumor volume, reduced angiogenesis and inflammation, and potentiated the action of established anticancer medicines and radiation (Table I.5).

Table I.5 – Examples of studies on the anticancer action of SODm.

Cancer	**SODm**	**Model**	**References**
Skin	MnTE-2-PyP^{5+}	Mouse	(Zhao et al., 2005)
Brain	MnTnHex-2-PyP^{5+}	Mouse	(Keir et al., 2011)
Lymphoma	MnTE-2-PyP^{5+} (+ *Dexamethasone*)	Murine cells	(Jaramillo et al., 2009)
Breast	MnTE-2-PyP^{5+}	Mouse	(Rabbani et al., 2009)
Colon	Tempol (+ *Doxorubicin*)	Cells	(Ravizza et al., 2004)
Prostate	MnTDE-2-ImP^{5+} (+ *IR*) MnTE-2-PyP^{5+} (+ *IR*) Tempol	Mouse Mouse Cells	(Gridley et al., 2007) (Makinde et al., 2010) (Thomas and Sharifi, 2012)

Due to the promising results obtained in experimental models, some SODm had already entered Clinical Trials. Some examples are given in Table I.6.

Table I.6 – Examples of **S**ODm in clinical trials.

SODm	**Description**	**Date**	**Phase**	**Sponsor**	**References**
BMX-001	Glioma (Combination with radiation or temozolomide)	Ongoing (2016)	I/II	BioMimetix	(BioMimetix JV, 2016)
GC4419	Head and neck cancer (combination with chemoradiation)	2013-16	I	Galera Therapeutics	(Galera, 2013)
	Head and neck cancer (prior to radiation therapy)	Ongoing (2015)	II	Galera Therapeutics	(Galera, 2015)
AEOL-10150	Lung acute radiation syndrome	2016	I	Aeolus Pharmaceuticals	(Pharmaceuticals, 2017)

SODm could be potentially used as anticancer agents alone, decreasing cell proliferation or combined with established anticancer treatments, increasing the RS levels induced by chemo- and radiotherapy.

1.2. Renal Cancer

1.2.1. Epidemiology

The prevalence of cancer is increasing and cancer represents one of the major causes of death worldwide. There are more than 200 different tumor types with own subtypes. Therefore, the interest in the research and development of anticancer drugs is growing (American Cancer Society, 2017).

The renal cell carcinoma (RCC) accounts for 3-5 % of all adult malignancies (Escudier et al., 2016) and comprises approximately 85 % of all kidney malignances (Bukowski et al., 2015). In 2012, 338 000 new cases of kidney cancer were estimated worldwide. The kidney cancer is the 9th most common cancer in men and the 14th most common in women worldwide (Znaor et al., 2015). RCC is approximately 1.75 times more common in men when compared with women and tends to be more elevated at older ages (Siegel et al., 2017). The incidence of RCC varies demographically, with the highest rates registered in developed countries, namely in North America and European Balkan countries, and the lowest rates in African countries and South-East Asia (Fig. 1.9; Ervik et al., 2016; Znaor et al., 2015). Approximately 64 000 new cancer cases affecting the kidney and renal pelvis, which include RCC, and 14 500 deaths are estimated in the USA in 2017 (Siegel et al., 2017). The incidence rates of kidney cancer registered in Europe in 2012 were 15.5/100 000. The highest incidence rates of kidney cancer are found in the Czech Republic (31.4/100 000), as well as the highest mortality rates (10.4/100 000). The renal cancer incidence rates increased over the past decades, in part related with the better diagnosis capability, but it seems to be stabilized in recent years, at least in most developed countries.

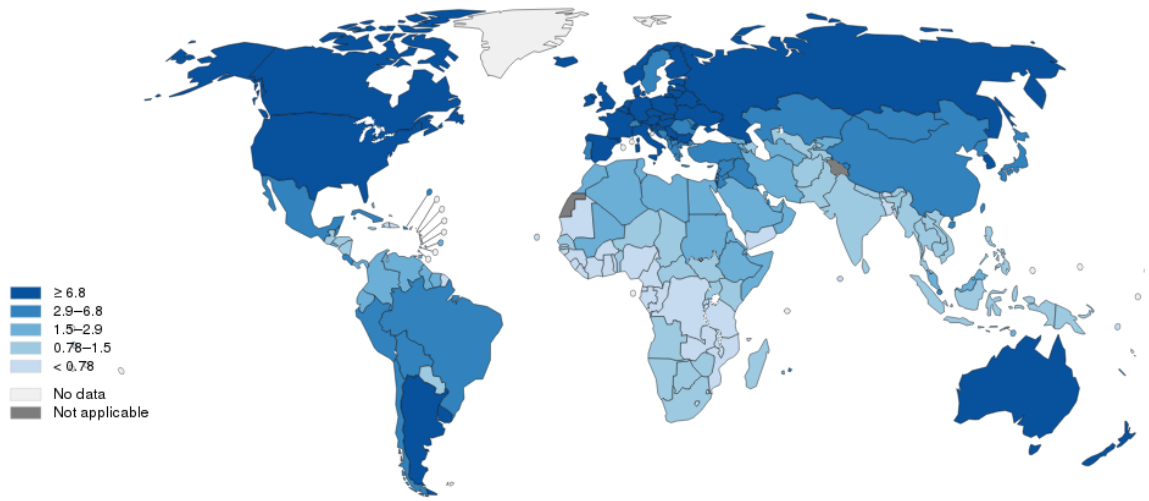

Fig. 1.9 – Age-standardized incidence rates for kidney cancer worldwide in 2012 (adapted from Ervik et al., 2016).

1.2.2. Classification

The RCC represents a group of distinct diseases that can be distinguished histologically and genetically (Keefe et al., 2015). Morphological criteria are used to classify renal tumors into different histological subtypes. The pathologic classification of RCC has changed over the years. While RCC appears to arise from the nephron, different histologic subtypes are believed to originate from different cells (Shuch et al., 2015). The main RCC subtypes include the clear cell (70-80%), papillary (10-15%), chromophobe (~5%), and collecting duct (<1%; Sanchez et al., 2017; Shuch et al., 2015).

Clinically, the clear cell RCC (ccRCC) is the most common renal neoplasm seen in adults. The size can be as small as 1 cm or less and, in this case it can be discovered accidentally, or can have several kilograms (Diaz et al., 1999). Actually, more than 50

% of RCCs are detected incidentally (Escudier et al., 2016). The ccRCC are normally golden yellow, well circumscribed and can have distinct areas with hemorrhages and necrosis (Diaz et al., 1999; Shuch et al., 2015). On microscopic evaluation, these tumor cells have abundant clear cytoplasm due to deposition of lipid and glycogen (Fig. 1.10). The ccRCC appears to originate from the proximal tubule (Shen et al., 2005).

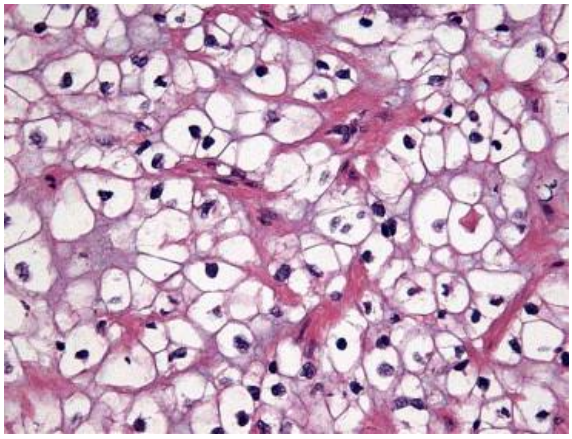

Fig. 1.10 – Clear cell renal carcinoma (reproduced from Algaba et al., 2011).

1.2.3. Etiology

The etiology of RCC is not yet fully understood. Nevertheless there are some stablished risk factors, which resulted from case-control studies conducted during the last three decades along with clinical data (Table I.7).

Cigarette smoking is considered a well-established risk factor for RCC by the International Agency for Research on Cancer (IARC) with an increase up to 50 % of risk in smokers (Hunt et al., 2005; IARC, 2012). A high body mass index (BMI) has also been associated with an increased risk of RCC development, in a dose-response manner (Renehan et al., 2008; Wilson and Cho, 2016). The mechanisms that might explain this connection between obesity and RCC include chronic tissue hypoxia,

insulin resistance and compensatory hyperinsulinemia, endocrine alterations, inflammatory response, lipid peroxidation and oxidative stress (Chow et al., 2010).

Table I.7 – Risk factors for renal cell carcinoma (RCC).

Risk Factors ***Well-Established***	**Association with RCC**	**Risk Factors** ***Suspected***	**Association with RCC**
Cigarette smoking	+	Diabetes mellitus	+
Excess body weight	+	End−stage renal disease	+
Hypertension	+	Parity in women	+
Familiar cancer syndromes	+	Genetic predisposition	+
		Physical activity	-
		Alcohol consumption	-

+ positive; - inverse

Adapted from Chow et al., 2010.

Hypertension is one of the major chronic diseases that affects almost one third of the world's population (Mills et al., 2016). When hypertension is uncontrolled, it can lead to chronic kidney disease and renal function reduction, which may predispose to the development of RCC. Several studies showed an association between a clinical history of long-term hypertension and the development of RCC (Chow et al., 2010; Weikert et al., 2008). Although there is a strong relationship between obesity and hypertension, these disorders seem to be independently related with the risk of RCC (Chow et al., 2010). The biological mechanisms underlying the association between hypertension and RCC are still unclear, but might include chronic renal hypoxia, lipid peroxidation and the formation of ROS (Gago-Dominguez et al., 2002). In what concern ROS it is important to note that many chemicals that lead to kidney injury and /or renal cancer have at least partially oxidative stress related mechanisms involved. This issue will be further developed in the present chapter.

Despite the majority of RCC cases seem to be sporadic, there are relevant genetic defects associated with 2-3 % of RCC cases (Ljungberg et al., 2011). In this context, some familiar cancer syndromes have a well-stablished association with RCC. A two-fold increased risk was reported for a first-degree relative of a patient having RCC, suggesting a hereditary component (Ljungberg et al., 2011). The most common hereditary RCC syndromes are the von Hippel-Lindau (VHL), hereditary papillary RCC (HPRCC), hereditary leiomyomatosis RCC and Birt-Hogg-Dubé (Table I.8; Chow et al., 2010; Ljungberg et al., 2011; Ridge et al., 2014). RCC accounts for 50% of deaths in patients with VHL, which is an autosomal dominant condition with high penetrance (Ridge et al., 2014). VHL arises as a result of a mutation in the VHL tumor suppressor gene in chromosome 3. HPRCC is a rare autosomal dominant syndrome due to mutations in the C-met proto-oncogene in chromosome 7 (Ridge et al., 2014).

Table I.8 – Familial renal cell carcinoma (RCC) syndromes with respective genetic changes.

Syndrome	**Genetic element**	**Function**
von Hippel-Lindau	*VHL* gene Chromosome 3p25-26	Tumor suppressor
Hereditary Papillary RCC	C-*met* proto-oncogene Chromosome 7q31	Oncogene
Familial leiomyomatosis RCC	*Fumarate hydratase* Chromosome 1q42	Tumor suppressor
Birt-Hogg-Dubé	*Folliculin* Chromosome 17p11.2	Tumor suppressor

Adapted from Coleman, 2008; Ljungberg et al., 2011.

Although the main genetic alterations present in RCC are associated with mutations in VHL gene and in other well characterized tumor-suppressor genes, in the last decade other important genes involved in RCC etiology have been uncovered. Many of those genes are dysregulated or silenced via epigenetic mechanisms, mostly through methylation of promoter CpG islands or dysregulation of specific microRNAs (Morris and Latif, 2016). In the last years, several new genes that are also mutated in RCC were identified, such as polybromo 1 (PBRM1), breast cancer 1 (BRCA1) associated protein-1 (BAP1), SET domain-containing protein 2 (SETD2) and jumonji, AT-rich interactive domain 1A (JARID1A; Creighton et al., 2013; Gossage et al., 2014; Keefe et al., 2015; F Piva et al., 2015; Francesco Piva et al., 2015).

There are also some disorders, hormonal factors or social behaviors associated with the risk of RCC. A previous history of diabetes mellitus or renal diseases, as well as the increasing parity among women, have been linked with RCC, although with some inconclusive results (Chow et al., 2010). Diet can also have an important role in RCC. A diet rich in fruits and vegetables can be important to reduce the risk of RCC, due to antioxidant properties. Nevertheless this association is variable (Chow et al., 2010). Several studies showed an inverse association between the practice of physical activity or the alcohol consumption and RCC (Lew et al., 2011; WCRF/AICR, 2015).

It is important to highlight that many of the established risk factors of RCC, such as smoking, obesity, hypertension or chronic kidney diseases are known to increase the oxidative stress in kidneys.

The kidney is a complex and heterogeneous organ. It has a high vulnerability to the toxic effects of many chemical compounds due its important physiological functions. This organ is responsible for the maintenance of the electrolytic balance, synthesis, filtration and concentration of waste products from the systemic circulation. In addition, the kidney has metabolizing capability with the inherent risk of bio-activate pro-carcinogens. Therefore, the epithelium of the nephron is more susceptible than other epithelial tissues. The kidney has also a transcellular transport system that remove

directly some xenobiotics from the blood into the lumen, which could be relevant for the toxicity of some compounds as well (Radford et al., 2013). The probable renal carcinogens include organic solvents, pesticides, heavy metals, food and medicines contaminants, industry products and pharmaceutical drugs. Several studies have evaluated the genotoxicity and carcinogenesis potential of different compounds in experimental renal models and in epidemiological studies (Table I.9).

Carcinogenesis is a multifactorial process. The chemicals that participate in this process may act as genotoxic or non-genotoxic carcinogens. The genotoxic carcinogens interact directly with DNA and induce genetic damage. Most of that genotoxic compounds needs a previous metabolic activation that results in highly electrophilic compounds. Non-genotoxic xenobiotics may act as promoting tumor agents and can participate in all carcinogenesis stages through diverse mechanisms. As aforementioned, some of the kidney carcinogens described in Table I.9 have a mode of action directly or indirectly related with oxidative stress. Aristolochic acid (AA) is a natural alkaloid found in extracts of *Aristolochia* species of plants, present in some herbal medicines. AA is considered one of the most potent human carcinogens. It is known that AA toxic mechanisms include oxidative stress (Pozdzik et al., 2008; Wu et al., 2015; Yu et al., 2011).

Trichloroethylene (TCE) has been widely used as a degreasing agent in many manufacturing industries (Kim et al., 2014). TCE is a well-known carcinogen and recently was categorized in class 1 by IARC due to the sufficient evidence for its association with kidney cancer (Guha et al., 2012; Kelsh et al., 2010). The oxidative stress is one of its mechanisms: TCE causes lipid peroxidation, oxidation of proteins and depletion of antioxidant enzymes (Lash et al., 2000).

Table I.9 – Carcinogenic compounds that might contribute to RCC etiology.

Compound	IARC classification *	References
Aristolochic acid	1	(Hoang et al., 2016; Nesslany et al., 2007)
Trichloroethylene	1	(Kim et al., 2014; Robbiano et al., 2004)
Arsenic	1	(Radford et al., 2013; Yuan et al., 2010)
Cadmium	1	(Il'yasova and Schwartz, 2005; Song et al., 2015)
Benzo[a]pyrene	1	(Asha and Girija, 2011; Radford et al., 2013)
Cisplatin	2A	(Nesslany et al., 2007)
Captafol	2A	(Mačkić and Ahmetović, 2011; Robbiano et al., 2004)
Acrylamide	2A	(Hogervorst et al., 2008; Pelucchi et al., 2015)
Lead	2B	(Robbiano et al., 1999; Steenland et al., 1992)
Ochratoxin A	2B	(Costa et al., 2015; Robbiano et al., 2004)
Chlorothalonil	2B	(Lock and Hard, 2004; Radford et al., 2013)
Bromodichloromethane	2B	(Radford et al., 2013; Robbiano et al., 2004)
N-Nitrosomorpholine	2B	(Korr et al., 2001; Radford et al., 2013)
2-Nitrofluorene	2B	(Cui et al., 1999; Radford et al., 2013)
Streptozotocin	2B	(Nesslany et al., 2007; Radford et al., 2013)
Potassium Bromate	2B	(Nesslany et al., 2007; Robbiano et al., 1999)
Nitrilotriacetic acid	2B	(Robbiano et al., 1999)
Nitrobenzene	2B	(Hsu et al., 2007; Robbiano et al., 2004)

* (IARC, 2017) | IARC classes: 1-known to cause cancer in humans; 2A-probably carcinogenic to humans; 2B-possibly carcinogenic to humans.

Heavy metals, such as arsenic, cadmium or lead are known to be nephrotoxic at high levels of exposure. Additionally, some studies suggested an association with kidney cancer. There is strong epidemiologic evidence that arsenic causes increased

rates of kidney cancer (Yuan et al., 2010). The exposure to cadmium is also associated with kidney cancer (Il'yasova and Schwartz, 2005). A recent meta-analysis showed that a high cadmium exposure significantly increased renal cancer up to approximately 1.5 times. Additionally, this association was higher for occupational exposure compared with non-occupational exposure (Song et al., 2015). Generally, there is a weak evidence of human kidney cancer induced by lead exposure (Steenland and Boffetta, 2000). Nonetheless, an up to 2-fold higher risk was observed in some studies (Steenland et al., 1992). There are several evidences concerning the induction of oxidative stress by heavy metals. They are able to increase ROS by Fenton-like reactions and by their electron-sharing affinities that result in the formation of covalent bindings with proteins, some of them important for the cellular antioxidant defense (Nuran Ercal et al., 2001). Potassium bromate ($KBrO_3$) is another example of a nephrotoxic and renal carcinogen that has the oxidative stress as an important mechanism to induce toxicity (Khan and Sultana, 2004; Radford et al., 2013).

Acrylamide is used in the industry to produce polyacrylamides and is also generated during the cooking process. Some studies did not find an association between the exposure to acrylamide and the risk of kidney cancer (Mucci et al., 2003; Swaen et al., 2007). Nevertheless, most recent data showed a significant increase in kidney cancer in subjects exposed to dietary acrylamide (Hogervorst et al., 2008; Pelucchi et al., 2015). Thus, its association with RCC remains open to discussion.

One of the nephrotoxic agents most studied is the mycotoxin ochratoxin A (OTA). The OTA is a well-known food contaminant and an animal carcinogen. The deleterious effects induced by this nephrotoxic agent have been extensively revised in the literature and different mechanisms of toxicity pointed out, including the occurrence of oxidative stress (EFSA, 2006; O'Brien and Dietrich, 2005; Pfohl-Leszkowicz and Manderville, 2007; Ringot et al., 2006). However, the contribution of ROS and other reactive species (RS) to the mode of action of OTA has been a matter of discussion. In this context, several lines of evidence regarding the involvement of oxidative stress are

summarized in Fig. 1.11. OTA increases ROS and depletes the levels of antioxidant enzymes leading to a wide range of cellular lesions (Costa et al., 2015; EFSA, 2006; Marin-Kuan et al., 2011; O'Brien and Dietrich, 2005; Pfohl-Leszkowicz and Manderville, 2007; Ringot et al., 2006; Schaaf et al., 2002). OTA-induced ROS production preceded the loss of cell viability particularly in renal cells, indicating that these RS may contribute to OTA cytotoxicity rather than being a consequence of cell death processes (O'Brien and Dietrich, 2005; Schaaf et al., 2002). OTA also increases lipid peroxidation and induces oxidative stress-related proteins. Additionally, several *in vitro* and *in vivo* studies assessed the impact of different antioxidants in OTA-induced toxicity, generally showing protective effects (Costa et al., 2015; Marin-Kuan et al., 2011; O'Brien and Dietrich, 2005; Pfohl-Leszkowicz and Manderville, 2007; Sorrenti et al., 2013). OTA was also found to stimulate the formation of NO through an NF-κB–dependent induction of iNOS. High levels of NO may cause nitrosative stress with the formation of peroxynitrite, nitrogen dioxide and hydroxyl radicals (Cavin et al., 2009).

The genotoxicity of OTA has been reported using complementary endpoints, namely micronuclei, chromosomal aberrations and DNA breaks (comet assay). This genotoxic potential has also been associated with oxidative DNA damage, as assessed by the formation of 8-oxoguanine or using modified versions of the comet assay (e.g. with FPG; Costa et al., 2015, 2013; Schaaf et al., 2002). Oxidative stress has been proposed as an indirect mechanism of genotoxicity displayed by many chemicals, including OTA (Marin-Kuan et al., 2011; Pfohl-Leszkowicz and Manderville, 2012). Recent studies propose an alternative mechanism for OTA genotoxicity, describing a pathway in which OTA can be metabolized into electrophilic species that directly bind DNA bases (Pfohl-Leszkowicz and Manderville, 2012).

The question whether ROS are responsible or not for the deleterious effects induced by OTA is therefore still open to discussion. Despite some authors found no changes in the abovementioned oxidative stress biomarkers, most data clearly supports an involvement of ROS. However, regarding the overall conclusions of many available

studies and the effective impact of these species upon OTA exposure, ROS do not seem to play a pivotal role in OTA toxicity, and other mechanisms should also be considered.

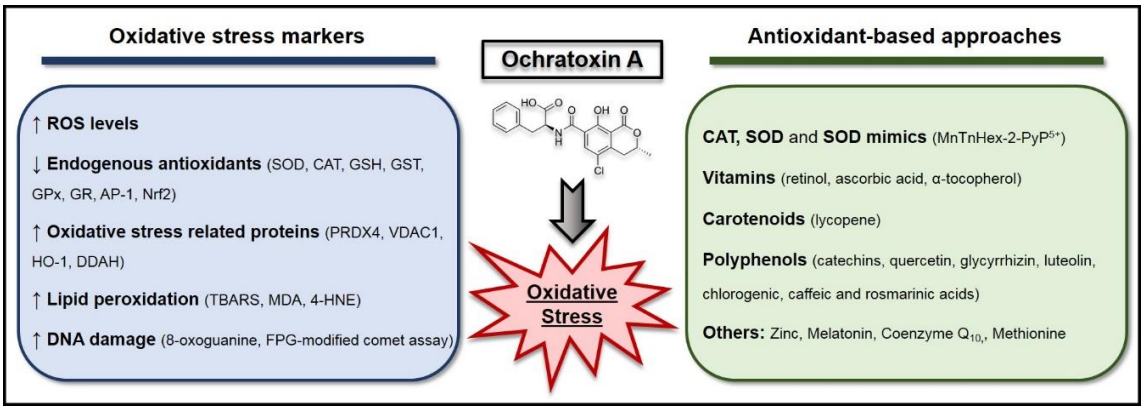

Fig. 1.11 – Lines of evidence supporting the induction of oxidative stress by Ochratoxin A (OTA). **Abbreviations** - SOD: superoxide dismutase, CAT: catalase, GSH: glutathione, GST: Glutathione S-transferase, GPx: glutathione peroxidase, GR: Glutathione reductase, AP-1: activator protein 1, Nrf2: nuclear factor E2-related factor 2, PRDX4: peroxiredoxin-4, VDAC1: voltage-dependent anion channel 1, HO-1: heme-oxygenase 1, DDAH: dimethylarginine dimethylaminohydrolase, TBARS: thiobarbituric acid reactive substances, MDA: malondialdehyde, 4-HNE: 4-Hydroxynonenal, FPG: formamidopyrimidine DNA glycosylase.

1.2.4. Molecular Pathogenesis

The study of familiar RCCs has identified the involvement of diverse molecular pathways. The main pathophysiological pathways of ccRCC include a hypoxic status with the activation of angiogenesis and the mammalian target of rapamycin (mTOR) pathway (Algaba, 2015; Bratslavsky et al., 2007).

The majority of sporadic ccRCC is associated with defects in the VHL tumor suppressor gene (Keefe et al., 2015; Linehan et al., 2009; Linehan and Ricketts, 2016).

The mutation in VHL gene is present in more than 80 % of sporadic ccRCC and is less common in other RCC subtypes (Algaba, 2015; Gomy and Silva Jr., 2012). The VHL gene encodes the pVHL protein, which regulates hypoxia inducible factors (HIF), involved in the cellular response to the changes in oxygen levels (Algaba, 2015). The HIF regulate the expression of genes related with energy metabolism, angiogenesis, erythropoiesis, cell proliferation and apoptosis, among other biologic processes (Gomy and Silva Jr., 2012). The HIF1-α and HIF2-α mediate the transcription of a number of downstream genes thought to be important in cancer, including transforming growth factor alpha (TGF-α), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF; Linehan et al., 2010). In a hypoxic state, HIF not degraded and become available to activate those genes that will be important to inhibit tumor cell apoptosis and stimulate the angiogenesis. The augmented tumor vasculature provides additional nutrients and oxygen to promote the growth of tumor cells. Moreover, the upregulation of TGF-α stimulates autocrine cell growth or activate energy supply factors such as glucose transporter protein-1 (GLUT-1) and erythropoietin (EPO; Fig. 1.12).

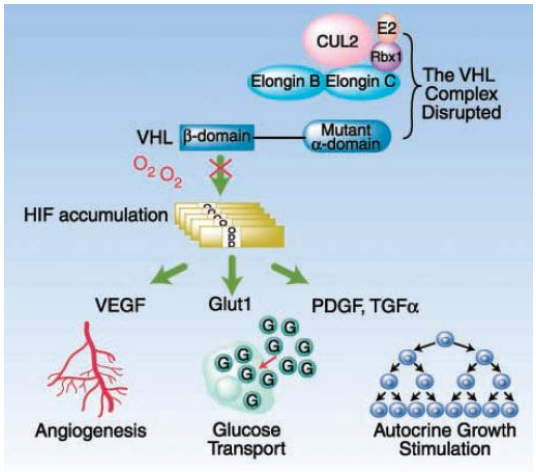

Fig. 1.12 – The von Hippel-Lindau (VHL) pathway in the ccRCC progression (reproduced from Bratslavsky et al., 2007).

Therefore, the absence of pVHL mimics a hypoxia status and results in a constitutive upregulation of HIF that produces a transcriptional activation of several target genes, such as VEGF, PDGF, EPO, TGF-α and GLUT-1 (Gomy and Silva Jr., 2012).

When HIF-α levels are increased the mTOR pathway can also be upregulated. The mTOR is an intracellular serine/threonine protein kinase with several cellular functions, such as protein translation, metabolism, angiogenesis, cell growth, migration and apoptosis (Koul et al., 2011; Zong et al., 2014). The mTOR is positively regulated by phosphatidylinositide 3-kinase (PI3K) - protein kinase B (Akt) pathway, which is also important in the pathophysiology of ccRCC (Keefe et al., 2015; Koul et al., 2011). The PI3K/Akt signaling pathway is involved in several cellular functions, such as proliferation, cell-cycle progression and invasion (Fresno Vara et al., 2004; Martini et al., 2014). Recently, was reported that this pathway is modestly mutated but highly activated in RCC (Guo et al., 2015).

1.2.5. Oxidative stress in RCC

ROS have a significant role in initiation, development and progression of cancer. ROS are important in tumorigenicity due to their potential to induce DNA damage and also to sustain tumor progression. It is known that ROS have effects on signal transduction pathways and may increase cell proliferation, survival and cellular migration. (Gius and Spitz, 2006; Valko et al., 2004; Waris and Ahsan, 2006).

In RCC, as well as in other cancers, oxidative stress is considered a key factor. Oxidative stress, by itself, has the ability to change the characteristics of kidney cells. Recently, was observed that chronic exposure to high levels of oxidative stress alone is

sufficient to induce malignant transformation of kidney epithelial cells, potentially through acquisition of stem cell characteristics (Mahalingaiah et al., 2015). Additionally, the aberrant expression of epigenetic regulatory genes involved in DNA methylation and histone modifications could have an important role in oxidative stress-induced malignant transformation (Mahalingaiah et al., 2016). Those epigenetic changes have also been implicated in renal cancer development and progression (Ellinger et al., 2010).

The pathophysiological pathways of RCC previous described are closely related with oxidative stress, showing the importance of the cellular redox imbalance in the development of kidney cancer. The loss of VHL contributes to an enhanced oxidative stress, which is mediated in large part by NOX complex (Block, 2012; Block et al., 2010; Szatrowski and Nathan, 1991). Oxidative stress can induce growth factors, namely HIF, and thereby accelerate tumor growth. Importantly, both the regulation and the effects induced by HIF are modulated by the cellular redox state (Kinnula and Crapo, 2004). Moreover, the regulation of the PI3K/Akt/mTOR signaling is also redox-sensitive (Block, 2012). Therefore, oxidative stress not only causes direct and irreversible oxidative damage to macromolecules but also disrupts key redox-dependent signaling processes. In patients with RCC, was observed significant increase of different ROS, such as H_2O_2, $OH^{\bullet}$, $ONOO^-$ and $O_2^{\bullet -}$, as well as nitric oxide (NO), compared to controls and patients with benign tumors (Block, 2012; Ganesamoni et al., 2012).

Several studies showed decreased expression / activities of antioxidant enzymes. The lower expression of SOD was described in RCC (Oberley et al., 1996; Šverko et al., 2011) along with lower enzymatic activity when compared with the adjacent tissues (Robbins and Zhao, 2014; Zhao et al., 2017). Furthermore, low activity of glutathione peroxidase (Sarto et al., 1999), catalase (Ganesamoni et al., 2012; Šverko et al., 2011) or both (Pljesa-Ercegovac et al., 2008) was reported. The absent of peroxisomes, organelles that mediate a wide variety of biosynthetic and biodegradative reactions,

including the metabolism of H_2O_2 and other ROS (Terlecky, 2012), was also reported in renal cancer cells (Frederiks et al., 2010).

The presence of lipid peroxidation (Gago-Dominguez and Castelao, 2006; Ganesamoni et al., 2012) and the formation of adducts with proteins by reactive lipid mediators, such as 4-HNE was also detected in RCC (Oberley et al., 1999). Moreover, in ccRCC oxidative alterations of proteins and DNA were also observed (Pljesa-Ercegovac et al., 2008; Šverko et al., 2011). In RCC, as in other cancers, ROS levels increase with tumor progression. It was reported that patients with metastatic disease had persistently increased oxidative stress parameters (Ganesamoni et al., 2012).

1.2.6. Diagnosis

The diagnosis and the clinical stage of the patients is based on computed tomography or magnetic resonance imaging (Sanchez et al., 2017). Staging for RCC has evolved from the Robson classification into the TNM system, developed by the International Union Against Cancer and the American Joint Committee on Cancer (Table I.10; Ridge et al., 2014; Sanchez et al., 2017).

Table I.10 – The TNM staging of Malignant Tumours (TNM).

Stage	Tumor size	Localization	Description
T1	Diameter ≤ 7 cm	Localized	Tumor confined to the kidney
T2	Diameter > 7 cm	Localized	Tumor confined to the kidney
T3	Any size	Regional	Tumor extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond Gerota fascia
T4	Any size	Metastatic	Tumor invades beyond the Gerota fascia

Adapted from Sanchez et al., 2017.

RCC is characterized by a lack of early-warning signs, which leads to a high proportion of patients diagnosed with metastases (Motzer et al., 1996). The progression of metastatic disease is a sequential process where cancer cells leave the primary tumor via blood or lymphatic circulations and deposit in other tissues or organs. The advanced and metastatic disease has an enormous impact for the mortality rates in patients with RCC (Rini et al., 2009). At the time of the diagnosis, 15 - 20 % of the patients have already metastatic disease and. Moreover, at least one third of the patients already treated for localized carcinomas or submitted to surgery will develop metastatic disease (Bukowski et al., 2015; Hillman et al., 2007; Zerbi et al., 2008). The median survival of patients with metastases is only eight months, with a five-year survival rate of less than 10 % (Hillman et al., 2007; Zerbi et al., 2008). Tumors greater than 7 cm and those with locally invasive characteristics have increased potential for being malignant (Rini et al., 2009). The behavior of metastatic RCC is difficult to predict. Generally, RCC starts to invade and metastasizes into ipsilateral adrenal (Diaz et al., 1999) and afterwards into more distant organs, mainly lungs, bone, liver, intestines and brain (Sahi et al., 2010; Sountoulides et al., 2011; Zerbi et al., 2008). In general, the pathways that drive metastasis are considered different from those involved in the tumorigenesis initiation process (Nguyen et al., 2009). Nevertheless, the VHL pathway previously described in the initiation process, seems to be also an important key factor for cancer metastization particularly in ccRCC (Gossage, 2015). Additionally, the mTOR protein is also determinant in both cancer initiation and progression, by its role in cell migration (Finlay et al., 2012; Zong et al., 2014).

1.2.7. Treatment

The initial treatment provided to patients with RCC depends of its clinical stage. For localized kidney cancers with no metastasis, the standard of care is nephrectomy. Nevertheless, several studies showed an increase in patients' morbidity due to the risk of chronic renal disease induced by radical nephrectomy (Capitanio and Montorsi, 2016; Sanchez et al., 2017). Therefore, nephron-sparing surgery (NSS) or partial nephrectomy, along with active surveillance and minimally invasive techniques, have been introduced into daily clinical practice (Sun et al., 2012). These approaches are less invasive and limit renal function impairment and overtreatment (Capitanio and Montorsi, 2016).

The rationale for active surveillance is justified by the growth kinetics, since the median growth rate of RCC is usually slow (2–3 mm/year; Mehrazin et al., 2015). Although surgery still represents the standard of care for RCC, the use of minimally invasive techniques, such as cryotherapy or radiofrequency ablation has increased.

In the past, patients with metastatic ccRCC were treated mainly with systemic therapy based on immune modulators (e.g. interferon α and interleukin-2). In the last years, after the discovery of the molecular pathways involved in RCC, the management of patients with metastatic RCC suffered dramatic changes with new therapeutic approaches. These new therapies include antiangiogenic drugs targeting VEGF and its receptors, mTOR inhibitors and an immune checkpoint inhibitor, with established superiority over cytokine therapies (Table I.11; Fig. 1.13). In 2005 and 2006 the antiangiogenic drugs sorafenib and sunitinib were approved, followed by pazopanib, axitinib, bevacizumab, cabozantinib, and lenvatinib, with identical biological targets. Two mTOR inhibitors, temsirolimus and everolimus and the immune checkpoint inhibitor nivolumab were also approved by the Food and Drug Administration (FDA), after showing beneficial in phase 3 clinical trials (Choueiri and Motzer, 2017).

Table I.11 – Targeted therapies for management of Metastatic Renal-Cell Carcinoma (mRCC).

Drug	Mechanism
Sorafenib	VEGFR-2, PDGFR and MAPK/MEK/ERK inhibitor
Sunitinib	VEGFR (1–3), PDGFR and c-Kit inhibitor
Pazopanib	VEGFR, PDGFR and c-Kit inhibitor
Axitinib	VEGFR (1–3), PDGFR and c-Kit inhibitor
Cabozantinib	VEGFR, c-MET and AXL inhibitor
Lenvatinib	VEGFR (1-3) and FGFR inhibitor
Bevacizumab	Recombinant humanized monoclonal antibody directed against VEGF-A
Temsirolimus	mTOR inhibitor
Everolimus	mTOR inhibitor
Nivolumab	Anti-programmed death 1 (PD-1) inhibitor

Adapted from Capitanio and Montorsi, 2016; Choueiri and Motzer, 2017; Escudier et al., 2016.

In addition to the drug efficacy, the individual patient factors and possible comorbidities are also important to avoid toxicity. According to the Europe's leading medical oncology society (ESMO) clinical practice guidelines, the first-line treatment of patients with good or intermediate prognosis includes three treatments that have demonstrated efficacy in pivotal phase III trials: bevacizumab (combined with interferon), sunitinib, and pazopanib (Escudier et al., 2010; Motzer et al., 2007; Sternberg et al., 2010). The treatment with sorafenib, high-dose interleukin-2 and low-dose interferon combined with bevacizumab are alternative options (Escudier et al., 2016).

The temsirolimus is currently the only drug tested in a phase III study, which demonstrated beneficial effects as first-line treatment in patients with poor prognosis (Escudier et al., 2016; Hudes et al., 2007).

The second-line treatment of ccRCC in patients with low or intermediate risk, post cytokines, include axitinib, sorafenib or pazopanib with sunitinib as therapeutic options. In patients with poor risk, the second-line treatment was recently dramatically modified, after two clinical trials showed an increased overall survival with nivolumab and cabozantinib (Choueiri et al., 2015; Motzer et al., 2015). Other therapeutic options include axitinib, everolimus and sorafenib (Escudier et al., 2016).

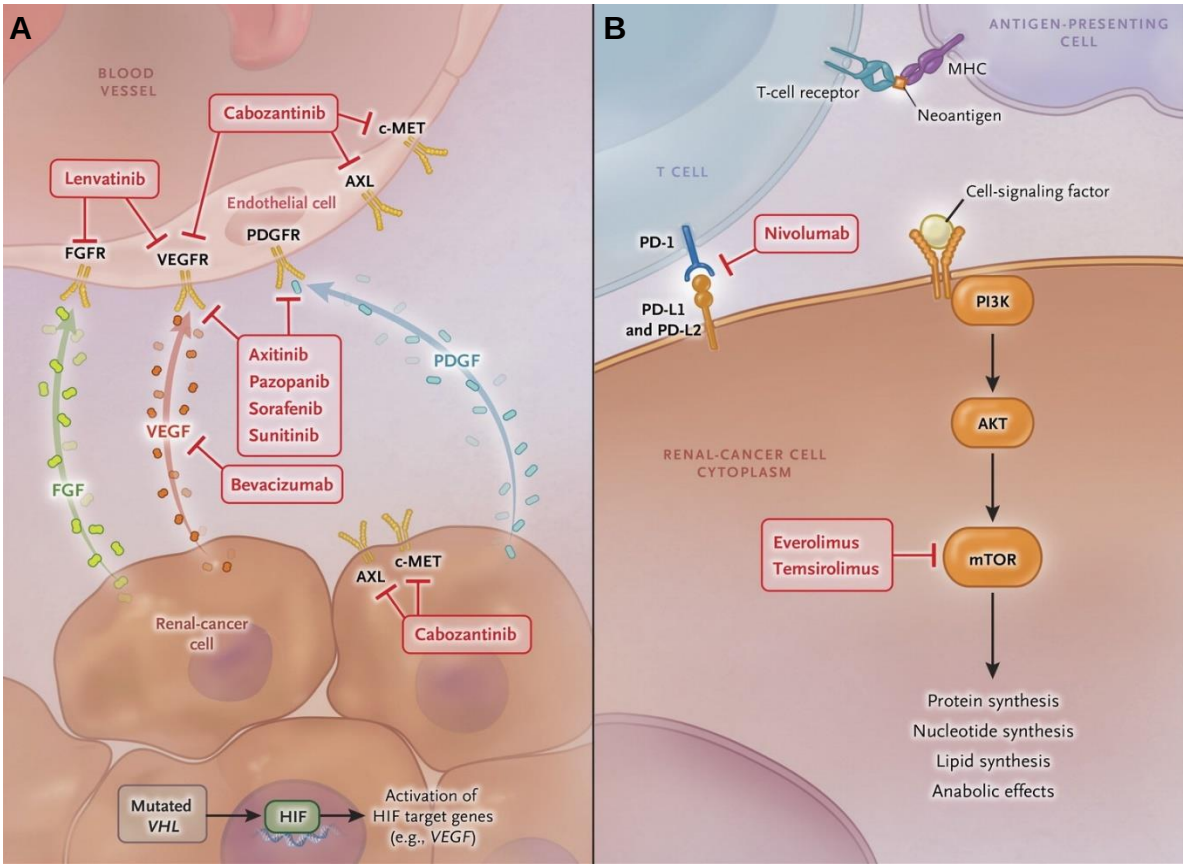

Fig. 1.13 – Pathways and Current Drugs in Metastatic Renal-Cell Carcinoma (mRCC). (A) VEGF and tyrosine kinase inhibitors and (B) PD-1 and mTOR inhibitors (reproduced from Choueiri and Motzer, 2017).

1.3. References

Algaba, F., 2015. Renal Cell Carcinoma: Pathologic and Molecular Assessment of Targets, in: Renal Cell Carcinoma. Springer New York, New York, NY, 15–38.

Algaba, F., Akaza, H., López-Beltrán, A., Martignoni, G., Moch, H., Montironi, R., Reuter, V., 2011. Current pathology keys of renal cell carcinoma. Eur. Urol. 60, 634–643.

American Cancer Society, 2017. Cancer Facts & Figures 2017. American Cancer Society, Atlanta.

Aquilano, K., Baldelli, S., Ciriolo, M.R., 2014. Glutathione: New roles in redox signalling for an old antioxidant. Front. Pharmacol. 5, 1–12.

Armstrong, D., Stratton, R.D., 2016. Oxidative Stress and Antioxidant Protection: The Science of Free Radical Biology and Disease. Wiley, Hoboken.

Asha, S., Girija, D., 2011. Benzo[a]pyrene induced liver and kidney cancer in swiss albino mice. Res. Pharm. 4, 22–27.

Ashcraft, K.A., Boss, M.K., Tovmasyan, A., Roy Choudhury, K., Fontanella, A.N., Young, K.H., Palmer, G.M., Birer, S.R., Landon, C.D., Park, W., *et al.*, 2015. Novel manganese-porphyrin superoxide dismutase-mimetic widens the therapeutic margin in a preclinical head and neck cancer model. Int. J. Radiat. Oncol. Biol. Phys. 93, 892–900.

Batinić-Haberle, I., Ndengele, M.M., Cuzzocrea, S., Rebouças, J.S., Spasojević, I., Salvemini, D., 2009. Lipophilicity is a critical parameter that dominates the efficacy of metalloporphyrins in blocking the development of morphine antinociceptive tolerance through peroxynitrite-mediated pathways. Free Radic. Biol. Med. 46, 212–219.

Batinić-Haberle, I., Rebouças, J.S., Spasojević, I., 2010. Superoxide dismutase mimics: chemistry, pharmacology, and therapeutic potential. Antioxid. Redox Signal. 13, 877–918.

Batinić-Haberle, I., Spasojevic, I., Tse, H.M., Tovmasyan, A., Rajic, Z., St. Clair, D.K., Vujaskovic, Z., Dewhirst, M.W., Piganelli, J.D., 2012. Design of Mn porphyrins for treating oxidative stress injuries and their redox-based regulation of cellular transcriptional activities. Amino Acids 42, 95–113.

Batinić-Haberle, I., Tovmasyan, A., Roberts, E.R.H., Vujaskovic, Z., Leong, K.W., Spasojevic, I., 2014. SOD Therapeutics: Latest Insights into Their Structure-Activity Relationships and Impact on the Cellular Redox-Based Signaling Pathways. Antioxid. Redox Signal. 20, 2372–2415.

Batinic-Haberle, I., Tovmasyan, A., Spasojevic, I., 2015. An educational overview of the chemistry, biochemistry and therapeutic aspects of Mn porphyrins - From superoxide dismutation to H2O2-driven pathways. Redox Biol. 5, 43–65.

Batinić-Haberle, I., Tovmasyan, A., Spasojević, I., 2016. Mn Porphyrin-Based Redox-Active Therapeutics, in: Batinic-Haberle, I., Reboucas, J.S., Spasojevic, I. (Eds.), Redox-Active Therapeutics, 165–212.

Benatar, M., 2007. Lost in translation: Treatment trials in the SOD1 mouse and in human ALS. Neurobiol. Dis. 26, 1–13.

BioMimetix JV, LLC; Duke Cancer Institute. Trial of Newly Diagnosed High Grade Glioma Treated With Concurrent Radiation Therapy, Temozolomide and BMX-001 (BMX-HGG). In ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2016- [cited 2017 Feb 15]. Available from: https://clinicaltrials.gov/ct2/ show/ NCT02655601. NLM Identifier: NCT02655601.

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative Stress and Antioxidant Defense. World Allergy Organ. J. 5, 9–19.

Block, K., 2012. Oxidative Stress and Redox-Signaling in Renal Cell Cancer, in: Amato, R.J. (Ed.), Emerging Research and Treatments in Renal Cell Carcinoma. InTech.

Block, K., Gorin, Y., New, D.D., Eid, A., Chelmicki, T., Reed, A., Choudhury, G.G., Parekh, D.J., Abboud, H.E., 2010. The NADPH oxidase subunit p22phox inhibits the function of the tumor suppressor protein tuberin. Am. J. Pathol. 176, 2447–55.

Bottino, R., Balamurugan, A.N., Bertera, S., Pietropaolo, M., Trucco, M., Piganelli, J.D., 2002. Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound. Diabetes 51, 2561–2567.

Bratslavsky, G., Sudarshan, S., Neckers, L., Linehan, W.M., 2007. Pseudohypoxic pathways in renal cell carcinoma. Clin. Cancer Res. 13, 4667–4671.

Brieger, K., Schiavone, S., Miller, F.J., Krause, K.H., 2012. Reactive oxygen species: from health to disease. Swiss Med Wkly 142, 1-14.

Bukowski, R.M., Figlin, R.A., Motzer, R.J., 2015. Targeted Therapy for Metastatic Renal Cell Carcinoma: Introduction, in: Renal Cell Carcinoma. Springer New York, New York, NY, 1–14.

Capitanio, U., Montorsi, F., 2016. Renal cancer. Lancet 387, 894–906.

Cavin, C., Delatour, T., Marin-Kuan, M., Fenaille, F., Holzhäuser, D., Guignard, G., Bezençon, C., Piguet, D., Parisod, V., Richoz-Payot, *et al*., 2009. Ochratoxin A - Mediated DNA and protein damage: Roles of nitrosative and oxidative stresses. Toxicol. Sci. 110, 84–94.

Celic, T., Spanjol, J., Bobinac, M., Tovmasyan, A., Vukelic, I., Reboucas, J.S., Batinic-Haberle, I., Bobinac, D., 2014. Mn porphyrin-based SOD mimic, MnTnHex-2-PyP^{5+}, and non-SOD mimic, MnTBAP(3-), suppressed rat spinal cord ischemia/reperfusion injury via NF-kappa B pathways. Free Radic. Res. 48, 1426–1442.

Chatterjee, P.K., Patel, N.S.A., Kvale, E.O., Brown, P.A.J., Stewart, K.N., Mota-Filipe, H., Sharpe, M.A., Di Paola, R., Cuzzocrea, S., Thiemermann, C., 2004. EUK-134 reduces renal dysfunction and injury caused by oxidative and nitrosative stress of the kidney. Am. J. Nephrol. 24, 165–177.

Choueiri, T., Escudier, B., Powles, T., Mainwaring, P., Rini, B., Donskov, F., Hammers, H., Hutson, T., 2015. Cabozantinib versus Everolimus in Advanced Renal-Cell Carcinoma. N. Engl. J. Med. 373, 1814–1823.

Choueiri, T.K., Motzer, R.J., 2017. Systemic Therapy for Metastatic Renal-Cell Carcinoma. N. Engl. J. Med. 376, 354–366.

Chow, W.-H., Dong, L.M., Devesa, S.S., 2010. Epidemiology and risk factors for kidney cancer. Nat. Rev. Urol. 7, 245–257.

Coleman, J.A., 2008. Familial and Hereditary Renal Cancer Syndromes. Urol. Clin. North Am. 35, 563–572.

Costa, J.G., Fernandes, A.S., Guerreiro, P.S., Filipe, E., Miranda, J.P., Castro, M., Oliveira, N.G., 2013. Induction of micronuclei and cytotoxic effects of ochratoxin A in Vero cells. Toxicol. Lett. 221, S121.

Costa, J.G., Saraiva, N., Guerreiro, P.S., Louro, H., Silva, M.J., Miranda, J.P., Castro, M., Batinic-Haberle, I., Fernandes, A.S., Oliveira, N.G., 2015. Ochratoxin A-induced cytotoxicity, genotoxicity and reactive oxygen species in kidney cells: an integrative approach of complementary endpoints. Food Chem. Toxicol. 87, 65–76.

Cotrim, A.P., Hyodo, F., Matsumoto, K.I., Sowers, A.L., Cook, J.A., Baum, B.J., Krishna, M.C., Mitchell, J.B., 2007. Differential radiation protection of salivary glands versus tumor by tempol with accompanying tissue assessment of tempol by magnetic resonance imaging. Clin. Cancer Res. 13, 4928–4933.

Creighton, C.J., Morgan, M., Gunaratne, P.H., Wheeler, D. a., Gibbs, R. a., Gordon Robertson, A., Chu, A., Beroukhim, R., Cibulskis, K., Signoretti, S., *et al.*., 2013. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 499, 43–49.

Crow, J.P., 2006. Catalytic antioxidants to treat amyotropic lateral sclerosis. Expert Opin. Investig. Drugs 15, 1383–1393.

Crow, J.P., Calingasan, N.Y., Chen, J., Hill, J.L., Beal, M.F., 2005. Manganese porphyrin given at symptom onset markedly extends survival of ALS mice. Ann. Neurol. 58, 258–265.

Cui, X.S., Eriksson, L.C., Möller, L., 1999. Formation and persistence of DNA adducts during and after a long-term administration of 2-nitrofluorene. Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 442, 9–18.

Cuzzocrea, S., Mazzon, E., Dugo, L., Serraino, I., Di Paola, R., Britti, D., De Sarro, A., Pierpaoli, S., Caputi, A.P., Masini, E., Salvemini, D., 2002. A role for superoxide in gentamicin-mediated nephropathy in rats. Eur. J. Pharmacol. 450, 67–76.

Dedon, P.C., Tannenbaum, S.R., 2004. Reactive nitrogen species in the chemical biology of inflammation. Arch. Biochem. Biophys. 423, 12–22.

Dhar, S.K., St. Clair, D.K., 2012. Manganese superoxide dismutase regulation and cancer. Free Radic. Biol. Med. 52, 2209–2222.

Diaz, J.I., Mora, L.B., Hakam, A., 1999. The Mainz classification of renal cell tumors. Cancer Control 6, 571–579.

Doyle, T., Chen, Z., Muscoli, C., Bryant, L., Esposito, E., Cuzzocrea, S., Dagostino, C., Ryerse, J., Rausaria, S., Kamadulski, A., *et al.*, 2012. Targeting the overproduction of peroxynitrite for the prevention and reversal of paclitaxel-induced neuropathic pain. J. Neurosci. 32, 6149–60.

Dröge, W., 2002. Free Radicals in the Physiological Control of Cell Function. Physiol. Rev. 82, 47–95.

EFSA, 2006. Opinion Of The Scientific Panel On Contaminants In The Food Chain On A Request From The Commission Related To Ochratoxin A In Food. EFSA J. 365(EFSA-Q-2005-154) 1–56.

Ellinger, J., Kahl, P., Mertens, C., Rogenhofer, S., Hauser, S., Hartmann, W., Bastian, P.J., Büttner, R., Müller, S.C., Von Ruecker, A., 2010. Prognostic relevance of global histone H3 lysine 4 (H3K4) methylation in renal cell carcinoma. Int. J. Cancer 127, 2360–2366.

Ervik, M., Lam, F., Ferlay, J., Mery, L., Soerjomataram, I., Bray, F., 2016. GLOBOCAN 2012, cancer incidence and mortality worldwide. Cancer Today. Lyon, France: International Agency for Research on Cancer. Cancer Today. Available from: http://gco.iarc.fr/today, accessed [23/02/2017].

Escudier, B., Bellmunt, J., Négrier, S., Bajetta, E., Melichar, B., Bracarda, S., Ravaud, A., Golding, S., Jethwa, S., Sneller, V., 2010. Phase III Trial of Bevacizumab Plus Interferon Alfa-2a in Patients With Metastatic Renal Cell Carcinoma (AVOREN): Final Analysis of Overall Survival. J. Clin. Oncol. 28, 2144–2150.

Escudier, B., Porta, C., Schmidinger, M., Rioux-Leclercq, N., Bex, A., Khoo, V., Gruenvald, V., Horwich, A., on behalf of the ESMO Guidelines Committee, 2016. Renal cell carcinoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann. Oncol. 27, v58–v68.

Fernandes, A.S., Costa, J., Gaspar, J., Rueff, J., Cabral, M.F., Cipriano, M., Castro, M., Oliveira, N.G., 2012. Development of pyridine-containing macrocyclic copper(II) complexes: potential role in the redox modulation of oxaliplatin toxicity in human breast cells. Free Radic Res 46, 1157–1166.

Fernandes, A.S., Flórido, A., Saraiva, N., Cerqueira, S., Ramalhete, S., Cipriano, M., Cabral, M.F., Miranda, J.P., Castro, M., Costa, J., Oliveira, N.G., 2015. Role of the Copper(II) Complex Cu[15]pyN5 in Intracellular ROS and Breast Cancer Cell Motility and Invasion. Chem. Biol. Drug Des. 86, 578–588.

Fernandes, A.S., Saraiva, N., Oliveira, N.G., 2016. Redox Therapeutics in Breast Cancer: Role of SOD Mimics, in: Batinic-Haberle, I., Reboucas, J.S., Spasojevic, I. (Eds.), Redox-Active Therapeutics, 451–467.

Finlay, D.K., Rosenzweig, E., Sinclair, L. V, Feijoo-Carnero, C., Hukelmann, J.L., Rolf, J., Panteleyev, A.A., Okkenhaug, K., Cantrell, D.A., 2012. PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of $CD8^+$ T cells. J. Exp. Med. 209, 2441–2453.

Frederiks, W.M., Bosch, K.S., Hoeben, K.A., van Marle, J., Langbein, S., 2010. Renal cell carcinoma and oxidative stress: The lack of peroxisomes. Acta Histochem. 112, 364–371.

Fresno Vara, J.A., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., Gonzalez-Baron, M., 2004. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev 30, 193–204.

Fujii, T., Takaoka, M., Ohkita, M., Matsumura, Y., 2005. Tempol protects against ischemic acute renal failure by inhibiting renal noradrenaline overflow and endothelin-1 overproduction. Biol. Pharm. Bull. 28, 641–645.

Fukai, T., Ushio-Fukai, M., 2011. Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxid. Redox Signal. 15, 1583–1606.

Fulda, S., 2010. Evasion of apoptosis as a cellular stress response in cancer. Int. J. Cell Biol. 2010.

Gago-Dominguez, M., Castelao, J.E., 2006. Lipid peroxidation and renal cell carcinoma: Further supportive evidence and new mechanistic insights. Free Radic. Biol. Med. 40, 721–733.

Gago-Dominguez, M., Castelao, J.E., Yuan, J.M., Ross, R.K., Yu, M.C., 2002. Lipid peroxidation: A novel and unifying concept of the etiology of renal cell carcinoma (United States). Cancer Causes Control 13, 287–293.

Galera Therapeutics, Inc. A Phase 1 Dose Escalation Study of GC4419 in Combination With Chemoradiation for Squamous Cell Cancer of the Head & Neck. In ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2013-16 [cited 2017 Feb 15]. Available from: https://clinicaltrials.gov/ct2/show/ NCT01921426. NLM Identifier: NCT01921426.

Galera Therapeutics, Inc; Clinical Assistance Programs, LLC; Novella Clinical. A Study of the Effects of GC4419 on Radiation Induced Oral Mucositis in Patients With Head/Neck Cancer. In ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2015- [cited 2017 Feb 15]. Available from: https://clinicaltrials.gov/ct2 /show/ NCT02508389. NLM Identifier: NCT02508389.

Ganesamoni, R., Bhattacharyya, S., Kumar, S., Chauhan, A., Mete, U.K., Agarwal, M.M., Mavuduru, R., Kaushik, G., Mandal, A.K., Singh, S.K., 2012. Status of oxidative stress in patients with renal cell carcinoma. J. Urol. 187, 1172–1176.

Gauter-Fleckenstein, B., Fleckenstein, K., Owzar, K., Jiang, C., Batinic-Haberle, I., Vujaskovic, Z., 2008. Comparison of two Mn porphyrin-based mimics of superoxide dismutase in pulmonary radioprotection. Free Radic. Biol. Med. 44, 982–989.

Gauter-Fleckenstein, B., Fleckenstein, K., Owzar, K., Jiang, C., Rebouças, J.S., Batinic-Haberle, I., Vujaskovic, Z., 2010. Early and late administration of MnTE-2-PyP^{5+} in mitigation and treatment of radiation-induced lung damage. Free Radic. Biol. Med. 48, 1034–1043.

Gauter-Fleckenstein, B., Reboucas, J.S., Fleckenstein, K., Tovmasyan, A., Owzar, K., Jiang, C., Batinic-Haberle, I., Vujaskovic, Z., 2014. Robust rat pulmonary radioprotection by a lipophilic Mn N-alkylpyridylporphyrin, MnTnHex-2-PyP^{5+}. Redox Biol. 2, 400–410.

Gius, D., Spitz, D.R., 2006. Redox Signaling in Cancer Biology. Antioxid. Redox Signal. 8, 1249–1252.

Gomy, I., Silva Jr., W.A., 2012. Molecular Pathogenesis of Renal Cell Carcinoma: A Review, in: Emerging Research and Treatments in Renal Cell Carcinoma. InTech.

Gossage, L., 2015. VHL and HIF in Clear Cell Renal Cell Carcinoma: Molecular Abnormalities and Potential Clinical Applications, in: Renal Cell Carcinoma. Springer New York, New York, NY, 57–110.

Gossage, L., Murtaza, M., Slatter, A.F., Lichtenstein, C.P., Warren, A., Haynes, B., Marass, F., Roberts, I., Shanahan, S.J., Claas, A., *et al*., 2014. Clinical and pathological impact of VHL, PBRM1, BAP1, SETD2, KDM6A, and JARID1c in clear cell renal cell carcinoma. Genes, Chromosom. Cancer 53, 38–51.

Gridley, D.S., Makinde, A.Y., Luo, X., Rizvi, A., Crapo, J.D., Dewhirst, M.W., Moeller, B.J., Pearlstein, R.D., Slater, J.M., 2007. Radiation and a metalloporphyrin radioprotectant in a mouse prostate tumor model. Anticancer Res. 27, 3101–3109.

Guha, N., Loomis, D., Grosse, Y., Lauby-Secretan, B., Ghissassi, F. El, Bouvard, V., Benbrahim-Tallaa, L., Baan, R., Mattock, H., Straif, K., 2012. Carcinogenicity of trichloroethylene, tetrachloroethylene, some other chlorinated solvents, and their metabolites. Lancet Oncol. 13, 1192–1193.

Guo, H., German, P., Bai, S., Barnes, S., Guo, W., Qi, X., Lou, H., Liang, J., Jonasch, E., Mills, G.B., Ding, Z., 2015. The PI3K/AKT Pathway and Renal Cell Carcinoma. J. Genet. Genomics 42, 343–353.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: The next generation. Cell 144, 646–674.

Hanahan, D., Weinberg, R.A., 2000. The hallmarks of cancer. Cell 100, 57–70.

Hillman, G.G., Wang, Y., Che, M., Raffoul, J.J., Yudelev, M., Kucuk, O., Sarkar, F.H., 2007. Progression of renal cell carcinoma is inhibited by genistein and radiation in an orthotopic model. BMC Cancer 7(4), 1-12.

Hines, I.N., Hoffman, J.M., Scheerens, H., Day, B.J., Harada, H., Pavlick, K.P., Bharwani, S., Wolf, R., Gao, B., Flores, S., *et al*., 2003. Regulation of postischemic liver injury following different durations of ischemia. AJP -Gastrointestinal and Liver Physiology. Am J Physiol Gastrointest Liver Physiol 284, 536–545.

Hoang, M.L., Chen, C.-H., Chen, P.-C., Roberts, N.J., Dickman, K.G., Yun, B.H., Turesky, R.J., Pu, Y.-S., Vogelstein, B., Papadopoulos, N., *et al*., 2016. Aristolochic Acid in the Etiology of Renal Cell Carcinoma. Cancer Epidemiol. Prev. Biomarkers, 25, 1600-1608.

Hogervorst, J.G., Schouten, L.J., Konings, E.J., Brandt, P.A. van den, Goldbohm, R.A., 2008. Dietary acrylamide intake and the risk of renal cell, bladder, and prostate cancer. Am. J. Clin. Nutr. 87, 1428–1438.

Holley, A.K., St. Clair, D.K., 2016. Manganese Superoxide Dismutase (MnSOD) and Its Importance in Mitochondrial Function and Cancer, 11–50.

Hsu, C.-H., Stedeford, T., Okochi-Takada, E., Ushijima, T., Noguchi, H., Muro-Cacho, C., Holder, J.W., Banasik, M., 2007. Framework Analysis for the Carcinogenic Mode of Action of Nitrobenzene. J. Environ. Sci. Heal. Part C 25, 155–184.

Hubens, W., Okado-Matsumoto, A., 2016. Redox Regulation and Misfolding of SOD1: Therapeutic Strategies for Amyotrophic Lateral Sclerosis, 605–626.

Hudes, G., Carducci, M., Tomczak, P., Dutcher, J., Figlin, R., Kapoor, A., Staroslawska, E., Sosman, J., McDermott, D., Bodrogi, I., *et al*., 2007. Temsirolimus, Interferon Alfa, or Both for Advanced Renal-Cell Carcinoma. N. Engl. J. Med. 356, 2271–2281.

Hunt, J.D., Van Der Hel, O.L., McMillan, G.P., Boffetta, P., Brennan, P., 2005. Renal cell carcinoma in relation to cigarette smoking: Meta-analysis of 24 studies. Int. J. Cancer 114, 101–108.

IARC, 2017. Monographs on the Evaluation of Carcinogenic Risks to Humans. List Classif. Vol. 1–117. Available from: http://monographs.iarc.fr/ENG/Classification/ latest_classif.php, accessed [15/01/2017].

IARC, 2012. Personal habits and indoor combustions. Volume 100 E. A review of human carcinogens. IARC Monogr. Eval. Carcinog. Risks Hum. 100, 1–538.

Il'yasova, D., Schwartz, G.G., 2005. Cadmium and renal cancer. Toxicol. Appl. Pharmacol. 207, 179–186.

Izumi, M., McDonald, M.C., Sharpe, M.A., K, P.C., 2002. Superoxide dismutase mimetics with catalase activity reduce the organ injury in hemorrhagic shock. Shock 18, 230–235.

Jackson, I.L., Zhang, X., Hadley, C., Rabbani, Z.N., Zhang, Y., Marks, S., Vujaskovic, Z., 2012. Temporal expression of hypoxia-regulated genes is associated with early changes in redox status in irradiated lung. Free Radic. Biol. Med. 53, 337–346.

Jaramillo, M.C., Briehl, M.M., Batinic-Haberle, I., Tome, M.E., 2015. Manganese (III) meso-tetrakis N-ethylpyridinium-2-yl porphyrin acts as a pro-oxidant to inhibit electron transport chain proteins, modulate bioenergetics, and enhance the response to chemotherapy in lymphoma cells. Free Radic. Biol. Med. 83, 89–100.

Jaramillo, M.C., Frye, J.B., Crapo, J.D., Briehl, M.M., Tome, M.E., 2009. Increased Manganese Superoxide Dismutase Expression or Treatment with Manganese Porphyrin Potentiates Dexamethasone-Induced Apoptosis in Lymphoma Cells. Cancer Res. 69, 5450–5457.

Johnson, F., Giulivi, C., 2005. Superoxide dismutases and their impact upon human health. Mol. Aspects Med. 26, 340–352.

Keefe, S.M., Rathmell, W.K., Nathanson, K.L., 2015. Genomic Assessment of Renal Cancer, in: Renal Cell Carcinoma. Springer New York, New York, NY, 39–56.

Kehrer, J.P., Klotz, L.-O., 2015. Free radicals and related reactive species as mediators of tissue injury and disease: implications for Health. Crit. Rev. Toxicol. 45, 765–98.

Keir, S.T., Dewhirst, M.W., Kirkpatrick, J.P., Bigner, D.D., Batinic-Haberle, I., 2011. Cellular Redox Modulator, ortho Mn(III) meso-tetrakis(N-n-Hexylpyridinium-2-yl)porphyrin, MnTnHex-2-PyP^{5+} in the Treatment of Brain Tumors. Anticancer. Agents Med. Chem. 11, 202–212.

Kelsh, M.A., Alexander, D.D., Mink, P.J., Mandel, J.H., 2010. Occupational Trichloroethylene Exposure and Kidney Cancer. Epidemiology 21, 95–102.

Khan, N., Sultana, S., 2004. Abrogation of potassium bromate-induced renal oxidative stress and subsequent cell proliferation response by soy isoflavones in Wistar rats. Toxicology 201, 173–184.

Kim, I., Ha, J., Lee, J., Yoo, K., Rho, J., 2014. The Relationship between the Occupational Exposure of Trichloroethylene and Kidney Cancer. Ann. Occup. Environ. Med. 26, 12.

Kinnula, V.L., Crapo, J.D., 2004. Superoxide dismutases in malignant cells and human tumors. Free Radic. Biol. Med. 36, 718–744.

Korr, H., Botzem, B., Schmitz, C., Enzmann, H., 2001. N-Nitrosomorpholine induced alterations of unscheduled DNA synthesis, mitochondrial DNA synthesis and cell proliferation in different cell types of liver, kidney, and. Chem. Biol. Interact. 134, 217–233.

Koul, H., Huh, J.-S., Rove, K.O., Crompton, L., Koul, S., Meacham, R.B., Kim, F.J., 2011. Molecular aspects of renal cell carcinoma: a review. Am. J. Cancer Res. 1, 240–254.

Kruck, S., Merseburger, A.S., Stenzl, A., Bedke, J., 2014. How far is the horizon? From current targets to future drugs in advanced renal cancer. World J. Urol. 32, 69–77.

Lash, L.H., Parker, J.C., Scott, C.S., 2000. Modes of action of trichloroethylene for kidney tumorigenesis. Environ. Health Perspect. 108, 225–240.

Lew, J.Q., Chow, W.-H., Hollenbeck, A.R., Schatzkin, A., Park, Y., 2011. Alcohol consumption and risk of renal cell cancer: the NIH-AARP diet and health study. Br. J. Cancer 104, 537–541.

Linehan, W.M., Ricketts, C.J., 2016. Kidney cancer in 2016: RCC — advances in targeted therapeutics and genomics. Nat. Rev. Urol. 14(2), 76–78.

Linehan, W.M., Rubin, J.S., Bottaro, D.P., 2009. VHL loss of function and its impact on oncogenic signaling networks in clear cell renal cell carcinoma. Int. J. Biochem. Cell Biol. 41, 753–756.

Linehan, W.M., Srinivasan, R., Schmidt, L.S., 2010. The genetic basis of kidney cancer: a metabolic disease. Nat Rev Urol 7, 277–285.

Liou, G.-Y., Storz, P., 2010. Reactive oxygen species in cancer. Free Radic. Res. 44, 479–496.

Ljungberg, B., Campbell, S.C., Cho, H.Y., Jacqmin, D., Lee, J.E., Weikert, S., Kiemeney, L.A., 2011. The epidemiology of renal cell carcinoma. Eur. Urol. 60, 615–621.

Lock, E. a, Hard, G.C., 2004. Chemically induced renal tubule tumors in the laboratory rat and mouse: review of the NCI/NTP database and categorization of renal carcinogens based on mechanistic information. Crit. Rev. Toxicol. 34(3), 211–299.

Lushchak, V.I., 2014. Free radicals, reactive oxygen species, oxidative stress and its classification. Chem. Biol. Interact. 224, 164–175.

Mackensen, G.B., Patel, M., Sheng, H., Calvi, C.L., Batinic-Haberle, I., Day, B.J., Liang, L.P., Fridovich, I., Crapo, J.D., Pearlstein, R.D., *et al*., 2001. Neuroprotection from delayed postischemic administration of a metalloporphyrin catalytic antioxidant. J. Neurosci. 21, 4582–4592.

Mačkić, S., Ahmetović, N., 2011. Toxicological Profiles of Highly Hazardous. Herbologia 12, 55–60.

Mahalingaiah, P.K.S., Ponnusamy, L., Singh, K.P., 2015. Chronic oxidative stress leads to malignant transformation along with acquisition of stem cell characteristics, and epithelial to mesenchymal transition in human renal epithelial cells. J. Cell. Physiol. 230, 1916–1928.

Mahalingaiah, P.K.S., Ponnusamy, L., Singh, K.P., Mahalingaiah, P.K.S., Ponnusamy, L., Singh, K.P., 2016. Oxidative stress-induced epigenetic changes associated with malignant transformation of human kidney epithelial cells. Oncotarget 5, 11127–11143.

Mahmood, J., Jelveh, S., Calveley, V., Zaidi, A., Doctrow, S.R., Hill, R.P., 2011. Mitigation of radiation-induced lung injury by genistein and EUK-207. Int. J. Radiat. Biol. 87, 889–901.

Makinde, A.Y., Rizvi, A., Crapo, J.D., Pearlstein, R.D., Slater, J.M., Gridley, D.S., 2010. A metalloporphyrin antioxidant alters cytokine responses after irradiation in a prostate tumor model. Radiat. Res. 173, 441–452.

Marcus, D.L., Thomas, C., Rodriguez, C., Simberkoff, K., Tsai, J.S., Strafaci, J. a, Freedman, M.L., 1998. Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease. Exp. Neurol. 150, 40–44.

Marin-Kuan, M., Ehrlich, V., Delatour, T., Cavin, C., Schilter, B., 2011. Evidence for a role of oxidative stress in the carcinogenicity of ochratoxin A. J. Toxicol. 2011.

Martini, M., De Santis, M.C., Braccini, L., Gulluni, F., Hirsch, E., 2014. PI3K/AKT signaling pathway and cancer: an updated review. Ann. Med. 34, 1–12.

Masini, E., Cuzzocrea, S., Mazzon, E., Marzocca, C., Mannaioni, P.F., Salvemini, D., 2002. Protective effects of M40403, a selective superoxide dismutase mimetic, in myocardial ischaemia and reperfusion injury in vivo. Br. J. Pharmacol. 136, 905–917.

Mehrazin, R., Smaldone, M.C., Egleston, B., Tomaszewski, J.J., Concodora, C.W., Ito, T.K., Abbosh, P.H., Chen, D.Y.T., Kutikov, A., Uzzo, R.G., 2015. Is anatomic complexity associated with renal tumor growth kinetics under active surveillance? Urol. Oncol. Semin. Orig. Investig. 33, 167.e7-167.e12.

Miller, A.F., 2012. Superoxide dismutases: Ancient enzymes and new insights. FEBS Lett. 586, 585–595.

Mills, K.T., Bundy, J.D., Kelly, T.N., Reed, J.E., Kearney, P.M., Reynolds, K., Chen, J., He, J., 2016. Global Disparities of Hypertension Prevalence and ControlClinical Perspective. Circulation 134, 441–450.

Morris, M.R., Latif, F., 2016. The epigenetic landscape of renal cancer. Nat. Rev. Nephrol. 13, 47–60.

Motzer, R.J., Bander, N.H., Nanus, D.M., 1996. Renal-Cell Carcinoma. N. Engl. J. Med. 335, 865–875.

Motzer, R.J., Escudier, B., McDermott, D.F., George, S., Hammers, H.J., Srinivas, S., Tykodi, S.S., Sosman, J.A., Procopio, G., Plimack, E.R., *et al*., 2015. Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. N. Engl. J. Med. 1803–1813.

Motzer, R.J., Hutson, T.E., Tomczak, P., Michaelson, M.D., Bukowski, R.M., Rixe, O., Oudard, S., Negrier, S., Szczylik, C., Kim, S.T., *et al*., 2007. Sunitinib versus Interferon Alfa in Metastatic Renal-Cell Carcinoma. N. Engl. J. Med. 356, 115–124.

Mucci, L. a, Dickman, P.W., Steineck, G., Adami, H.-O., Augustsson, K., 2003. Dietary acrylamide and cancer of the large bowel, kidney, and bladder: absence of an association in a population-based study in Sweden. Br. J. Cancer 88, 84–89.

Nesslany, F., Zennouche, N., Simar-Meintières, S., Talahari, I., NKili-Mboui, E.N., Marzin, D., 2007. In vivo Comet assay on isolated kidney cells to distinguish genotoxic carcinogens from epigenetic carcinogens or cytotoxic compounds. Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 630, 28–41.

Nguyen, D.X., Bos, P.D., Massagué, J., 2009. Metastasis: from dissemination to organ-specific colonization. Nat. Rev. Cancer 9, 274–284.

Nuran Ercal, B.S.P., Hande Gurer-Orhan, B.S.P., Nukhet Aykin-Burns, B.S.P., 2001. Toxic Metals and Oxidative Stress Part I: Mechanisms Involved in Me-tal induced Oxidative Damage. Curr. Top. Med. Chem. 1, 529–539.

O'Brien, E., Dietrich, D.R., 2005. Ochratoxin A: the continuing enigma. Crit. Rev. Toxicol. 35, 33–60.

Oberley-Deegan, R.E., Steffan, J.J., Rove, K.O., Pate, K.M., Weaver, M.W., Spasojevic, I., Frederick, B., Raben, D., Meacham, R.B., Crapo, J.D., *et al*., 2012. The Antioxidant, MnTE-2-PyP, Prevents Side-Effects Incurred by Prostate Cancer Irradiation. PLoS One 7, 1–8.

Oberley, T.D., Sempf, J.M., Oberley, L.W., 1996. Histology and lmmunogold analysis of antioxidant enzymes in common renal cancers. Histol. Histopathol. 11(1), 153-160.

Oberley, T.D., Toyokuni, S., Szweda, L.I., 1999. Localization of hydroxynonenal protein adducts in normal human kidney and selected human kidney cancers. Free Radic. Biol. Med. 27, 695–703.

Pearlstein, R.D., Higuchi, Y., Moldovan, M., Johnson, K., Fukuda, S., Gridley, D.S., Crapo, J.D., Warner, D.S., Slater, J.M., 2010. Metalloporphyrin antioxidants ameliorate normal tissue radiation damage in rat brain. Int. J. Radiat. Biol. 86, 145–163.

Pelucchi, C., Bosetti, C., Galeone, C., La Vecchia, C., 2015. Dietary acrylamide and cancer risk: An updated meta-analysis. Int. J. Cancer 136, 2912–2922.

Pfohl-Leszkowicz, A., Manderville, R.A., 2012. An update on direct genotoxicity as a molecular mechanism of ochratoxin a carcinogenicity. Chem. Res. Toxicol. 25, 252–262.

Pfohl-Leszkowicz, A., Manderville, R.A., 2007. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. Mol. Nutr. Food Res. 51, 61–99.

Pharmaceuticals, A., 2017. AEOL 10150 Available from: http://www.aolsrx.com/ product-pipeline/aeol-10150, accessed [17/02/2017].

Piva, F., Giulietti, M., Occhipinti, G., Santoni, M., Sotte, V., Iacovelli, R., Burattini, L., Santini, D., Montironi, R., Cascinu, S., Principato, G., 2015. Computational analysis of the mutations in BAP1 , PBRM1 and SETD2 genes reveals the impaired molecular processes in Renal Cell Carcinoma. Oncotarget 6, 32161–32168.

Piva, F., Santoni, M., Matrana, M.R., Satti, S., Giulietti, M., Occhipinti, G., Massari, F., Cheng, L., AntonioLopez, B., Scarpelli, M., *et al*., 2015. BAP1, PBRM1 and SETD2 in clear-cell renal cell carcinoma: molecular diagnostics and possible targets for personalized therapies. Expert Rev. Mol. Diagn. 15, 1201–1210.

Pljesa-Ercegovac, M., Mimic-Oka, J., Dragicevic, D., Savic-Radojevic, A., Opacic, M., Pljesa, S., Radosavljevic, R., Simic, T., 2008. Altered antioxidant capacity in human renal cell carcinoma: role of glutathione associated enzymes. Urol. Oncol. 26, 175–181.

Pozdzik, A.A., Salmon, I.J., Debelle, F.D., Decaestecker, C., Van den Branden, C., Verbeelen, D., Deschodt-Lanckman, M.M., Vanherweghem, J.-L., Nortier, J.L., 2008. Aristolochic acid induces proximal tubule apoptosis and epithelial to mesenchymal transformation. Kidney Int. 73, 595–607.

Rabbani, Z.N., Spasojevic, I., Zhang, X., Moeller, B.J., Haberle, S., Vasquez-Vivar, J., Dewhirst, M.W., Vujaskovic, Z., Batinic-Haberle, I., 2009. Antiangiogenic action of redox-modulating Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin, MnTE-2-PyP^{5+}, via suppression of oxidative stress in a mouse model of breast tumor. Free Radic. Biol. Med. 47, 992–1004.

Radford, R., Frain, H., Ryan, M.P., Slattery, C., McMorrow, T., 2013. Mechanisms of chemical carcinogenesis in the kidneys. Int. J. Mol. Sci. 14, 19416–19433.

Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., Dhama, K., 2014. Oxidative Stress, Prooxidants, and Antioxidants: The Interplay. Biomed Res. Int. 2014, 1–19.

Ramdial, K., Rossi, F.H., Franco, M.C., Estevez, A.G., 2016. Amyotrophic Lateral Sclerosis: Present Understanding of the Role of SOD, 597–603.

Ravizza, R., Gariboldi, M.B., Passarelli, L., Monti, E., 2004. Role of the p53/p21 system in the response of human colon carcinoma cells to Doxorubicin. BMC Cancer 4, 92.

Ray, P.D., Huang, B.W., Tsuji, Y., 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell. Signal. 24, 981–990.

Renehan, A.G., Tyson, M., Egger, M., Heller, R.F., Zwahlen, M., 2008. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet 371, 569–578.

Reuter, S., Gupta, S.C., Chaturvedi, M.M., Aggarwal, B.B., 2010. Oxidative stress, inflammation, and cancer: How are they linked? Free Radic. Biol. Med. 49, 1603–1616.

Ridge, C.A., Pua, B.B., Madoff, D.C., 2014. Epidemiology and staging of renal cell carcinoma. Semin. Intervent. Radiol. 31, 3–8.

Ringot, D., Chango, A., Schneider, Y.J., Larondelle, Y., 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chem. Biol. Interact. 159, 18–46.

Rini, B.I., Campbell, S.C., Escudier, B., 2009. Renal cell carcinoma. Lancet 373, 1119–1132.

Robbiano, L., Baroni, D., Carrozzino, R., Mereto, E., Brambilla, G., 2004. DNA damage and micronuclei induced in rat and human kidney cells by six chemicals carcinogenic to the rat kidney. Toxicology 204, 187–195.

Robbiano, L., Carrozzino, R., Puglia, C.P., Corbu, C., Brambilla, G., 1999. Correlation between Induction of DNA Fragmentation and Micronuclei Formation in Kidney Cells from Rats and Humans and Tissue-Specific Carcinogenic Activity. Toxicol. Appl. Pharmacol. 161, 153–159.

Robbins, D., Zhao, Y., 2014. Manganese superoxide dismutase in cancer prevention. Antioxid. Redox Signal. 20, 1628–1645.

Saba, H., Batinic-Haberle, I., Munusamy, S., Mitchell, T., Lichti, C., Megyesi, J., MacMillan-Crow, L.A., 2007. Manganese porphyrin reduces renal injury and mitochondrial damage during ischemia/reperfusion. Free Radic. Biol. Med. 42, 1571–1578.

Sahi, C., Knox, J.J., Clemons, M., Joshua, A.M., Broom, R., 2010. Renal cell carcinoma bone metastases: clinical advances. Ther. Adv. Med. Oncol. 2, 75–83.

Sanchez, A., Allard, C.B., Wilson, K.M., 2017. Epidemiology of Renal Cell Carcinoma, in: Pathology and Epidemiology of Cancer. Springer International Publishing, Cham, 313–334.

Sarto, C., Frutiger, S., Cappellano, F., Sanchez, J.-C., Doro, G., Catanzaro, F., Hughes, G.J., Hochstrasser, D.F., Mocarelli, P., 1999. Modified expression of plasma glutathione peroxidase and manganese superoxide dismutase in human renal cell carcinoma. Electrophoresis 20, 3458–3466.

Schaaf, G.J., Nijmeijer, S.M., Maas, R.F.M., Roestenberg, P., De Groene, E.M., Fink-Gremmels, J., 2002. The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. Biochim. Biophys. Acta - Mol. Basis Dis. 1588, 149–158.

Shen, S.S., Krishna, B., Chirala, R., Amato, R.J., Truong, L.D., 2005. Kidney-specific cadherin, a specific marker for the distal portion of the nephron and related renal neoplasms. Mod. Pathol. 18, 933–940.

Sheng, H., Enghild, J.J., Bowler, R., Patel, M., Batinić-Haberle, I., Calvi, C.L., Day, B.J., Pearlstein, R.D., Crapo, J.D., Warner, D.S., 2002. Effects of metalloporphyrin catalytic antioxidants in experimental brain ischemia. Free Radic. Biol. Med. 33, 947–961.

Sheng, H., Spasojevic, I., Tse, H.M., Jung, J.Y., Hong, J., Zhang, Z., Piganelli, J.D., Batinic-Haberle, I., Warner, D.S., 2011. Neuroprotective Efficacy from a Lipophilic Redox-Modulating Mn(III) N-Hexylpyridylporphyrin, MnTnHex-2-PyP: Rodent Models of Ischemic Stroke and Subarachnoid Hemorrhage. J. Pharmacol. Exp. Ther. 338, 906–916.

Sheng, H., Spasojevic, I., Warner, D.S., Batinic-Haberle, I., 2004. Mouse spinal cord compression injury is ameliorated by intrathecal cationic manganese(III) porphyrin catalytic antioxidant therapy. Neurosci. Lett. 366, 220–225.

Sheng, H., Warner, D.S., 2016. Metalloporphyrin in CNS Injuries, in: Batinic-Haberle, I., Reboucas, J.S., Spasojevic, I. (Eds.), Redox-Active Therapeutics, 541–561.

Shibamoto, T., Bjeldanes, L., 2009. Introduction to Food Toxicology, 2nd ed. Academic Press.

Shuch, B., Amin, A., Armstrong, A.J., Eble, J.N., Ficarra, V., Lopez-Beltran, A., Martignoni, G., Rini, B.I., Kutikov, A., 2015. Understanding pathologic variants of renal cell carcinoma: Distilling therapeutic opportunities from biologic complexity. Eur. Urol. 67, 85–97.

Siegel, R., Miller, K., Jemal, A., 2017. Cancer statistics, 2017 . CA Cancer J Clin 67, 7–30.

Sies, H., 2015. Oxidative stress: A concept in redox biology and medicine. Redox Biol. 4, 180–183.

Sies, H., 1985. Oxidative Stress. Academic Press, London.

Sies, H., Jones, D., 2007. Oxidative Stress, in: Encyclopedia of Stress. Elsevier, 45–48.

Smith, K.R., Uyeminami, D.L., Kodavanti, U.P., Crapo, J.D., Chang, L.-Y., Pinkerton, K.E., 2002. Inhibition of tobacco smoke-induced lung inflammation by a catalytic antioxidant. Free Radic. Biol. Med. 33, 1106–1114.

Song, J.K., Luo, H., Yin, X.H., Huang, G.L., Luo, S.Y., Lin, D.R., Yuan, D.B., Zhang, W., Zhu, J.G., 2015. Association between cadmium exposure and renal cancer risk: a meta-analysis of observational studies. Sci. Rep. 5, 17976.

Sorrenti, V., Di Giacomo, C., Acquaviva, R., Barbagallo, I., Bognanno, M., Galvano, F., 2013. Toxicity of ochratoxin A and its modulation by antioxidants: A review. Toxins (Basel). 5, 1742–1766.

Sosa, V., Moliné, T., Somoza, R., Paciucci, R., Kondoh, H., LLeonart, M.E., 2013. Oxidative stress and cancer: An overview. Ageing Res. Rev. 12, 376–390.

Sountoulides, P., Metaxa, L., Cindolo, L., 2011. Atypical presentations and rare metastatic sites of renal cell carcinoma: a review of case reports. J. Med. Case Rep. 5, 429.

Steenland, K., Boffetta, P., 2000. Lead and cancer in humans: Where are we now? Am. J. Ind. Med. 38, 295–299.

Steenland, K., Selevan, S., Landrigan, P., 1992. The mortality of lead smelter workers: An update. Am. J. Public Health 82, 1641–1644.

Sternberg, C.N., Davis, I.D., Mardiak, J., Szczylik, C., Lee, E., Wagstaff, J., Barrios, C.H., Salman, P., Gladkov, O.A., Kavina, A., *et al*., 2010. Pazopanib in locally advanced or metastatic renal cell carcinoma: Results of a randomized phase III trial. J. Clin. Oncol. 28, 1061–1068.

Storz, P., 2013. Oxidative Stress in Cancer, in: Oxidative Stress and Redox Regulation. Springer Netherlands, Dordrecht, 427–447.

Sun, M., Abdollah, F., Bianchi, M., Trinh, Q.-D., Jeldres, C., Thuret, R., Tian, Z., Shariat, S.F., Montorsi, F., Perrotte, P., Karakiewicz, P.I., 2012. Treatment Management of Small Renal Masses in the 21st Century: A Paradigm Shift. Ann. Surg. Oncol. 19, 2380–2387.

Šverko, A., Sobočanec, S., Kušić, B., Mačak-Šafranko, Ž., Šarić, A., Leniček, T., Kraus, O., Andrišić, L., Korolija, M., Balog, T., Šunjić, S.B., Marotti, M., 2011. Superoxide dismutase and cytochrome P450 isoenzymes might be associated with higher risk of renal cell carcinoma in male patients. Int. Immunopharmacol. 11, 639–645.

Swaen, G.M.H., Haidar, S., Burns, C.J., Bodner, K., Parsons, T., Collins, J.J., Baase, C., 2007. Mortality study update of acrylamide workers. Occup. Environ. Med. 64, 396–401.

Szatrowski, T.P., Nathan, C.F., 1991. Production of Large Amounts of Hydrogen Peroxide by Human Tumor Cells. Cancer Res. 51, 794–799.

Terlecky, S.R., 2012. Peroxisomes, oxidative stress, and inflammation. World J. Biol. Chem. 3, 93.

Thomas, R., Sharifi, N., 2012. SOD Mimetics: A Novel Class of Androgen Receptor Inhibitors That Suppresses Castration-Resistant Growth of Prostate Cancer. Mol. Cancer Ther. 11, 87–97.

Tovmasyan, A., Maia, C.G.C., Weitner, T., Carballal, S., Sampaio, R.S., Lieb, D., Ghazaryan, R., Ivanovic-Burmazovic, I., Ferrer-Sueta, G., Radi, R., *et al*., 2015a. A comprehensive evaluation of catalase-like activity of different classes of redox-active therapeutics. Free Radic. Biol. Med. 86, 308–321.

Tovmasyan, A., Sampaio, R.S., Boss, M.K., Bueno-Janice, J.C., Bader, B.H., Thomas, M., Reboucas, J.S., Orr, M., Chandler, J.D., Go, Y.M., *et al*., 2015b. Anticancer therapeutic potential of Mn porphyrin/ascorbate system. Free Radic. Biol. Med. 89, 1231–1247.

Valentine, J.S., Doucette, P.A., Zittin Potter, S., 2005. Copper-zinc superoxide dismutase and amyotrophic lateral sclerosis. Annu. Rev. Biochem. 74, 563–593.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C.J.C.J., Telser, J., 2004. Role of oxygen radicals in DNA damage and cancer incidence. Mol. Cell. Biochem. 266, 37–56.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M., Mazur, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem. Biol. Interact. 160, 1–40.

Verbon, E.H., Post, J.A., Boonstra, J., 2012. The influence of reactive oxygen species on cell cycle progression in mammalian cells. Gene 511, 1–6.

Wang, J., Yi, J., 2008. Cancer cell killing via ROS: To increase or decrease, that is a question. Cancer Biol. Ther. 7, 1875–1884.

Wang, Z.Q., Porreca, F., Cuzzocrea, S., Galen, K., Lightfoot, R., Masini, E, Muscoli, C., Mollace, V., Ndengele, M., Ischiropoulos, H., *et al*., 2004. A newly identified role for superoxide in inflammatory pain. J. Pharmacol. Exp. Ther. 309, 869–878.

Wangila, G.W., Nagothu, K.K., Steward, R., Bhatt, R., Iyere, P.A., Willingham, W.M., Sorenson, J.R.J., Shah, S. V, Portilla, D., 2006. Prevention of cisplatin-induced kidney epithelial cell apoptosis with a Cu superoxide dismutase-mimetic [copper2II(3,5-ditertiarybutylsalicylate)4(ethanol)4]. Toxicol. In Vitro 20, 1300–1312.

Waris, G., Ahsan, H., 2006. Reactive oxygen species: role in the development of cancer and various chronic conditions. J. Carcinog. 5, 14.

WCRF/AICR, 2015. Continuous Update Project Report: Diet, Nutrition, Physical Activity and Kidney Cancer.

Wei, Q., Zhou, Q., Chen, Y., Ou, R., Cao, B., Xu, Y., Yang, J., Shang, H.-F., 2017. Analysis of SOD1 mutations in a Chinese population with amyotrophic lateral sclerosis: a case-control study and literature review. Sci. Rep. 7, 44606.

Weidinger, A., Kozlov, A. V, 2015. Biological Activities of Reactive Oxygen and Nitrogen Species: Oxidative Stress versus Signal Transduction. Biomolecules 5, 472–484.

Weikert, S., Boeing, H., Pischon, T., Weikert, C., Olsen, A., Tjonneland, A., Overvad, K., Becker, N., Linseisen, J., Trichopoulou, A., *et al.*, 2008. Blood pressure and risk of renal cell carcinoma in the European prospective investigation into cancer and nutrition. Am. J. Epidemiol. 167, 438–446.

Weitzel, D.H., Tovmasyan, A., Ashcraft, K.A., Rajic, Z., Weitner, T., Liu, C., Li, W., Buckley, A.F., Prasad, M.R., Young, K.H., *et al.*, 2015. Radioprotection of the Brain White Matter by Mn(III) N-Butoxyethylpyridylporphyrin-Based Superoxide Dismutase Mimic MnTnBuOE-2-PyP^{5+}. Mol. Cancer Ther. 14, 70–79.

Wilson, K.M., Cho, E., 2016. Obesity and Kidney Cancer, in: Pischon, T., Nimptsch, K. (Eds.), Obesity and Cancer. Springer International Publishing, Cham, 81–93.

World Cancer Research Fund International/American Institute for Cancer Research, 2015. Continuous Update Project Report: Diet, Nutrition, Physical Activity and Kidney Cancer. Available from: http://wcrf.org/kidney-cancer-2015, accessed [23/02/2017].

Wu, T.K., Wei, C.W., Pan, Y.R., Cherng, S.H., Chang, W.J., Wang, H.F., Yu, Y.L., 2015. Vitamin C attenuates the toxic effect of aristolochic acid on renal tubular cells via decreasing oxidative stress mediated cell death pathways. Mol. Med. Rep. 12, 6086–6092.

Xu, Y., Armstrong, S.J., Arenas, I. a, Pehowich, D.J., Davidge, S.T., 2004. Cardioprotection by chronic estrogen or superoxide dismutase mimetic treatment in the aged female rat. Am. J. Physiol. Heart Circ. Physiol. 287, H165–H171.

Yu, F.-Y., Wu, T.-S., Chen, T.-W., Liu, B.-H., 2011. Aristolochic acid I induced oxidative DNA damage associated with glutathione depletion and ERK1/2 activation in human cells. Toxicol. In Vitro 25, 810–816.

Yuan, Y., Marshall, G., Ferreccio, C., Steinmaus, C., Liaw, J., Bates, M., Smith, A.H., 2010. Kidney Cancer Mortality. Epidemiology 21, 103–108.

Zelko, I.N., Mariani, T.J., Folz, R.J., 2002. Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic. Biol. Med. 33, 337–349.

Zerbi, A., Ortolano, E., Balzano, G., Borri, A., Beneduce, A.A., Di Carlo, V., 2008. Pancreatic metastasis from renal cell carcinoma: which patients benefit from surgical resection? Ann. Surg. Oncol. 15, 1161–1168.

Zhang, Y., Zhang, X., Rabbani, Z.N., Jackson, I.L., Vujaskovic, Z., 2012. Oxidative Stress Mediates Radiation Lung Injury by Inducing Apoptosis. Int. J. Radiat. Oncol. 83, 740–748.

Zhao, Y., Chaiswing, L., Oberley, T.D., Batinic-Haberle, I., St. Clair, W., Epstein, C.J., St. Clair, D., 2005. A mechanism-based antioxidant approach for the reduction of skin carcinogenesis. Cancer Res. 65, 1401–1405.

Zhao, Z., Azadzoi, K., Choi, H.-P., Jing, R., Lu, X., Li, C., Wang, F., Lu, J., Yang, J.-H., 2017. LC-MS/MS Analysis Unravels Deep Oxidation of Manganese Superoxide Dismutase in Kidney Cancer. Int. J. Mol. Sci. 18, 319.

Znaor, A., Lortet-Tieulent, J., Laversanne, M., Jemal, A., Bray, F., 2015. International variations and trends in renal cell carcinoma incidence and mortality. Eur. Urol. 67, 519–530.

Zong, H., Yin, B., Zhou, H., Cai, D., Ma, B., Xiang, Y., 2014. Inhibition of mTOR pathway attenuates migration and invasion of gallbladder cancer via EMT inhibition. Mol. Biol. Rep. 41, 4507–4512.

Chapter 2

Aim

Hypothesis

Can SODm modulate renal cancer etiology (A) and progression (B)?

General aim

The general aim of this thesis is the study of the potential effect of SODm on the redox modulation of renal cancer etiology and progression using *in vitro* cell models.

MnPs are SODm with remarkable beneficial effects in different pathological models, including cancer. Previous studies have highlighted the interest of MnPs both as mechanistic tools and as prospective drugs for cancer treatment. However, there is only scarce data on the involvement of oxidative stress and on its modulation by SODm in renal cancer at initiation and progression stages. In this regard, this thesis aims to contribute to clarify the importance of oxidative stress in the initiation and progression of renal cancer, as well as to elucidate the potential of MnPs for renal cancer treatment. For this purpose, one of the most promising SODm, the manganese porphyrin MnTnHex-2-PyP (MnP), was used throughout this work. The SODm was firstly used in a case study focused on the cellular effects of the well-known nephrotoxic and carcinogenic agent, the ochratoxin A (OTA; **Chapter 3**). Afterwards, the MnP was used in a human renal cancer cell model to assess different aspects of cell viability and migration (**Chapter 4**).

Specific objectives

(A) Chapter 3

The aim of the work presented in Chapter 3 was to take advantage of the usefulness of MnP as a tool to unravel the influence of oxidative stress in nephrotoxicity and in renal cancer initiation. For this purpose, the mycotoxin OTA was chosen as a case study, since it is nephrotoxic, carcinogenic and the influence of oxidative stress in its toxicity is still not well understood. The Vero E6 non-human primate kidney cells were selected as a recognized non--tumor renal model, and different objectives were pursued:

- To characterize OTA cytotoxicity through complementary methodologies: crystal violet (CV), neutral red (NR), and lactate dehydrogenase (LDH).
- To assess the MnP cytotoxicity profile and its influence on OTA-induced cytotoxicity.
- To study OTA genotoxicity thought the cytokinesis-block micronucleus (CBMN) and the comet assays.
- To assess the influence of MnP in cell cycle distribution and apoptosis in OTA-exposed cells.
- To evaluate the effect of MnP on OTA-induced intracellular ROS, using dihydrorhodamine 123 (DHR) and dihydroethidium (DHE) fluorescence probes.

The data obtained within Chapter 3 will contribute to the Toxicology and Oncology fields by clarifying the involvement of oxidative stress in OTA-induced nephrotoxic effects. Moreover, this work will give further insights on the knowledge of MnP mode of action, highlighting its importance as a mechanistic tool in experimental *in vitro* models.

(B) Chapter 4

In Chapter 4, the main objective was to evaluate the effects of MnP in an established human clear cell renal cancer model, the 786-O cells. As this chapter aims to provide knowledge on the impact of SODm in an advanced stage of renal cancer, it will focus on endpoints of cell proliferation and migration, two aspects closely related with metastases formation. Therefore, the following specific objectives were sought:

- To assess the influence of MnP in intracellular ROS, using the DHR fluorescence probe.
- To study the MnP influence in cell viability, by complementary methodologies: CV and MTS assays.
- To characterize the cell cycle distribution of MnP treated cells.
- To assess the MnP effect in cell migration, using different methodologies: wound--healing and chemotaxis transwell assays.

The data obtained within the Chapter 4 of this thesis will contribute to the renal cancer field by clarifying the involvement of oxidative stress on the proliferation and migration of cancer cells. In addition, this work would provide *in vitro* data on the possible beneficial effect of MnP in renal cancer treatment, reinforcing this strategy to cope with cancer deleterious effects.

Chapter 3

Case study: Ochratoxin A, ROS and SODm

This Chapter was adapted from:

Costa, João G.; Saraiva, Nuno; Guerreiro, Patrícia S.; Louro, Henriqueta; Silva, Maria J.; Miranda, Joana P.; Castro, Matilde; Batinic-Haberle, Ines; Fernandes, Ana S.; Oliveira, Nuno G.. Ochratoxin A induced cytotoxicity, genotoxicity and reactive oxygen species in kidney cells: an integrative approach of complementary endpoints. Food and Chemical Toxicology (2016), 87, 65-76.

Abstract

Ochratoxin A (OTA) is a well-known nephrotoxic and potential carcinogenic agent but no consensus about the molecular mechanisms underlying its deleterious effects has been reached yet. The aim of this study is to integrate several endpoints concerning OTA-induced toxicological effects in Vero kidney cells in order to obtain additional mechanistic data, especially regarding the influence of reactive oxygen species (ROS). One innovative aspect of this work is the use of the superoxide dismutase mimic (SODm) MnTnHex-2-PyP as a mechanistic tool to clarify the involvement of oxidative stress in OTA toxicity. The results showed concentration and time-dependent cytotoxic effects of OTA (crystal violet, neutral red and LDH leakage assays). While the SODm mildly increased cell viability, trolox and ascorbic acid had no effect with regards to this endpoint. OTA induced micronuclei formation. Using the FPG modified comet assay, OTA modestly increased the % of DNA in tail, revealing the presence of oxidative DNA lesions. This mycotoxin increased apoptosis, which was attenuated by SODm. In addition, the SODm decreased the ROS accumulation observed in DHE assay. Taken together, our data suggest that ROS partially contribute to the cytotoxicity and genotoxicity of OTA, although other mechanisms may be relevant in OTA-induced deleterious effects.

3.1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several species of fungi from Penicillium and Aspergillus genera that commonly contaminate animal feed and food, such as cereals, coffee, cocoa or dried fruit worldwide (EFSA, 2006). OTA is a stable compound that is not destroyed by common food processing (EFSA, 2006). The chemical structure of OTA comprises a dihydrocoumarin moiety linked to a molecule of L-b-phenylalanine via an amide bond as shown in Fig. 3.1.

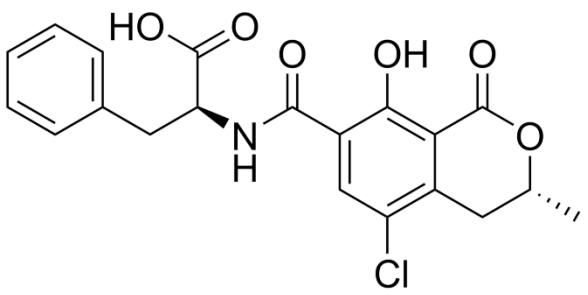

Fig. 3.1 – Chemical structure of ochratoxin A (OTA).

Exposure to OTA has been associated with a number of diseases in both animals and humans, predominantly affecting the kidney. Besides nephrotoxicity, other toxic effects have been associated with OTA exposure such as neurotoxicity, immunotoxicity, myelotoxicity, reproductive toxicity and teratogenicity (WHO, 2001; O'Brien and Dietrich, 2005; Pfohl-Leszkowicz and Manderville, 2007). Moreover, OTA has been classified as a possible carcinogen to humans – Group 2B (IARC, 1993), based on its known tumourigenicity in rodents. Nevertheless, uncertainties remain about its mode of action, particularly, whether it acts primarily through genotoxic or non-genotoxic mechanisms. It is thus important to further characterize the genotoxic potential of this compound as well as its mechanisms of action to fully evaluate other related effects,

namely outcomes associated with OTA-induced cytotoxicity and cell cycle modifications.

Several potential mechanisms are described in literature for OTA toxicity. These mechanisms include the inhibition of protein synthesis, interference with metabolic pathways involving phenylalanine, disruption of calcium homeostasis, promotion of membrane lipid peroxidation, inhibition of mitochondrial respiration, DNA damage and the modulation of gene expression (Ringot et al., 2006; Arbillaga et al., 2007; Pfohl-Leszkowicz and Manderville, 2007, 2012; Cavin et al., 2009). However, there is still no consensus reached on the exact mechanisms and their relevance for OTA toxicity. Despite the number of studies favoring the involvement of oxidative stress, its relevance remains debatable in the context of OTA toxicity (Mally, 2012; Vettorazzi et al., 2013; Gayathri et al., 2015). Some studies have shown the importance of oxidative stress in renal cell toxicity after OTA exposure through a significant increase of reactive oxygen species (ROS), oxidative DNA, lipid and protein damage and by the depletion of cellular antioxidant defenses, along with other redox-driven alterations (Schaaf et al., 2002; Cavin et al., 2009; Marin-Kuan et al., 2011; Ramyaa and Padma, 2013). For this reason, *in vitro* and *in vivo* studies have been performed using antioxidants in an attempt to prevent these adverse effects of ROS generated by OTA. The most used antioxidants include vitamin E, phenolic compounds (catechins and quercetin), melatonin, zinc and N-acetylcysteine, alone or combined (Meki and Hussein, 2001; Costa et al., 2007; Fusi et al., 2010; Ramyaa and Padma, 2013; Sorrenti et al., 2013; Zheng et al., 2013; Yang et al., 2014b). The use of such antioxidants, however have not always altered significantly the toxicity induced by OTA. Nevertheless, the use of superoxide dismutase (SOD) and catalase (CAT) to prevent renal lesions in cases of ochratoxicosis was previously suggested (Baudrimont et al., 1994, 1997). Despite several studies carried out with antioxidants, manganese(III) porphyrins (MnPs) with high superoxide dismutase mimetic activity have never been studied in the context of OTA toxicity. These compounds have the ability to mimic the natural SOD enzymes and to scavenge a

plethora of different ROS, modulating the cellular redox status (Batinic-Haberle et al., 2014, 2015). MnPs have been developed as potential drugs for different pathologies and are also useful mechanistic tools to assess the involvement of oxidative stress in pathological and toxicological conditions (Batinic-Haberle et al., 2014, 2015). MnTnHex-2-PyP (Fig. 3.2; Batinic-Haberle et al., 2002) is considered one of the most promising superoxide dismutase mimics (SODm) with a very high *in vivo* potency along with a large therapeutic window in pre-clinical trials (non-human primates; Tovmasyan et al., 2013).

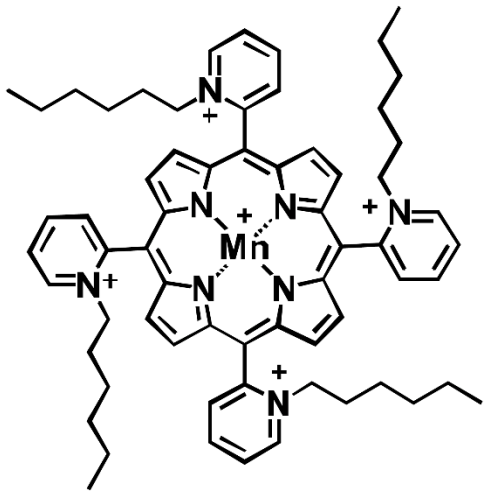

Fig. 3.2 – Chemical structure of the manganese porphyrin (MnP) MnTnHex-2-PyP^{5+}.

The present report aims at providing new mechanistic insights into OTA mode of action with a special focus on oxidative stress induction, using an integrated approach of several endpoints related to cytotoxicity, genotoxicity, apoptosis and ROS generation in a representative model of renal epithelial cells. We used MnTnHex-2-PyP as a mechanistic tool to further explore OTA toxicity.

3.2. Material and methods

3.2.1. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS; 0.01 M, pH 7.4), trypsin, penicillin-streptomycin (pen/strep) solution, crystal violet, neutral red, lactate dehydrogenase (LDH) Tox7 kit, cytochalasin-B, L-ascorbic acid, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), dimethylsulfoxide (DMSO), ethyl methanesulfonate (EMS), low melting point agarose, RNAse and ochratoxin A (purity ≥ 98 %) were obtained from Sigma-Aldrich (St. Louis, MO, USA). OTA stock solutions were prepared in DMSO. The final DMSO concentration in cell culture was 0.5 % (v/v) for all assays. Giemsa dye, methanol, ethanol, acetic acid and propidium iodide (PI) were purchased from Merck (Darmstadt, Germany). Dihydroethidium (DHE), dihydrorhodamine 123 (DHR) and the Alexa Fluor® 488 Annexin V/PI kit were acquired from Molecular Probes (Eugene, OR, USA). The 10 mM stock solutions of DHE and DHR were prepared in DMSO, aliquoted, and stored under nitrogen at –20 ºC. Formamidopyrimidine DNA glycosylase (FPG) was kindly provided by A. R. Collins (University of Oslo, Norway). MnTnHex-2-PyP^{5+} was synthetized as described previously (Batinic-Haberle et al., 2002; charges are omitted for clarity throughout the manuscript).

3.2.2. Cell culture

The green monkey kidney cells (Vero-E6 cell line) was obtained from ATCC (Manassas, VA, USA). Vero-E6 cells were cultured in DMEM medium, containing 10 % FBS and 1 % pen/strep. Cells were maintained at 37 ºC, under a humidified air atmosphere containing 5 % of CO_2.

3.2.3. Crystal violet (CV) staining assay

Cell viability was first evaluated with the CV staining assay. Vero cells were seeded at a density of 1 x 10^4 or 5 x 10^3 per well in 200 mL culture medium in 96-well plates and incubated for 24 h. Cells were then incubated for 24 or 48 h with OTA (5–50 µM) and/or antioxidants (L-ascorbic acid, trolox and MnTnHex-2-PyP). The CV assay was then carried out according to Fernandes et al., 2010a, 2010b and Guerreiro et al., 2013. Absorbance values for untreated control cells correspond to 100 % cell viability. For this assay two to four independent experiments were carried out. Eight replicate cultures were used in each independent experiment. The half maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism Statistical Software (version 6).

3.2.4. Neutral red (NR) assay

The NR assay was carried out as a confirmatory assay to assess cell viability of OTA treated cells. Vero cells were seeded at a density of 1 x 10^4 or 6 x 10^3 cells per well in 200 mL culture medium in 96-well plates and incubated for 24 h. Cells were then incubated for 24 or 48 h with OTA (5–50 µM). The NR was adapted from a previously described protocol (Repetto et al., 2008). Cells were incubated for 2.5 h with NR at a

final concentration of 40 mg/mL. Absorbance values for untreated control cells correspond to 100 % cell viability. Three independent experiments were performed, each one comprising eight replicate cultures. The IC_{50} was calculated using GraphPad Prism Statistical Software (version 6).

3.2.5. Lactate dehydrogenase (LDH) leakage assay

The media from cell cultures of the abovementioned CV and NR assays (48 h treatment) were collected and used for the LDH assay using the LDH Tox7 kit. The technique was performed as described in the manufacturer's protocol. Three independent experiments were performed.

3.2.6. Cytokinesis-block micronucleus (CBMN) assay

Approximately 5 x 10^3 cells were seeded in 500 mL culture medium per well of a 8-well Lab-Tek™ II Chamber Slide™ System (Nunc) and incubated for 24 h. Afterwards, the cells were incubated with OTA (7.5–100 µM) for 24 h and culture medium with 0.5 % (v/v) DMSO was used as vehicle control. The cells were then washed with culture medium and cytochalasin B was added at a final concentration of 4.5 mg/mL to arrest cytokinesis (Fenech, 2007). Cells were allowed to grow for 28 h and then rinsed with PBS. The slides were fixed with ice-cold methanol for 20 min at –20 ºC. After air drying, the Lab-Tek™ II Chamber Slide™ Systems were dismantled and slides were stained with Giemsa (4 % v/v in 0.01 M phosphate buffer, pH 6.8) for 8 min. The slides were then coded for microscope analysis. Three independent experiments were performed for each treatment. Two replicate cultures were used in each independent experiment. For the assessment of micronuclei (MN) frequency, 1000 binucleated (BN)

cells with well-preserved cytoplasm were scored using 1000 x magnification on a light microscope (Leitz), according to described criteria (Fenech, 2007). The frequency of micronucleated binucleated cells (MNBN) was used as genotoxicity index. The total number of MN was also recorded (Bandarra et al., 2013). The decrease in cell proliferation was evaluated by two standard indices, the percentage of binucleated cells (% BN) and the nuclear division index (NDI; Fenech, 2000, 2007). For these indices, 500 cells were classified according to the number of nuclei using a 500 x magnification in a light microscope (Leitz).

3.2.7. Comet assay

Cells were plated in 24-well plates at a concentration of 1 x 10^4 cells/mL, 0.5 mL per well and incubated for 24 h before exposure to OTA. EMS (20 mM) was used as a positive control while culture medium with 0.5 % (v/v) DMSO was the vehicle control. Cells were exposed for 24 h to OTA and for 1 h to EMS, washed with PBS and harvested by trypsinization. Cell suspension was centrifuged (1200 rpm, 10 min, 4 ºC) and the pellet was resuspended in 20 mL of PBS and embedded in 0.8 % low melting point agarose pre-warmed to 38 ºC, to prepare 4 gels for comet assay onto previously agarose coated microscope slides. The comet assay was performed according to Tice et al. (2000) and Collins (2004) with some modifications (Pinto et al., 2014). Briefly, slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10 % DMSO and 1 % Triton X-100, pH 10) for 1 h and rinsed twice (10 min) with enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8). The agarose-embedded cells were then treated either with enzyme buffer (conventional comet assay) or with 50 mL of FPG for 30 min at 37 ºC, to allow detection of oxidized and ring-opened purines or formamidopyrimidines. DNA was allowed to unwind under alkaline conditions by immersing slides into cold electrophoresis buffer (0.3 M NaOH,

1 mM $Na_2EDTA \cdot 2H_2O$; pH 13) for 20 min. Electrophoresis was run for 30 min at 0.8 V/cm. Following washing with PBS for neutralization, slides were dried overnight and stained with ethidium bromide (0.125 mg/mL) before analysis. Two independent assays were carried out, each one with 2 replicate cultures per exposure condition. In each experiment, a total of 200 randomly selected nucleoids were analyzed per culture (100 in gels with FPG treatment and the same number in untreated gels), using an Axioplan2 Imaging epifluorescence microscope equipped with a high resolution camera (Carl Zeiss Microscopy, Göttingen, Germany). Scoring was done with the Comet Imager 2.2 software (MetaSystems, Althlussheim, Germany). The percentage of DNA in tail, which is linearly related with the level of DNA strand breaks, was registered as a measure of DNA damage. The % of FPG-sensitive sites, corresponding to oxidative DNA lesions, was calculated using the following equation (Collins, 2014):

$$\textit{\% of FPG-sensitive sites} = (\%\ DNA)_{FPG} - (\%\ DNA)_{untreated},$$

Where $(\%\ DNA)_{FPG}$ refers to the percentage of DNA in the comet tail from FPG-treated cells and $(\%\ DNA)_{untreated}$, refers to the percentage of DNA in tail from the vehicle control.

Additionally, the data from the comet assay were analyzed according to the distribution of nucleoids per classes (Collins, 2004). According to the % of DNA in tail, the following classes were considered in this work: Class 0 (0–9 %); Class 1 (10–19 %); Class 2 (20–39 %); Class 3 (40–59 %); Class 4 (60–79 %); Class 5 (80–100 %) of DNA in tail.

3.2.8. Intracellular ROS evaluation

For cellular ROS analysis two different probes were used, DHE and DHR. Approximately 5 x 10^4 and 2.5 x 10^4 cells were cultured in 12-well plates with glass cover slips, for the short term and long term protocol, respectively. After 24 h, cells were exposed to OTA (30 and 50 µM), MnTnHex-2-PyP (5 µM) or both for 30 min or 24 h. Cells were then washed once with warm PBS and incubated with DHE (10 µM) or DHR (10 µM) in FBS-free media for 30 min at 37 ºC. Image acquisition was performed using a BX51 fluorescent Olympus microscope with a 40 x objective at specific excitation and emission wavelengths (DHE: 520–550 nm and <580 nm and DHR: 460–490 nm and <520 nm). Cell fluorescence and cell area were determined using ImageJ software (National Institutes of Health, Bethesda, MA, USA). At least 45 cells were measured per experiment and three independent experiments were carried out.

3.2.9. Cell DNA content analysis

Approximately 1.8 x 10^5 cells were cultured in 6-well plates for 24 h. Cells were then exposed to OTA (50 µM), MnTnHex-2-PyP (5 µM) or both for 48 h. For cell DNA content analysis, cells were harvested using 5 mM EDTA in PBS, washed with cold PBS and fixed with 80 % ethanol. After RNase A-treatment (20 mg/mL) and propidium iodide (10 mg/mL) staining, cells were analyzed using a FACSCalibur flow cytometer (BD). Data acquisition and analysis was performed using CellQuest software (BD) and FlowJo (Tree Star, San Carlos, Calif.), respectively.

3.2.10. Apoptosis assay

The percentage of apoptotic cells was determined using the dead cell apoptosis kit with Alexa® Fluor 488 Annexin V and PI for flow cytometry (Molecular Probes, Eugene, OR, USA), according to the manufacturers' instruction. Briefly 1.8 x 10^5 cells were cultured in 6-well plates for 24 h and were then exposed to OTA (50 µM), MnTnHex-2--PyP (5 µM) or both for 48 h. Cells were harvested by a soft trypsinization (0.5 mg/mL) and washed twice with cold PBS. Cells were analyzed by flow cytometry (FACSCalibur, BD). Data acquisition and analysis were performed using CellQuest software (BD) and FlowJo (Tree Star, San Carlos, Calif.), respectively.

3.2.11. Statistical analyses

Differences regarding OTA effects in cytotoxicity, CBMN, and intracellular ROS assays were evaluated with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The two-tailed Student's t-test was used in the comet assay to compare the differences in mean percentage of DNA in tail obtained with treated and control cells, as well as those obtained with and without FPG treatment. This statistical test was also used to assess the impact of antioxidants on cytotoxicity, cell cycle, apoptosis and intracellular ROS. The Chi-square test was used to analyze the differences in classes' distribution for the various OTA concentrations tested in the comet assay. All analyses were performed with the SPSS statistical package (version 22, SPSS Inc. Chicago, IL).

3.3. Results

3.3.1. OTA is cytotoxic and genotoxic to Vero cells

The impact of OTA treatment on cell viability was assessed employing different methodologies. Exposure to OTA (5–50 µM) induced a time- and concentration-dependent decrease in cell viability as assessed by the crystal violet staining (Fig. 3.3A) and the neutral red assays (Fig. 3.3B). Both assays revealed comparable concentration-response profiles after both 24 and 48 h-periods of incubation. The IC_{50} values calculated for a 48 h exposure were 31.8 µM for the CV assay and 21.9 µM for the NR assay. At the highest concentration level studied (50 µM; 48 h), the cell viability was 34.4 % and 20.7 % for the CV and NR assays, respectively. The results from the LDH leakage assay are depicted in Fig. 3.3C. OTA increased LDH leakage; cytotoxic effect was significant for the 50 µM concentration ($p < 0.05$).

The genotoxicity of OTA in Vero cells was evaluated using both the cytokinesis-block micronucleus assay and the comet assay. OTA exposure led to concentration-dependent reduction in the frequency of binucleated (BN) cells and the nuclear division index (NDI), revealing the impact of OTA on the normal cell division of this cell line (Fig. 3.4A and B). A significant increase in the frequency of micronucleated binucleated cells per 1000 BN cells (MNBN) and in the micronuclei total number per 1000 BN cells (MN) was observed, particularly at 25 µM (approximately two-fold increase, $p < 0.01$; Fig. 3.4C and D).

The percentage of DNA in the comet tail obtained with the standard and FPG-modified comet assays are presented in Table III.1 and Fig. 3.5. The exposure to all OTA concentrations caused a consistent increase in DNA damage (up to 1.7-fold) following FPG treatment, with a peak at 15 µM that resulted in a significant increase over the negative control ($p < 0.05$). When comparing FPG-treated and untreated cells,

a significant increase in the level of DNA damage was observed at 15 µM of OTA. Regarding the % of FPG sensitive sites index (Table III.1), an up to 3-fold (15 µM) increase *vs* control was observed, although without a concentration-effect relationship. The increase in the % of FPG sensitive sites was significant at 15 and 50 µM ($p < 0.05$). Significant differences were also found when the distribution of nucleoids per classes was taken into account, either considering data from the standard comet assay ($p < 0.05$) or the FPG-modified assay ($p < 0.001$). Treatment of cells with 7.5 µM of OTA caused a 2-fold increase in the percentage of class 2 nucleoids (20–39 % DNA in tail) *vs* negative controls in the conventional comet assay. In the FPG-modified version, no differences were observed in the distribution of nucleoids per class at 7.5 µM OTA concentration. However, the exposure to 15 µM yielded a 1.5-fold increase in class 1 and approximately 2.5-fold increase in classes 2 and 3 nucleoids, as compared to negative controls. In the latter assay, a 1.5-fold increase relative to control in classes 1, 2 and 3 nucleoids was observed at the concentration of 50 µM (data not shown). The positive controls, EMS and hydrogen peroxide, caused extensive DNA damage, which was accentuated following FPG treatment resulting in highly damage cells.

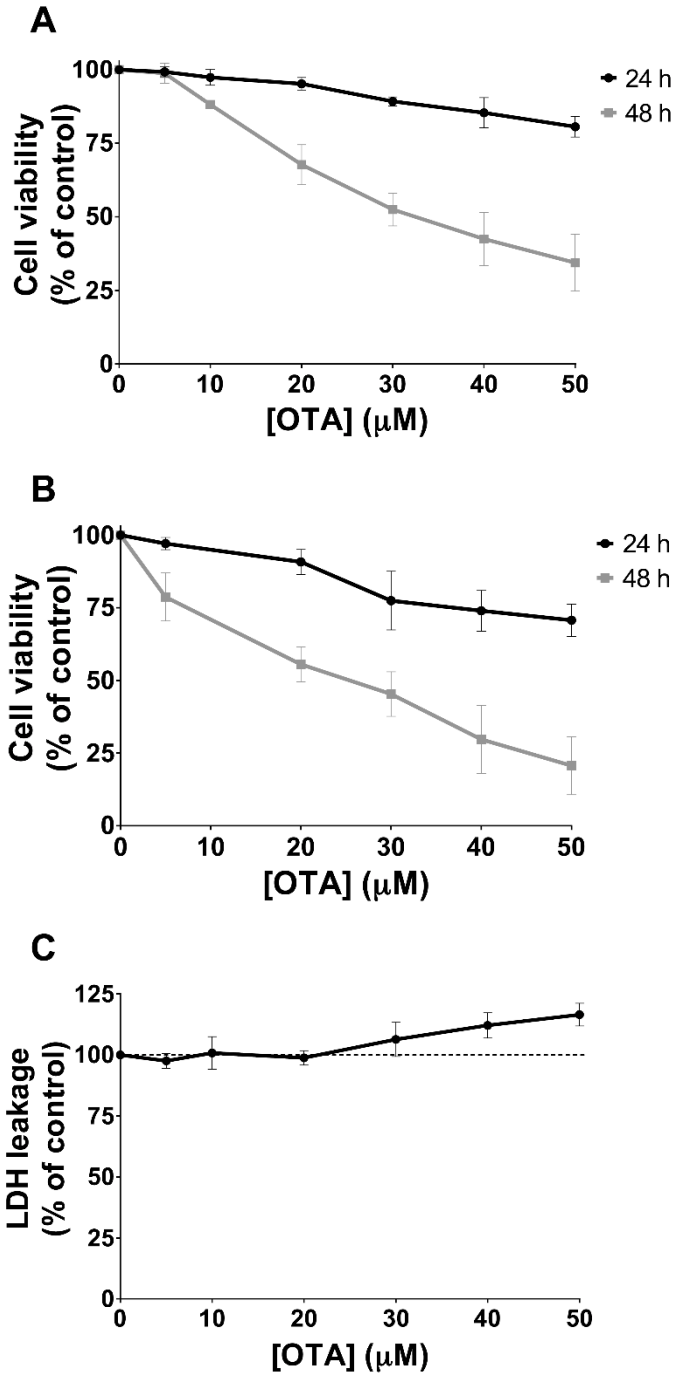

Fig. 3.3 – Cytotoxic effects of ochratoxin A (OTA; 5–50 μM) in Vero cells. The cell viability of OTA-exposed cells (24 h and 48 h) was evaluated by CV (A) and NR (B) assays. The LDH leakage (C) was analyzed after a 48 h exposure to OTA. Values represent mean ± SD (n = 2–4) and are expressed as percentages of the non-treated control cells.

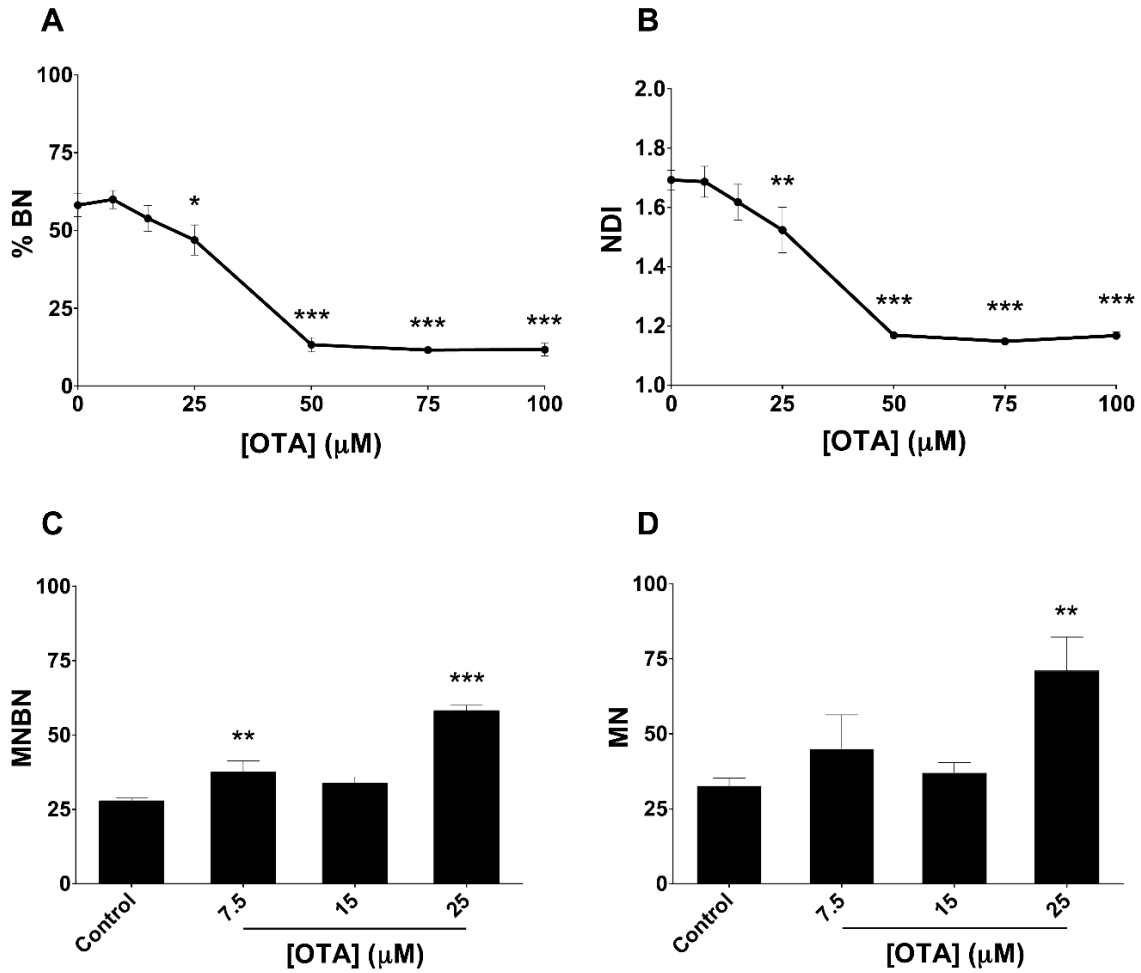

Fig. 3.4 – The effects of ochratoxin A (OTA) on Vero cell proliferative indices (A and B) and induction of micronuclei (C and D) were determined by the cytokinesis-block micronucleus (CBMN) assay. Percentage of binucleated cells (% BN) (A), nuclear division index (NDI) (B), frequency of micronucleated binucleated cells per 1000 BN cells (MNBN) (C) and frequency of micronuclei per 1000 binucleated cells (MN) (D) are shown. Values represent mean ± SD (n = 3); *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (ANOVA test) when compared with non-treated control cells.

Table III.1 – Results of the standard and FPG modified-comet assays following 24 h exposure of Vero cells to ochratoxin A (OTA).

OTA Concentration (µM)	Standard Comet assay % DNA in tail (Mean ± SD)	FPG-modified Comet assay % DNA in tail (Mean ± SD)	% FPG-sensitive sites (Mean ± SD)
0	5.56 ± 0.10	8.06 ± 0.23	2.50 ± 0.13
7.5	7.36 ± 1.65	9.41 ± 0.93	2.05 ± 2.58
15	5.84 ± 1.78	13.35 ± 1.51*,#	7.50 ± 0.27#
25	6.19 ± 1.71	8.79 ± 1.78	2.60 ± 3.49
50	6.15 ± 1.63	10.93 ± 0.97	4.78 ± 0.66#
H_2O_2 10 mM[a]	59.97 ± 2.82#	68.28 ± 15.64*,#	8.31 ± 10.0
EMS 20 mM[a]	47.23 ± 8.7#	59.1[b]	12.32

* Significantly different from Standard Comet assay values ($p < 0.05$).

Significantly different from negative controls ($p < 0.05$).

[a] The number of cells scored in each replicate was lower than 100 cells and only 2 replicates were analyzed.

[b] Only one sample was analyzable.

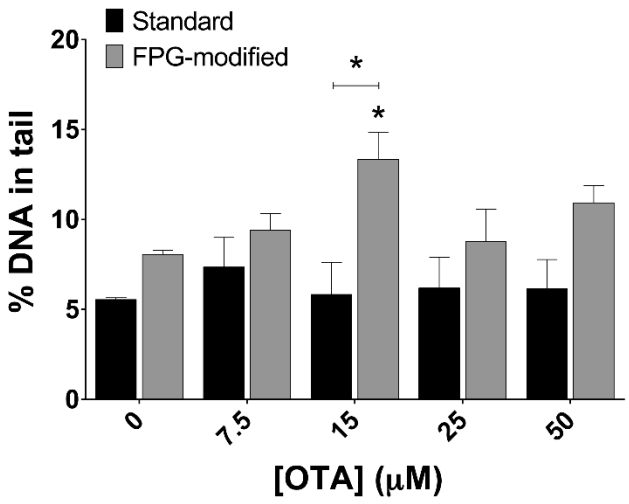

Fig. 3.5 – Results of the standard and FPG-modified comet assays in Vero cells exposed to OTA (7.5–50 µM). Values represent mean ± SD (n = 2); *$p < 0.05$ (Student's t-test).

3.3.2. OTA increases intracellular levels of reactive oxygen species (ROS) in Vero cells

The production of ROS was analyzed by microscopy using two fluorescence probes (DHE and DHR) with reactivity towards different ROS (Fig. 3.6). Both probes showed a ROS increase upon exposure to OTA (compared with non-treated controls). This increase was concentration-dependent and was observed both post short-term treatment (30 min) and longer exposure to OTA (24 h).

3.3.3. MnTnHex-2-PyP protects Vero cells against OTA-induced cytotoxicity

Since the abovementioned results point to an influence of ROS in OTA-treated cells, the cytotoxicity of OTA was evaluated in the presence of three antioxidants with distinct properties. Trolox (200 µM), L-ascorbic acid (100 µM) and MnTnHex-2-PyP (1–25 µM) *per se* did not considerably alter the viability of Vero cells after 48 h incubation (Fig. 3.7A and B). Trolox (200 µM) and L-ascorbic acid (100 µM) as well as their combined treatment did not prevent the cytotoxic effects of OTA (30 and 50 µM) after 48 h of incubation (Fig. 3.7A). Conversely, MnTnHex-2-PyP rendered some protection against OTA cytotoxicity (Fig. 3.7C), revealing a significant increase (up to ~13 %; $p < 0.05$) in cell viability as assessed by the CV assay (48 h). Such observation justified the additional studies performed to further investigate the impact of this SODm in OTA-treated cells.

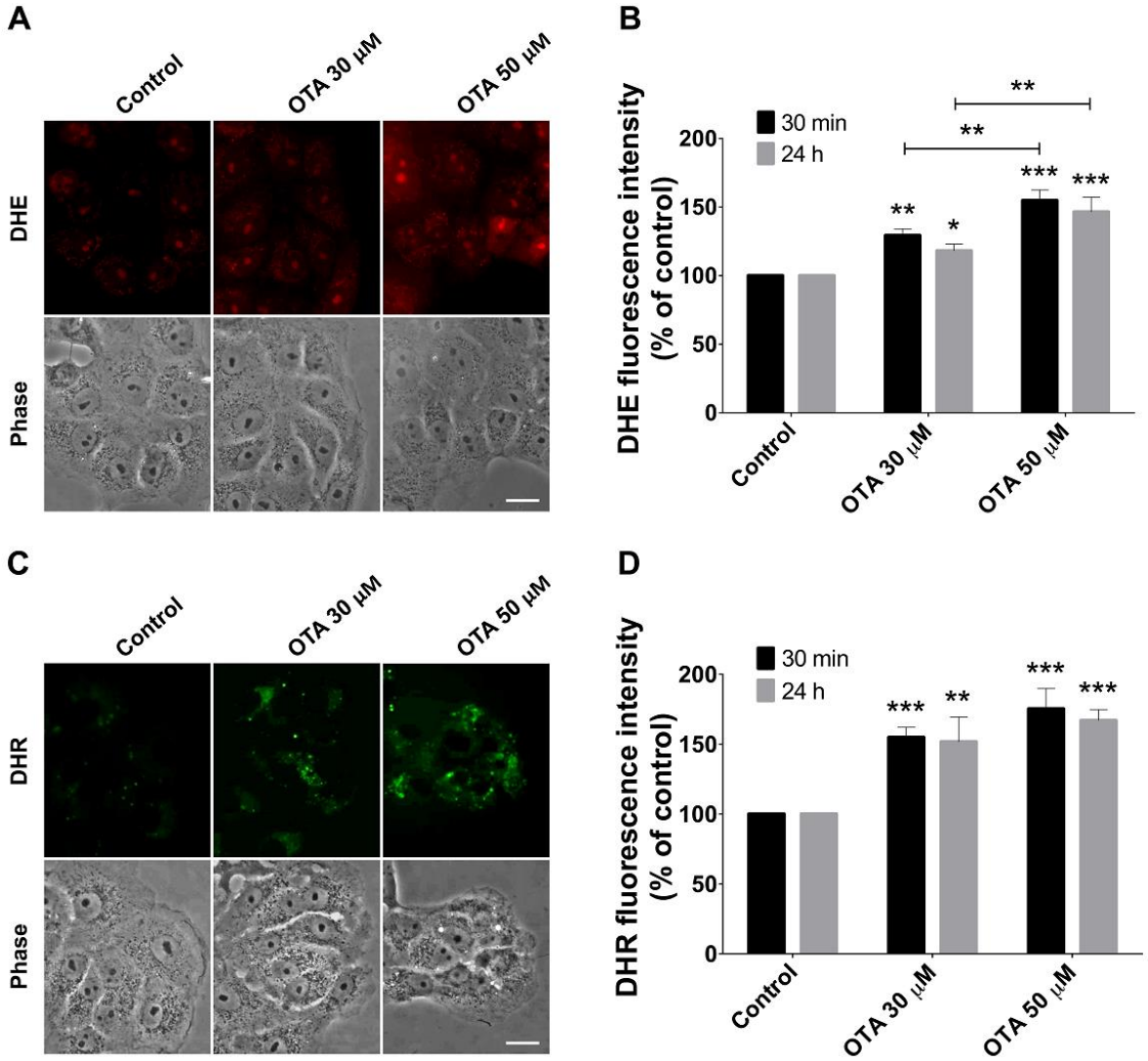

Fig. 3.6 – Exposure to ochratoxin A (OTA) led to an increase in intracellular ROS. The intracellular ROS levels in Vero cells exposed to OTA (30 and 50 µM) for 30 min and 24 h were detected by the DHE (A and B) and DHR (C and D) probes. Fluorescence microscopy images show representative cells after 30 min incubation with DHE (A) and DHR (C). Scale bars = 20 µm (A and C). Values represent mean ± SD (n = 3); *p < 0.05, **p < 0.01 and ***p < 0.001 (ANOVA test).

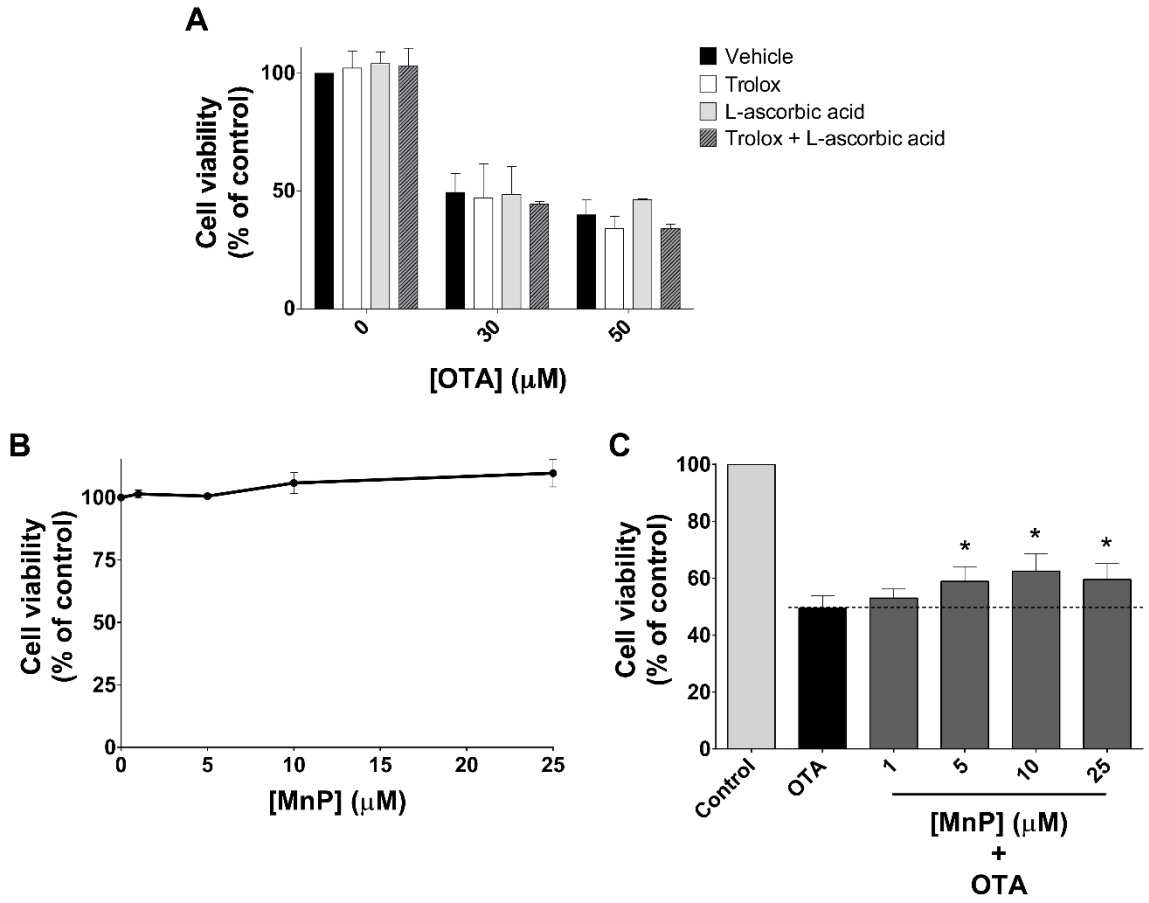

Fig. 3.7 – Effect of antioxidants in Vero cells exposed to ochratoxin A (OTA). The effect of trolox and L-ascorbic acid in Vero cells exposed to OTA (30 and 50 μM) for 48 h was assessed by the CV assay (A). The effect of the MnP on Vero cells viability after 48 h exposure was determined by the CV assay (B). The effect of MnP on viability of cells exposed to OTA (50 μM; 48 h) was evaluated by the CV assay (C). Values represent mean ± SD ($n \geq 3$) and are expressed as percentages of the non-treated control cells; *$p < 0.05$ (Student's t-test) when compared with cells exposed to OTA.

3.3.4. OTA increases the % of apoptotic cells: protective effect of MnTnHex-2-PyP

The impact of OTA in Vero cell cycle was also investigated by assessing the cell DNA content using PI stain in fixed cells. The exposure to OTA (50 µM; 48 h) led to a remarkable increase in sub-G1 population (40.4 %) when compared with the control cells (Fig. 3.8A-C). In addition, OTA decreased the G0/G1 population. The SODm MnTnHex-2-PyP alone (5 µM; 48 h) led to a cell cycle distribution similar to that of control cells. The combined exposure to OTA and MnP led to a significant reduction in sub-G1 population (8.8 %) and a slight increase in G0/G1 and G2/M populations when compared to OTA exposure. Representative histograms obtained by flow cytometric analysis are depicted in Fig. 3.8A. All three independent experiments carried out led to coherent results.

The occurrence of apoptosis was further confirmed by double staining of live cells with Annexin V and PI. The cells exposed to OTA (50 µM; 48 h) showed an increase in the % apoptosis of 56.6 % ($p < 0.001$ *vs* non-treated control cells; Fig. 3.8E). The MnP (5 µM; 48 h) alone did not change the % apoptosis compared with non-treated control cells. The combined exposure to this MnP and OTA led to a minor decrease in the % apoptosis (5.6 %) compared with cells exposed only to OTA. Representative graphs obtained by flow cytometric analysis of the cells are shown in Fig. 3.8D. All three independent experiments carried out led to coherent results.

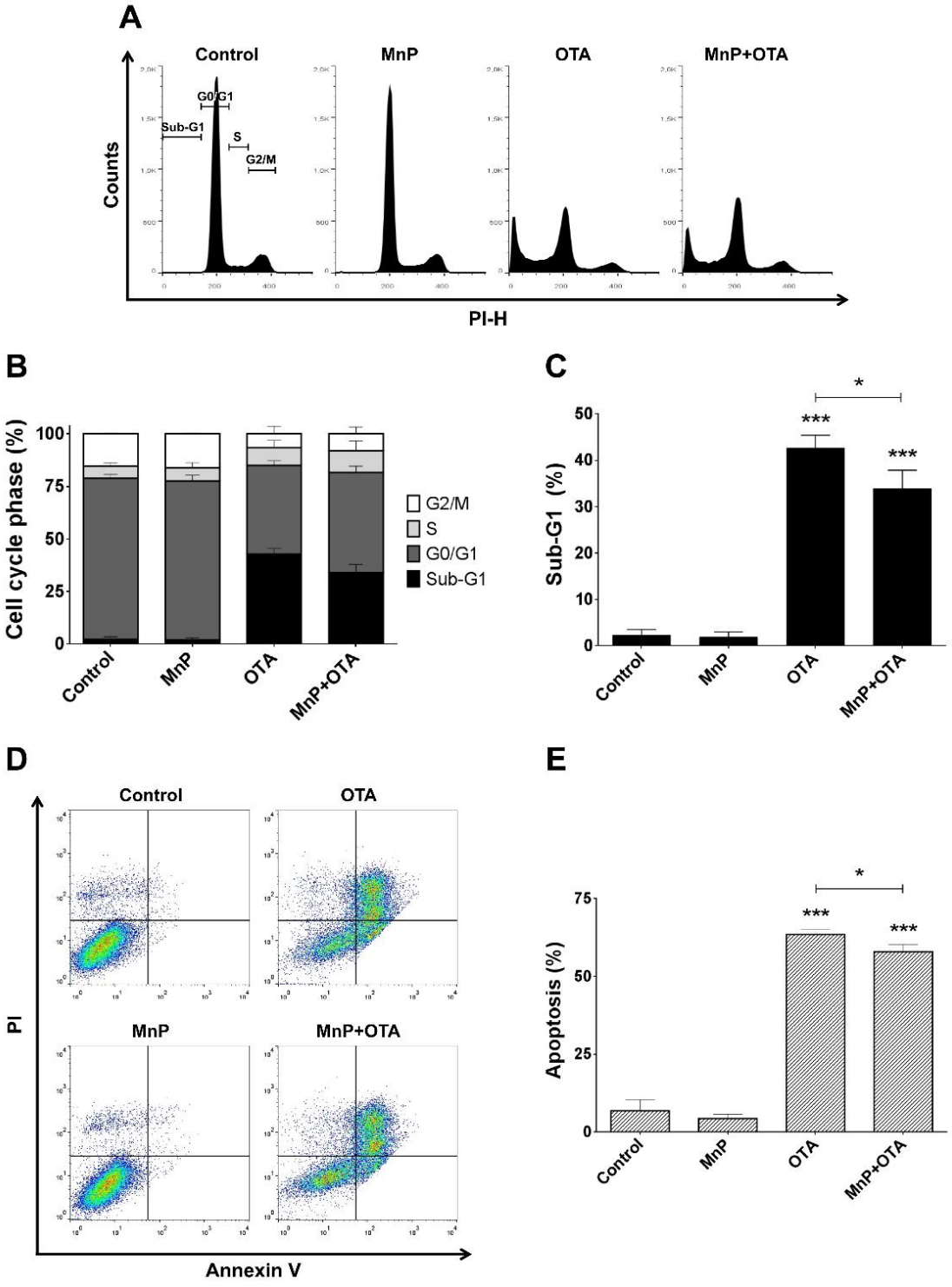

Fig. 3.8 – Effect of ochratoxin A (OTA) and manganese porphyrin (MnP) on cell cycle progression and apoptosis of Vero cells. Cellular DNA content analyzed by flow cytometry after 48 h incubation with OTA and MnP (A–C) with representative flow cytometry histograms (A), sub-G1, G0/G1, S and G2/M populations summary results

(B) and sub-G1 population percentage (C). Percentage of apoptotic cells determined by PI and Annexin V staining (D and E) with representative flow cytometry dot-plots (D) and summary results show percentage of apoptotic cells (E). Values represent mean ± SD (n = 3); *p < 0.05 and ***p < 0.001 (Student's t-test).

3.3.5. MnTnHex-2-PyP decreases the superoxide anion generation by OTA

The impact of MnTnHex-2-PyP in OTA-induced intracellular ROS accumulation is presented in Fig. 3.9. Using both probes DHE and DHR, the treatment with MnP (5 µM) did not markedly change the intracellular levels of ROS when compared with non-treated control cells for both periods of treatment (Fig. 3.9). In the DHE assay, when cells were simultaneously exposed to OTA (50 µM) and MnP, the intracellular ROS levels significantly decreased when compared with cells exposed only to OTA ($p < 0.01$) for either 30 min or 24 h (Fig. 3.9A). Conversely, in the DHR assay, the MnP did not afford protection against OTA-induced intracellular ROS (Fig. 3.9B).

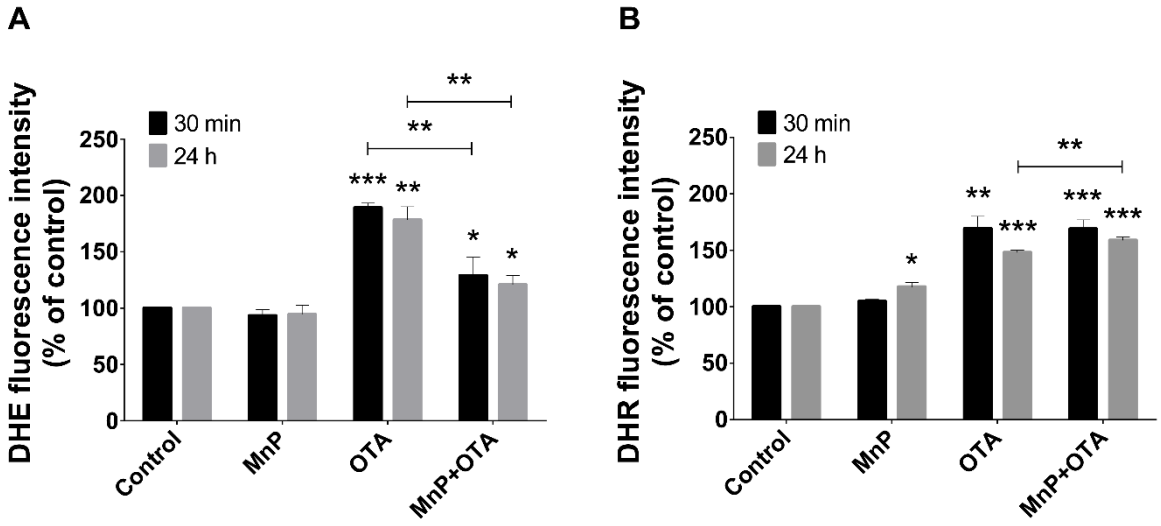

Fig. 3.9 – Effect of MnP on intracellular ROS levels. Intracellular ROS levels were detected in Vero cells exposed for 30 min or 24 h to MnP (5 µM), OTA (50 µM) or both using DHE (A) and DHR (B) fluorescence probes. Values represent mean ± SD (n = 3); *p < 0.05, **p < 0.01 and ***p < 0.001 (Student's t-test).

3.4. Discussion

Although many data have been published regarding the nephrotoxicity of OTA, the exact mechanisms involved, as well as the influence of oxidative stress in the deleterious effects of this mycotoxin, are still unclear.

OTA was shown to inhibit cell growth and induce cell death in different renal cell lines (Costa et al., 2007; Rached et al., 2008; Yang et al., 2014a). In this study, we observed a concentration-and time-dependent cytotoxicity effect in Vero cells exposed to OTA, as evaluated by mechanistically distinct and complementary methodologies. The concentrations used in this work, although above the usual *in vivo* human levels, are in the same order of magnitude of several previous *in vitro* reports that intend to provide mechanistic insights on OTA toxicity. It is also important to mention that all experiments were carried out using culture medium containing FBS, in order to better mimic the *in vivo* conditions of OTA exposure. In fact, OTA has high affinity for serum proteins, with protein binding percentages ≥ 99.9 % (EFSA, 2006). The IC_{50} values obtained herein for a 48 h-exposure period (31.8 µM and 21.9 µM, for the CV and NR assays respectively) are within the concentration range previously described for the same cells and exposure period (9.7–37 µM; Creppy et al., 2004; Bouslimi et al., 2008), although our experimental conditions differ from those already reported in the literature. The assays used revealed different profiles and the CV and the NR assays were the most sensitive to detect OTA cytotoxicity. The LDH leakage assay also revealed toxicity, but only following exposure to the highest OTA concentrations. Such finding can be explained by the mechanism underlying each assay. The CV stains adherent cells nuclei (Fernandes et al., 2010a), while the NR assay is mainly based on the uptake and subsequent lysosomal accumulation of the supravital dye NR by viable cells (Repetto et al., 2008). On the other hand, the LDH leakage assay is based on the measurement of lactate dehydrogenase activity in the extracellular medium (Decker and Lohmann-

Matthes, 1988). The loss of intracellular LDH and its release into the culture medium is an indicator of cell death due to cell membrane damage.

Another aspect of this work is to assess the genotoxicity of OTA in Vero cells. Despite several reports already published regarding the genotoxicity of OTA, only few data are available on these cells (Ramyaa and Padma, 2013). Herein, the genotoxicity of OTA was characterized through the CBMN and the comet assays. In the first case, OTA impacted the normal cell division and increased the MNBN frequency as well as the MN total number in Vero cells. The number of MN observed in control cells is within the range described in previous studies realized in this cell line (Ayed-Boussema et al., 2007; Chen et al., 2009; Dias et al., 2014). Concerning the genotoxicity of OTA, increases in MN frequency were also reported by other authors using different cell models and experimental conditions (Knasmuller et al., 2004; Ali et al., 2011; Follmann et al., 2014). In addition, the increase in the MN frequency observed herein (approximately two fold) is similar to the data previously reported for HepG2 cells exposed to OTA (25 µM; Ehrlich et al., 2002; Fuchs et al., 2008).

In the comet assay, exposure of Vero cells to a non-cytotoxic dose-range of OTA for 24 h raised the level of DNA damage modestly when compared with control cells. The comparison of data from FPG-modified and standard comet assays suggests the involvement of ROS generation and oxidative DNA damage in the formation of OTA-induced DNA breaks. Previous studies in other cell lines have shown conflicting results concerning the induction of DNA damage in standard or FPG-modified comet assay (e.g. Kamp et al., 2005; Ali et al., 2011; Corcuera et al., 2011; Liu et al., 2012; Zheng et al., 2013; Gonzalez-Arias et al., 2014). Despite different treatment protocols and the distinct sensitivities of the cell line used in the various studies, most data have supported the role of ROS-mediated induction of DNA breaks in OTA genotoxicity (Schilter et al., 2005; Zheng et al., 2013; Gonzalez-Arias et al., 2014).

When comparing the two genotoxicity methodologies used, the different sensitivities to detect an effect may rely on the different type of the lesions measurable

by each method. The comet assay allows the identification of primary DNA lesions, such as single- and double-strand breaks, or alkali-labile sites or intermediates of DNA repair that usually arise soon after exposure to genotoxic agents and may be easily repaired by the cells' DNA repair machinery. Micronuclei, however, may be generated through clastogenic or aneugenic events, that are irreversible and persistent. Thus, the results of the micronuclei assay are coherent with the moderate induction of DNA breaks detected by the comet assay. If those breaks are left unrepaired, they may have been converted into chromosome breaks pointing to a clastogenic mode of action. It is important to mention that OTA has been reported as an inducer of DNA adducts (Pfohl-Leszkowicz and Manderville, 2012). Although OTA may also damage actin stress fibres and induce aneugenic effects (Degen et al., 1997; Dopp et al., 1999) that might contribute to the MN formation, the available evidence points to a predominance of clastogenic lesions (Degen et al., 1997; Dopp et al., 1999; Knasmuller et al., 2004).

The data from the comet assay points to a moderate induction of oxidative DNA lesions by OTA. Therefore, we used two complementary probes (DHE and DHR) with reactivity towards different ROS to assess whether OTA alters the intracellular ROS levels. To the best of our knowledge, this is the first study in Vero cells using these fluorescence probes to study OTA effects. The DHE probe is mostly oxidized by superoxide anion (Tarpey et al., 2004; Fernandes et al., 2010b; Debowska et al., 2015). Conversely, DHR is considered unreactive with superoxide anion, but it can be oxidized by several other ROS (Henderson and Chappell, 1993; Fernandes et al., 2010b; Goncalves et al., 2012). Our data show an increase in intracellular ROS levels observed both after a short (30 min) and a long (24 h) treatment with OTA consistent with a slight induction of oxidative DNA lesions and a high cytotoxic effect observed at the same concentrations. Several previous studies that used different techniques, conditions or cell models also detected a ROS increase upon exposure to OTA (Schaaf et al., 2002; Shen et al., 2013; Ramyaa et al., 2014). However, there are also data showing no significant ROS increase (El Golli Bennour et al., 2009). Importantly, the previous

studies were mainly based on dichlorofluorescein probes, which have been criticized for presenting several draw-backs (Kalyanaraman et al., 2012).

Although some reports suggest the involvement of ROS, namely superoxide anion, hydroxyl radical and peroxide, in the mechanisms of toxicity of OTA (Baudrimont et al., 1994, 1997; Schaaf et al., 2002), techniques to identify specific ROS involved are still required. The use of fluorescence probes with different reactivity, along with the experiments using MnTnHex-2-PyP, may shed some light on this issue. In the DHE assay, the fluorescence increase by OTA was significantly decreased by the SODm. Therefore, we anticipate that superoxide anion was generated by this mycotoxin in Vero cells. Conversely, the ROS increase observed for OTA using the DHR probe was not affected by MnTnHex-2-PyP. While disproportionating superoxide anion, this SODm generates H_2O_2. In peroxidase-containing cells, H_2O_2 oxidizes DHR (Henderson and Chappell, 1993; Tarpey et al., 2004). This fact, along with the lack of catalase-like activity of MnPs (Tovmasyan et al., 2015), may justify the lack of protection of MnP in OTA-induced ROS observed with the DHR probe. To characterize the consequences of ROS generation on the toxicity of OTA, cytotoxicity studies were carried out in the presence of three antioxidants with distinct properties. Previous studies using vitamin E in mammalian cells exposed to OTA have shown dissimilar results. While some authors showed protective effects in other cells lines (Fusi et al., 2010; Gayathri et al., 2015), a previous report (El Golli Bennour et al., 2009) corroborates the negative results observed herein for trolox, an hydrophilic analogue of vitamin E. Despite the lesser information available in the literature, our results on ascorbic acid are in agreement with an *in vivo* study in a chick model (Hoehler and Marquardt, 1996). Besides these two classic antioxidants, our approach included the SODm MnTnHex-2-PyP, a catalytic polyfunctional redox-active compound. In addition to the catalysis of $O_2^{\bullet-}$ disproportionation in a SOD-like fashion, MnTnHex-2-PyP is reactive towards redox-active thiols of cellular signaling proteins involved in transcription, such as nuclear factor-kappaB (NF-κB). Oxidation of thiols of NF-κB would result in its inactivation.

Via such action, MnTnHex-2-PyP would reduce secondary oxidative stress and in turn would indirectly suppress levels of ROS or reactive nitrogen species (RNS). Both direct scavenging of ROS/RNS and indirect reduction of their levels via affecting cellular transcription may be involved in the effects observed herein (Tovmasyan et al., 2013; Batinic-Haberle et al., 2015). MnPs with similar properties have shown remarkable effects in experimental models of several diseases, and some of these are currently evaluated in clinical trials (Tovmasyan et al., 2013; Batinic-Haberle et al., 2014, 2015). Besides the potential benefits of MnPs in therapeutics, these compounds are also valuable mechanistic tools and have been frequently used to elucidate the involvement of ROS in pathological and toxicological conditions (Batinic-Haberle et al., 2015). In the present work, MnTnHex-2-PyP rendered some protection to Vero cells, increasing cell viability in a slight but significant way. This may be attributed to the polyfunctional redox-related activity of this compound and its capacity to modulate several redox pathways within cells (Batinic-Haberle et al., 2015). Moreover, it was also previously reported that the cellular activity of SOD and other antioxidant enzymes decrease upon exposure to OTA (Ozcelik et al., 2004; Zheng et al., 2013; Ramyaa et al., 2014; Yang et al., 2014b). The addition of a SODm might have compensated for such imbalance in SOD activity, improving cell viability.

Cytotoxicity and genotoxicity are frequently accompanied by cell cycle arrest and apoptosis. Therefore, we aimed to explore the contribution of OTA-induced ROS at these levels. Depending upon the cell type and OTA exposure conditions, different alterations in cell cycle were previously described, including arrests in G0/G1 (Kumar et al., 2012), S (Yang et al., 2014a) and G2/M phases (Cui et al., 2010; Follmann et al., 2014). Such alterations can be ascribed to DNA damage and subsequent biochemical changes induced by OTA, which preclude the progression through the cell cycle checkpoints (Sancar et al., 2004). In our study, this mycotoxin increased significantly the sub-G1 population, suggesting an increase in apoptosis. The increase in apoptosis of cells exposed to OTA was confirmed by the double staining with Annexin V and PI.

This finding agrees with previous reports that have also observed an increase in apoptosis in OTA-treated cells, using different methodologies (Ramyaa and Padma, 2013; Yang et al., 2014a). To investigate the role of ROS in the abovementioned events, the effects of a combined exposure to MnP and OTA were analyzed. The addition of the MnP modestly counteracted the increase in sub-G1 and Annexin V positive populations observed for OTA. This finding indicates a partial role of oxidative stress in OTA-induced apoptosis.

3.5. Conclusion

In conclusion, we performed an integrated approach to study OTA-induced toxicological effects in Vero cells. Our approach addressed different endpoints aiming to characterize cytotoxicity, genotoxicity and ROS generation by this mycotoxin. Among the different tools used to clarify the impact of oxidative stress on OTA toxicity, we explored the effect of MnTnHex-2-PyP, a useful SODm for mechanistic studies and therapeutic purposes. Our data suggest that mechanisms responsible for OTA deleterious effects include DNA damage and the generation of intracellular ROS. However, reactive species do not seem to play a central role in OTA toxicity to Vero kidney cells.

3.6. References

Ali, R., Mittelstaedt, R.A., Shaddock, J.G., Ding, W., Bhalli, J.A., Khan, Q.M., Heflich, R.H., 2011. Comparative analysis of micronuclei and DNA damage induced by Ochratoxin A in two mammalian cell lines. Mutat Res 723, 58-64.

Arbillaga, L., Azqueta, A., van Delft, J.H., Lopez de Cerain, A., 2007. In vitro gene expression data supporting a DNA non-reactive genotoxic mechanism for ochratoxin A. Toxicol Appl Pharmacol 220, 216-224.

Ayed-Boussema, I., Ouanes, Z., Bacha, H., Abid, S., 2007. Toxicities induced in cultured cells exposed to zearalenone: apoptosis or mutagenesis? J Biochem Mol Toxicol 21, 136-144.

Bandarra, S., Fernandes, A.S., Magro, I., Guerreiro, P.S., Pingarilho, M., Churchwell, M.I., Gil, O.M., Batinic-Haberle, I., Goncalves, S., Rueff, J., *et al*., 2013. Mechanistic insights into the cytotoxicity and genotoxicity induced by glycidamide in human mammary cells. Mutagenesis 28, 721-729.

Batinic-Haberle, I., Spasojevic, I., Stevens, R.D., Hambright, P., Fridovich, I., 2002. Manganese(iii) meso-tetrakis(ortho-N-alkylpyridyl)porphyrins. Synthesis, characterization, and catalysis of O2[radical dot]- dismutation. Journal of the Chemical Society, Dalton Transactions, 2689-2696.

Batinic-Haberle, I., Tovmasyan, A., Roberts, E.R., Vujaskovic, Z., Leong, K.W., Spasojevic, I., 2014. SOD therapeutics: latest insights into their structure-activity relationships and impact on the cellular redox-based signaling pathways. Antioxidants & redox signaling 20, 2372-2415.

Batinic-Haberle, I., Tovmasyan, A., Spasojevic, I., 2015. An educational overview of the chemistry, biochemistry and therapeutic aspects of Mn porphyrins - From superoxide dismutation to HO-driven pathways. Redox Biol 5, 43-65.

Baudrimont, I., Ahouandjivo, R., Creppy, E.E., 1997. Prevention of lipid peroxidation induced by ochratoxin A in Vero cells in culture by several agents. Chem Biol Interact 104, 29-40.

Baudrimont, I., Betbeder, A.M., Gharbi, A., Pfohl-Leszkowicz, A., Dirheimer, G., Creppy, E.E., 1994. Effect of superoxide dismutase and catalase on the nephrotoxicity induced by subchronical administration of ochratoxin A in rats. Toxicology 89, 101-111.

Bouslimi, A., Ouannes, Z., Golli, E.E., Bouaziz, C., Hassen, W., Bacha, H., 2008. Cytotoxicity and oxidative damage in kidney cells exposed to the mycotoxins ochratoxin a and citrinin: individual and combined effects. Toxicol Mech Methods 18, 341-349.

Cavin, C., Delatour, T., Marin-Kuan, M., Fenaille, F., Holzhauser, D., Guignard, G., Bezencon, C., Piguet, D., Parisod, V., Richoz-Payot, J., *et al*., 2009. Ochratoxin A-mediated DNA and protein damage: roles of nitrosative and oxidative stresses. Toxicol Sci 110, 84-94.

Chen, Q., Tang, S., Jin, X., Zou, J., Chen, K., Zhang, T., Xiao, X., 2009. Investigation of the genotoxicity of quinocetone, carbadox and olaquindox in vitro using Vero cells. Food Chem Toxicol 47, 328-334.

Collins, A.R., 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 26, 249-261.

Collins, A.R., 2014. Measuring oxidative damage to DNA and its repair with the comet assay. Biochim Biophys Acta 1840, 794-800.

Corcuera, L.A., Arbillaga, L., Vettorazzi, A., Azqueta, A., Lopez de Cerain, A., 2011. Ochratoxin A reduces aflatoxin B1 induced DNA damage detected by the comet assay in Hep G2 cells. Food Chem Toxicol 49, 2883-2889.

Costa, S., Utan, A., Cervellati, R., Speroni, E., Guerra, M.C., 2007. Catechins: natural free-radical scavengers against ochratoxin A-induced cell damage in a pig kidney cell line (LLC-PK1). Food Chem Toxicol 45, 1910-1917.

Creppy, E.E., Chiarappa, P., Baudrimont, I., Borracci, P., Moukha, S., Carratu, M.R., 2004. Synergistic effects of fumonisin B1 and ochratoxin A: are in vitro cytotoxicity data predictive of in vivo acute toxicity? Toxicology 201, 115-123.

Cui, J., Xing, L., Li, Z., Wu, S., Wang, J., Liu, J., Yan, X., Zhang, X., 2010. Ochratoxin A induces G(2) phase arrest in human gastric epithelium GES-1 cells in vitro. Toxicol Lett 193, 152-158.

Debowska, K., Debski, D., Hardy, M., Jakubowska, M., Kalyanaraman, B., Marcinek, A., Michalski, R., Michalowski, B., Ouari, O., Sikora, A., *et al*., 2015. Toward selective detection of reactive oxygen and nitrogen species with the use of fluorogenic probes - Limitations, progress, and perspectives. Pharmacological reports : PR 67, 756-764.

Decker, T., Lohmann-Matthes, M.L., 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. Journal of immunological methods 115, 61-69.

Degen, G.H., Gerber, M.M., Obrecht-Pflumio, S., Dirheimer, G., 1997. Induction of micronuclei with ochratoxin A in ovine seminal vesicle cell cultures. Arch Toxicol 71, 365-371.

Dias, E., Louro, H., Pinto, M., Santos, T., Antunes, S., Pereira, P., Silva, M.J., 2014. Genotoxicity of microcystin-LR in in vitro and in vivo experimental models. Biomed Res Int 2014, 949521.

Dopp, E., Muller, J., Hahnel, C., Schiffmann, D., 1999. Induction of genotoxic effects and modulation of the intracellular calcium level in syrian hamster embryo (SHE) fibroblasts caused by ochratoxin A. Food Chem Toxicol 37, 713-721.

EFSA, 2006. Opinion Of The Scientific Panel On Contaminants In The Food Chain On A Request From The Commission Related To Ochratoxin A In Food. The EFSA Journal 365, 1-56.

Ehrlich, V., Darroudi, F., Uhl, M., Steinkellner, H., Gann, M., Majer, B.J., Eisenbauer, M., Knasmuller, S., 2002. Genotoxic effects of ochratoxin A in human-derived hepatoma (HepG2) cells. Food Chem Toxicol 40, 1085-1090.

El Golli Bennour, E., Bouaziz, C., Ladjimi, M., Renaud, F., Bacha, H., 2009. Comparative mechanisms of zearalenone and ochratoxin A toxicities on cultured HepG2 cells: is oxidative stress a common process? Environ Toxicol 24, 538-548.

Fenech, M., 2000. The in vitro micronucleus technique. Mutat Res 455, 81-95.

Fenech, M., 2007. Cytokinesis-block micronucleus cytome assay. Nat Protoc 2, 1084-1104.

Fernandes, A.S., Gaspar, J., Cabral, M.F., Rueff, J., Castro, M., Batinic-Haberle, I., Costa, J., Oliveira, N.G., 2010a. Protective role of ortho-substituted Mn(III) N-alkylpyridylporphyrins against the oxidative injury induced by tert-butylhydroperoxide. Free Radic Res 44, 430-440.

Fernandes, A.S., Serejo, J., Gaspar, J., Cabral, F., Bettencourt, A.F., Rueff, J., Castro, M., Costa, J., Oliveira, N.G., 2010b. Oxidative injury in V79 Chinese hamster cells: protective role of the superoxide dismutase mimetic MnTM-4-PyP. Cell Biol Toxicol 26, 91-101.

Follmann, W., Behm, C., Degen, G.H., 2014. Toxicity of the mycotoxin citrinin and its metabolite dihydrocitrinone and of mixtures of citrinin and ochratoxin A in vitro. Arch Toxicol 88, 1097-1107.

Fuchs, S., Sontag, G., Stidl, R., Ehrlich, V., Kundi, M., Knasmuller, S., 2008. Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. Food Chem Toxicol 46, 1398-1407.

Fusi, E., Rebucci, R., Pecorini, C., Campagnoli, A., Pinotti, L., Saccone, F., Cheli, F., Purup, S., Sejrsen, K., Baldi, A., 2010. Alpha-tocopherol counteracts the cytotoxicity induced by ochratoxin a in primary porcine fibroblasts. Toxins (Basel) 2, 1265-1278.

Gayathri, L., Dhivya, R., Dhanasekaran, D., Periasamy, V.S., Alshatwi, A.A., Akbarsha, M.A., 2015. Hepatotoxic effect of ochratoxin A and citrinin, alone and in combination, and protective effect of vitamin E: In vitro study in HepG2 cell. Food Chem Toxicol 83, 151-163.

Gonçalves, S., Fernandes, A.S., Oliveira, N.G., Marques, J., Costa, J., Cabral, M.F., Miranda, J., Cipriano, M., Guerreiro, P.S., Castro, M., 2012. Cytotoxic effects of cadmium in mammary epithelial cells: protective role of the macrocycle [15]pyN5. Food Chem Toxicol 50, 2180-2187.

Gonzalez-Arias, C.A., Benitez-Trinidad, A.B., Sordo, M., Robledo-Marenco, L., Medina-Diaz, I.M., Barron-Vivanco, B.S., Marin, S., Sanchis, V., Ramos, A.J., Rojas-Garcia, A.E., 2014. Low doses of ochratoxin A induce micronucleus formation and delay DNA repair in human lymphocytes. Food Chem Toxicol 74, 249-254.

Guerreiro, P.S., Fernandes, A.S., Costa, J.G., Castro, M., Miranda, J.P., Oliveira, N.G., 2013. Differential effects of methoxyamine on doxorubicin cytotoxicity and genotoxicity in MDA-MB-231 human breast cancer cells. Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 757, 140–147.

Henderson, L.M., Chappell, J.B., 1993. Dihydrorhodamine 123: a fluorescent probe for superoxide generation? Eur J Biochem 217, 973-980.

Hoehler, D., Marquardt, R.R., 1996. Influence of vitamins E and C on the toxic effects of ochratoxin A and T-2 toxin in chicks. Poult Sci 75, 1508-1515.

IARC, 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 56, 489-521.

Kalyanaraman, B., Darley-Usmar, V., Davies, K.J., Dennery, P.A., Forman, H.J., Grisham, M.B., Mann, G.E., Moore, K., Roberts, L.J., 2nd, Ischiropoulos, H., 2012. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. Free Radic Biol Med 52, 1-6.

Kamp, H.G., Eisenbrand, G., Janzowski, C., Kiossev, J., Latendresse, J.R., Schlatter, J., Turesky, R.J., 2005. Ochratoxin A induces oxidative DNA damage in liver and kidney after oral dosing to rats. Mol Nutr Food Res 49, 1160-1167.

Knasmuller, S., Cavin, C., Chakraborty, A., Darroudi, F., Majer, B.J., Huber, W.W., Ehrlich, V.A., 2004. Structurally related mycotoxins ochratoxin A, ochratoxin B, and citrinin differ in their genotoxic activities and in their mode of action in human-derived liver (HepG2) cells: implications for risk assessment. Nutr Cancer 50, 190-197.

Kumar, R., Ansari, K.M., Chaudhari, B.P., Dhawan, A., Dwivedi, P.D., Jain, S.K., Das, M., 2012. Topical application of ochratoxin A causes DNA damage and tumor initiation in mouse skin. PLoS One 7, e47280.

Liu, J., Wang, Y., Cui, J., Xing, L., Shen, H., Wu, S., Lian, H., Wang, J., Yan, X., Zhang, X., 2012. Ochratoxin A induces oxidative DNA damage and G1 phase arrest in human peripheral blood mononuclear cells in vitro. Toxicol Lett 211, 164-171.

Mally, A., 2012. Ochratoxin a and mitotic disruption: mode of action analysis of renal tumor formation by ochratoxin A. Toxicol Sci 127, 315-330.

Marin-Kuan, M., Ehrlich, V., Delatour, T., Cavin, C., Schilter, B., 2011. Evidence for a role of oxidative stress in the carcinogenicity of ochratoxin a. J Toxicol 2011, 645361.

Meki, A.R., Hussein, A.A., 2001. Melatonin reduces oxidative stress induced by ochratoxin A in rat liver and kidney. Comp Biochem Physiol C Toxicol Pharmacol 130, 305-313.

O'Brien, E., Dietrich, D.R., 2005. Ochratoxin A: the continuing enigma. Crit Rev Toxicol 35, 33-60.

Ozcelik, N., Soyoz, M., Kilinc, I., 2004. Effects of ochratoxin a on oxidative damage in rat kidney: protective role of melatonin. J Appl Toxicol 24, 211-215.

Pfohl-Leszkowicz, A., Manderville, R.A., 2007. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. Mol Nutr Food Res 51, 61-99.

Pfohl-Leszkowicz, A., Manderville, R.A., 2012. An update on direct genotoxicity as a molecular mechanism of ochratoxin a carcinogenicity. Chem Res Toxicol 25, 252-262.

Pinto, M., Costa, P.M., Louro, H., Costa, M.H., Lavinha, J., Caeiro, S., Silva, M.J., 2014. Determining oxidative and non-oxidative genotoxic effects driven by estuarine sediment contaminants on a human hepatoma cell line. Sci Total Environ 478, 25-35.

Rached, E., Hoffmann, D., Blumbach, K., Weber, K., Dekant, W., Mally, A., 2008. Evaluation of putative biomarkers of nephrotoxicity after exposure to ochratoxin a in vivo and in vitro. Toxicol Sci 103, 371-381.

Ramyaa, P., Krishnaswamy, R., Padma, V.V., 2014. Quercetin modulates OTA-induced oxidative stress and redox signalling in HepG2 cells - up regulation of Nrf2 expression and down regulation of NF-kappaB and COX-2. Biochim Biophys Acta 1840, 681-692.

Ramyaa, P., Padma, V.V., 2013. Ochratoxin-induced toxicity, oxidative stress and apoptosis ameliorated by quercetin-modulation by Nrf2. Food Chem Toxicol 62, 205-216.

Repetto, G., del Peso, A., Zurita, J.L., 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 3, 1125-1131.

Ringot, D., Chango, A., Schneider, Y.J., Larondelle, Y., 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chem Biol Interact 159, 18-46.

Sancar, A., Lindsey-Boltz, L.A., Unsal-Kacmaz, K., Linn, S., 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 73, 39-85.

Schaaf, G.J., Nijmeijer, S.M., Maas, R.F., Roestenberg, P., de Groene, E.M., Fink-Gremmels, J., 2002. The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. Biochim Biophys Acta 1588, 149-158.

Schilter, B., Marin-Kuan, M., Delatour, T., Nestler, S., Mantle, P., Cavin, C., 2005. Ochratoxin A: potential epigenetic mechanisms of toxicity and carcinogenicity. Food Addit Contam 22 Suppl 1, 88-93.

Shen, X.L., Zhang, Y., Xu, W., Liang, R., Zheng, J., Luo, Y., Wang, Y., Huang, K., 2013. An iTRAQ-based mitoproteomics approach for profiling the nephrotoxicity mechanisms of ochratoxin A in HEK 293 cells. J Proteomics 78, 398-415.

Sorrenti, V., Di Giacomo, C., Acquaviva, R., Barbagallo, I., Bognanno, M., Galvano, F., 2013. Toxicity of ochratoxin a and its modulation by antioxidants: a review. Toxins (Basel) 5, 1742-1766.

Tarpey, M.M., Wink, D.A., Grisham, M.B., 2004. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. Am J Physiol Regul Integr Comp Physiol 286, R431-444.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35, 206-221.

Tovmasyan, A., Maia, C.G., Weitner, T., Carballal, S., Sampaio, R.S., Lieb, D., Ghazaryan, R., Ivanovic-Burmazovic, I., Ferrer-Sueta, G., Radi, R., *et al*., 2015. A comprehensive evaluation of catalase-like activity of different classes of redox-active therapeutics. Free Radic Biol Med 86, 308-321.

Tovmasyan, A., Sheng, H., Weitner, T., Arulpragasam, A., Lu, M., Warner, D.S., Vujaskovic, Z., Spasojevic, I., Batinic-Haberle, I., 2013. Design, mechanism of action, bioavailability and therapeutic effects of mn porphyrin-based redox modulators. Med Princ Pract 22, 103-130.

Vettorazzi, A., van Delft, J., Lopez de Cerain, A., 2013. A review on ochratoxin A transcriptomic studies. Food Chem Toxicol 59, 766-783.

WHO, 2001. Safety evaluation of certain mycotoxins in food. WHO Food Additives Series 47.

Yang, Q., He, X., Li, X., Xu, W., Luo, Y., Yang, X., Wang, Y., Li, Y., Huang, K., 2014a. DNA damage and S phase arrest induced by Ochratoxin A in human embryonic kidney cells (HEK 293). Mutat Res 765, 22-31.

Yang, Q., Shi, L., Huang, K., Xu, W., 2014b. Protective effect of N-acetylcysteine against DNA damage and S-phase arrest induced by ochratoxin A in human embryonic kidney cells (HEK-293). Food Chem Toxicol 70, 40-47.

Zheng, J., Zhang, Y., Xu, W., Luo, Y., Hao, J., Shen, X.L., Yang, X., Li, X., Huang, K., 2013. Zinc protects HepG2 cells against the oxidative damage and DNA damage induced by ochratoxin A. Toxicol Appl Pharmacol 268, 123-131.

Chapter 4

Influence of the SOD mimic MnTnHex-2-PyP on the viability and migration of human renal cancer cells

Abstract

The clear-cell renal carcinoma (ccRCC) is the most common type of renal cancer. It has an important genetic basis, which is closely related with oxidative stress. The importance of superoxide dismutase (SOD) in different pathological conditions led to the development of manganese(III) porphyrins (MnPs). These compounds have the ability to mimic the natural SOD enzymes and to scavenge a plethora of different reactive oxygen species (ROS), modulating the cellular redox status. MnTnHex-2-PyP (MnP) is considered one of the most promising superoxide dismutase mimics (SODm). In this study, the human renal cancer cells 786-O were treated with increasing concentrations of MnP (0.1-25 μM) for different exposure times (12-48 h). The Crystal Violet and MTS assays were used to evaluate the cell viability. The impact of MnP in the cell cycle and cell death was investigated by assessing the cellular DNA content using PI stain in fixed cells. Intracellular ROS were analyzed by flow cytometry using the fluorescence probe dihydrorhodamine 123. The impact of MnP (0.25 μM) in collective cell motility and chemotactic migration was evaluated by the wound-healing and the transwell assays, respectively. MnP exposure resulted in a concentration and time-dependent decrease in cell viability. The exposure to MnP (5 μM) led to a significant increase in sub-G1 population. Moreover, MnP induced a concentration-dependent increase in intracellular ROS, presumably due to the generation of H_2O_2 during the dismutation of $O_2^{\bullet-}$. The MnP did not lead to a reduction in collective cell motility. Nevertheless, the MnP significantly decreased the chemotactic migration of human renal cancer cells. Overall, these results suggest that MnTnHex-2-PyP may have a beneficial impact in reducing renal cancer cells viability and migration and warrant further studies regarding SODm-based therapeutic strategies against human renal cancer.

4.1. Introduction

Renal cell carcinoma (RCC) comprises up to 5 % of all malignant tumors (Block, 2012; Escudier et al., 2016; Koul et al., 2011). Over the past decade, a substantial amount of new information concerning the epidemiology, molecular and immunologic characteristics of RCC as well as novel therapies has appeared. Several studies suggested a genetic basis for renal cell carcinoma (Linehan and Ricketts, 2014; Shuch et al., 2015). Clear-cell renal carcinoma (ccRCC) is the most common type of renal cancer, accounting approximately for 75 % of renal epithelial malignancies (Linehan and Ricketts, 2016; Shuch et al., 2015). The main pathway of ccRCC involves a hypoxic status with the activation of angiogenesis. The majority of sporadic ccRCC is associated with defects in the Von Hippel-Lindau (VHL) tumor suppressor gene (Keefe et al., 2015; Linehan et al., 2009; Linehan and Ricketts, 2016). The loss of VHL function leads to a hypoxia response through hypoxia-inducible factors (HIF), which are unable to be degraded. Under those conditions, the HIF activates the transcription of a variety of genes that include the vascular endothelial growth factor (VEGF) and erythropoietin. Therefore, the loss of VHL is associated with an increase of many factors that regulate angiogenesis, tumorigenesis and metastasis (Keefe et al., 2015; Linehan et al., 2009; Ozcan et al., 2014). The activation of the mammalian/mechanistic target of rapamycin (mTOR), which is positively regulated by phosphatidylinositide 3-kinase (PI3K) - protein kinase B (Akt) pathway is also important in the physiopathology of ccRCC (Keefe et al., 2015; Koul et al., 2011). The mTOR protein has a key function in apoptosis, metabolism, cell growth, migration and tumor proliferation (Koul et al., 2011; Zong et al., 2014).

The reactive oxygen species (ROS) have an important role in initiation, development and progression of cancer (Gius and Spitz, 2006; Valko et al., 2004; Waris and Ahsan, 2006). The loss of VHL in particular contributes to an enhanced oxidative

stress, which is mediated in large part by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) (Block, 2012; Block et al., 2010; Szatrowski and Nathan, 1991). Importantly both the effects induced by HIF and its own regulation are modulated by the cellular redox state (Kinnula and Crapo, 2004). Moreover, the regulation of the PI3K/Akt/mTOR signaling is also redox-sensitive (Block, 2012). As aforementioned in Chapter 1, oxidative stress not only causes direct and irreversible oxidative damage to macromolecules, but also disrupts key redox-dependent signaling processes. The presence of ROS as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), peroxynitrite ($ONOO^-$) and superoxide ($O_2^{\bullet -}$) were described in RCC (Block, 2012). In ccRCC there are also oxidative alteration of lipids, proteins and DNA (Pljesa-Ercegovac et al., 2008; Šverko et al., 2011).

The cellular antioxidant defenses play a crucial role against oxidative stress. The superoxide dismutase enzymes (SOD) are part of these important natural antioxidant defenses. Many studies show a reduction in SOD expression in various types of cancer, when compared to normal tissues, suggesting a tumor suppressor role for this enzyme. Conversely, other studies report an elevation in MnSOD expression in cancer, supporting the progression of tumors to a more aggressive stage (Holley et al., 2014; Kinnula and Crapo, 2004; Robbins and Zhao, 2014; Šverko et al., 2011). These differences are probably related with the H_2O_2 levels and with the levels of MnSOD. Moreover, the MnSOD levels seem to be lower during early stages of cancer and higher at later stages of cancer progression, along with a persistent oxidative stress (Holley et al., 2014; Oberley and Buettner, 1979; Pani et al., 2010; Robbins and Zhao, 2014). In the ccRCC some authors mentioned low expression of SOD enzymes (Oberley et al., 1996; Šverko et al., 2011). However, other authors reported an up-regulation of SOD, although with no increase (Sarto et al., 1999) or with lower enzymatic activity when compared with the adjacent tissues (Robbins and Zhao, 2014; Zhao et al., 2017), probably due to some oxidative alterations that may affect the catalytic activity (Zhao et al., 2017). The MnSOD polymorphism (Ala16Ala), which results in lower activity of

SOD has been associated with an increased susceptibility to develop renal cancer (Atilgan et al., 2014).

The increasing knowledge of the SOD role in physiological and pathological conditions, resulted in the development of synthetic compounds with the capacity to mimic the native enzyme (Tovmasyan et al., 2013). SOD mimics (SODm) are able to catalytically remove $O_2^{\bullet -}$ through a dismutation process and scavenge a wide range of reactive species. The mode of action of SODm was initially considered to be highly specific towards $O_2^{\bullet -}$. Nevertheless, in the last years, with an increase in the knowledge of the cellular oxidative stress processes, SOD mimics have been pointed out as significant redox modulators in different redox-sensitive signaling pathways. Therefore, SODm have the ability to affect proliferation, differentiation and cell death (Batinić-Haberle et al., 2014). As described previously in Chapter 1, manganese(III) porphyrins (MnPs) are a particular group of SODm that have demonstrated a beneficial effect in different pathological conditions related with oxidative stress. Moreover, some of these compounds also presented a large therapeutic potential in cancer therapy as tumor radio- and chemosensitizers, as well as radioprotectors of normal tissue (Tovmasyan et al., 2015b). MnTnHex-2-PyP is considered one of the most promising SODm. Besides the lipophilicity and biocompatibility it has also a good bioavailability. The pharmacokinetic studies revealed an appropriate tissue penetration and retention with a preference to the mitochondria. MnTnHex-2-PyP has a large therapeutic window as demonstrated in *in vivo* studies (Batinić-Haberle et al., 2014).

Despite the promising results of MnPs in different types of cancer, as far as we know, there are not studies focused on renal cancer. Therefore, this work is a first attempt to study the potential benefic effect of a MnP in the treatment of renal cancer.

4.2. Material and methods

4.2.1. Chemicals

RPMI-1640 medium was obtained from ATCC (Manassas, VA, USA). Fetal bovine serum (FBS), phosphate buffered saline (PBS; 0.01 M, pH 7.4), trypsin, penicillin-streptomycin (pen/strep) solution, crystal violet, dimethylsulfoxide (DMSO) and RNAse were obtained from Sigma–Aldrich (St. Louis, MO, USA). The TrypLE™ Express Enzyme solution was obtained from Gibco, Invitrogen (UK). The [3-(4,5--dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H--Tetrazolium] - CellTiter 96® AQueous One Solution Reagent (MTS) was obtained from Promega Corp. (Madison, WI, USA). The ethanol, acetic acid and propidium iodide (PI) were purchased from Merck (Darmstadt, Germany). The dihydrorhodamine 123 (DHR) was acquired from Molecular Probes (Eugene, OR, USA). The 10 mM stock solution of DHR was prepared in DMSO, aliquoted, and stored under nitrogen at –20ºC. The MnTnHex-2-PyP^{5+} was synthetized as described previously (Batinić-Haberle et al., 2002) (charges are omitted for clarity throughout the manuscript).

4.2.2. Cell culture

The human renal cancer cell line 786-O was obtained from ATCC (Manassas, VA, USA). 786-O cells were cultured in RPMI-1640 medium, containing 10 % FBS and 1 % pen/strep. Cells were maintained at 37 ºC, under a humidified air atmosphere containing 5 % of CO_2.

4.2.3. Crystal Violet (CV) staining assay

Cell viability was evaluated with the CV staining assay. Approximately, 3–8 x 10^3 cells were seeded per well of a 96 well plate in culture medium and incubated for 24 h. After that, the culture medium was removed and cells were incubated for 16, 24 or 48 h with MnTnHex-2-PyP (0.1–25 μM) in RPMI-1640 culture medium containing 2 or 10 % FBS. H_2O_2 (10 mM) was used as a positive control. The CV assay was then carried out according to a previously described protocol (Ana S Fernandes et al., 2010; Ana S. Fernandes et al., 2010). Absorbance values for untreated control cells correspond to 100 % cell viability. For this assay two to three independent experiments were carried out. Three to six replicate cultures were used in each independent experiment.

4.2.4. MTS reduction assay

The MTS assay was carried out as a confirmatory assay to evaluate cell viability of MnTnHex-2-PyP treated cells, according to Guerreiro et al., 2016. 786-O cells were seeded at a density of 8 x 10^3 cells per well in 100 μL of culture medium in 96-well plates and incubated for 24 h. Culture medium was removed and cells were treated with MnTnHex-2-PyP (0.25 and 5 μM) for 12 or 24 h in culture medium containing 2 % FBS. Following the drug treatment, the medium was removed, each well was rinsed with PBS and cells were incubated for 2 h with 100 μL of culture medium and 20 μL of MTS substrate, according to the manufacturer's instructions. Absorbance was measured at 492 nm. Absorbance values for untreated control cells correspond to 100 % cell viability. H_2O_2 (10 mM) was used as a positive control. Two independent experiments were performed each one comprising six replicate cultures.

4.2.5. Cell DNA content analysis

The cell DNA content was analyzed according to Guerreiro et al., 2017 and Silva et al., 2016. Approximately 1.5 x 10^5 cells were cultured in 6-well plates for 24 h. Afterwards, cell culture medium was removed and cells were exposed to MnTnHex-2-PyP (0.25 and 5 µM) in RPMI-1640 with 2 % FBS for 24 h. For cell DNA content analysis, cells were harvested using 5 mM EDTA in PBS, washed with cold PBS and fixed with 80 % ethanol. After RNase A-treatment (20 µg/mL) and PI (10 µg/mL) staining, cells were analyzed using a FACSCalibur flow cytometer (BD). Data acquisition and analysis was performed using CellQuest software (BD) and FlowJo (Tree Star, San Carlos, Calif.), respectively. Three independent experiments were performed.

4.2.6. Intracellular ROS evaluation

Cellular ROS analysis was performed using the DHR probe. Approximately 2 x 10^5 cells were cultured in 6-well plates in complete culture medium. After 24 h, the medium was removed and 786-O cells were exposed to MnTnHex-2-PyP (0.25 and 5 µM) for 12 h in RPMI-1640 medium containing 2 % FBS. Following the drug treatment, the medium was removed, cells were rinsed with warm PBS and 786-O cells were detached with TrypLE™ Express Enzyme solution and then were incubated with DHR (10 µM) in FBS-free media for 30 min at 37 °C and 5 % CO_2. The intracellular ROS were analyzed using a FACSCalibur flow cytometer (BD). Data acquisition and analysis was performed using CellQuest software (BD) and FlowJo (Tree Star, San Carlos, Calif.), respectively. The median of DHR fluorescence intensity of approximately 2 x 10^4 cells per condition was used to compare the intracellular ROS levels. H_2O_2 (10 mM) was used as a positive control. Four independent experiments were performed.

4.2.7. In vitro wound-healing assay

The *in vitro* wound-healing assay was optimized according to Guerreiro et al., 2017 and Liang et al., 2007. Briefly, 2 x 10^5 cells were seeded in 24-well plates and cultured in complete medium. After 24 h, the medium was removed and each well was scratched using a 200 µL pipette tip, leaving a gap of approximately 0.8 mm in width. Cells were then rinsed twice with PBS to remove the detached cells and cell debris. Cells were incubated with MnTnHex-2-PyP (0.25 µM) in medium containing 2 % FBS. Wound closure was evaluated with an Olympus CKX41 inverted microscope. Photographs of the same areas of the scratch were taken using a 40 x objective with an Olympus SC20 camera. The scratches width was measured using ImageJ software (National Institutes of Health, Bethesda, MA, USA) at defined time points: 0, 8 and 12 h. At each time point, one picture was taken from the same scratch and three measures were made. Cellular motility was analyzed in relation to the initial distance between the two scratches edges which was considered as 0 % of wound closure. Three independent experiments were performed.

4.2.8. Chemotaxis

The chemotaxis assay was carried out according to Guerreiro et al., 2017. The chemotactic migration of 786-O cells was evaluated in 24-well plates with transwell inserts with transparent PET membranes containing 8-µm pores (BD Falcon, Bedford, MA, USA). Cells (1 x 10^5 cells in 2 % FBS medium) were seeded on the top of the insert, and complete medium was placed in the lower chamber of the culture well. The MnTnHex-2-PyP was added to both chambers, and cultures were incubated for 12 h. In order to distinguish chemotaxis and random individual cell migration, a control experiment was made using the same experimental conditions, but adding 10 % of FBS

to both chambers. After the incubation period, non-migrating cells were gently removed from the upper compartment with a cotton swab. Migrated cells present in the bottom of each membrane were fixed with cold 96 % ethanol and stained with 0.1 % crystal violet in 10 % ethanol. The number of cells was counted in five separate fields by light microscopy using a 10 x objective. The results were expressed as percentages relative to non-treated control cultures. Three independent experiments were performed.

4.2.9. Statistical analyses

Differences in mean values of the results were evaluated by the two-tailed Student's t--test. One-way analysis of variance (ANOVA) followed by Tukey test was performed to compare multiple mean values. All analyses were performed with the SPSS statistical package (version 22, SPSS Inc. Chicago, IL).

4.3. Results

4.3.1. MnTnHex-2-PyP increases intracellular levels of reactive oxygen species in 786-O cells

The production of ROS was analyzed by flow cytometry using the DHR fluorescence probe (Fig. 4.1). We observed a concentration-dependent ROS increase upon exposure to MnTnHex-2-PyP when compared with non-treated controls cells (Fig. 4.1A). For the lowest concentration of MnTnHex-2-PyP (0.25 µM) an increase in fluorescence intensity of approximately 16 % ($p = 0.05$) was observed. A considerably higher fluorescence increase of about 46.5 % ($p < 0.001$) was observed for the highest concentration tested (5 µM). The four independent experiments performed led to coherent results.

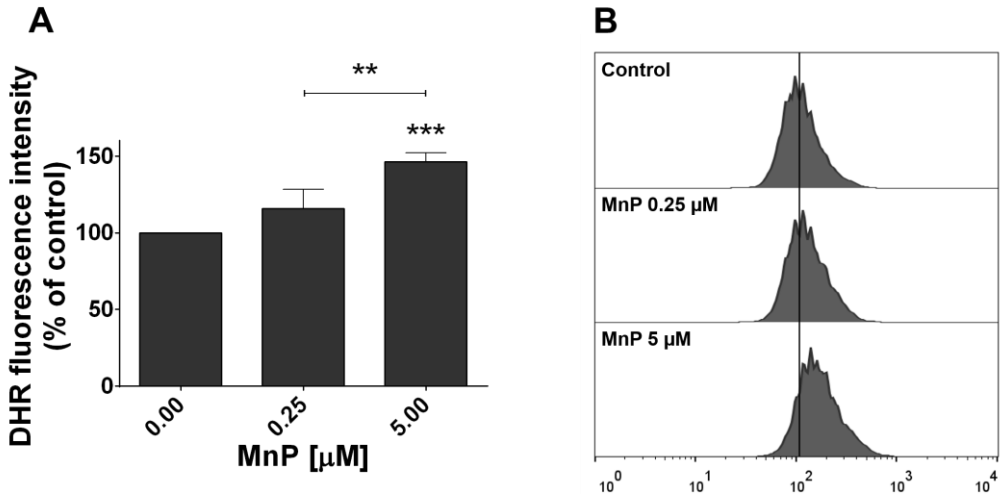

Fig. 4.1 – Exposure to manganese porphyrin (MnP) led to an increase in intracellular ROS in 786-O cells. The intracellular ROS levels after 12 h of exposure were detected by flow cytometry using the DHR probe. (A) Values represent mean ± SD (n = 4); **p < 0.01, ***p < 0.001 (ANOVA test). (B) Representative histograms from one representative assay are shown.

4.3.2. MnTnHex-2-PyP decreases 786-O cells viability

The effect of MnTnHex-2-PyP treatment on cell viability was assessed through different methodologies. The cells were exposed to MnTnHex-2-PyP at different FBS concentrations (2 and 10 %) and at different exposure times (16, 24 and 48 h). Exposure to MnTnHex-2-PyP (0.1–25 μM) induced a decrease in cell viability as assessed by the crystal violet (CV) staining assay (Fig. 4.2A-C). The results from the MTS reduction assay (Fig. 4.2D) revealed comparable results to the CV assay. The metabolic activity of cells exposed to 0.25 μM of MnTnHex-2-PyP (2 % FBS) remained unchanged for both exposure times (12 and 24 h). However, when 786-O cells were exposed to the highest concentration of MnTnHex-2-PyP (5 μM) a time-dependent cytotoxic effect was observed.

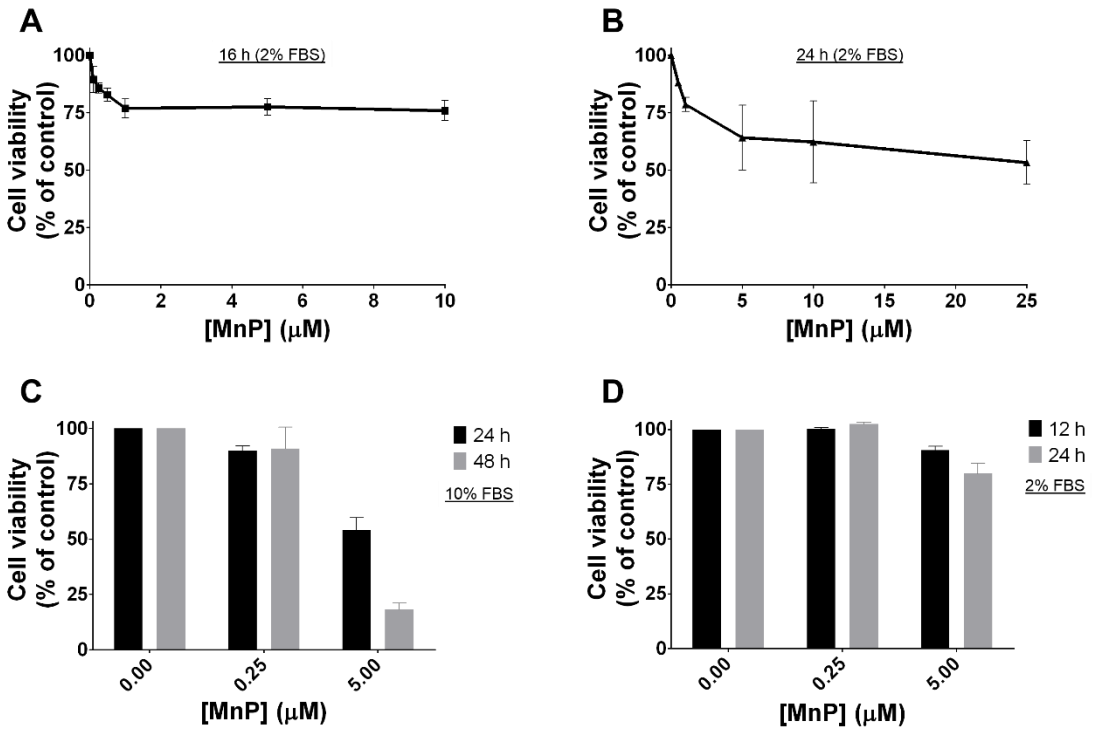

Fig. 4.2 – Cytotoxic effects of MnTnHex-2-PyP (MnP; 0.1-25 μM) in 786-O cells. The cell viability of MnP-exposed cells (12-48 h) was evaluated by CV (A-C) and MTS (D) assays. Values represent mean ± SD (n = 2-3) and are expressed as percentages of the non-treated control cells.

4.3.3. MnTnHex-2-PyP increases the cell death of 786-O cells

The impact of MnTnHex-2-PyP in the cell cycle and cell death of 786-O cells was also investigated by assessing the cellular DNA content using PI stain in fixed cells (Fig. 4.3). The exposure to MnTnHex-2-PyP (5 μM; 24 h) led to a significant increase of 19 % in sub-G1 population when compared with the untreated cells and, consequently decreased the S and G2/M populations (Fig. 4.3B-C). The lowest concentration of MnTnHex-2-PyP (0.25 μM; 24 h) led to a cell cycle distribution similar to that of control cells (Fig. 4.3A-B). All three independent experiments carried out led to coherent results.

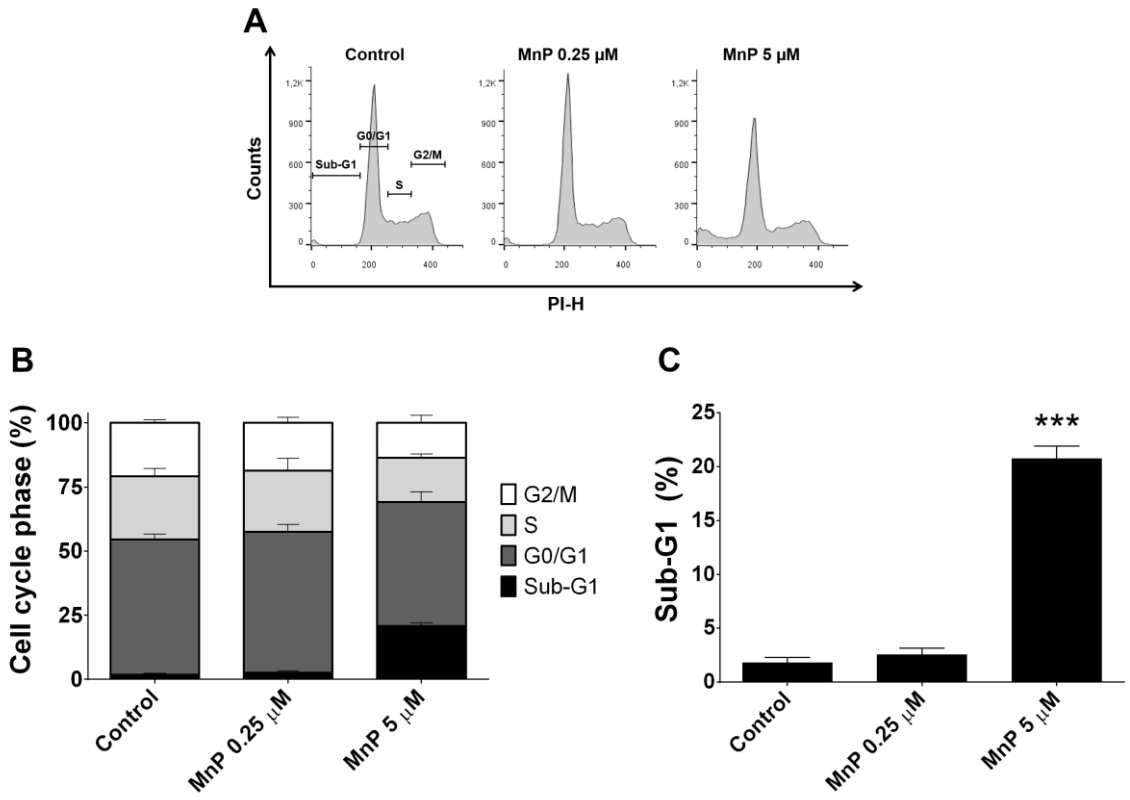

Fig. 4.3 – Effect of manganese porphyrin (MnP) on the cell cycle progression of 786-O cells. Cellular DNA content was analyzed by flow cytometry after 24 h incubation with MnP. (A) representative flow cytometry histograms. (B) sub-G1, G0/G1, S and G2/M populations summary results. (C) sub-G1 population percentage. Values represent mean ± SD (n=3); *** $p < 0.001$ (ANOVA test).

4.3.4. MnTnHex-2-PyP reduces 786-O cells' migration

Collective cell migration was initially assessed by the wound-healing assay. Treatment of 786-O cells with MnTnHex-2-PyP (0.25 µM) did not lead to a reduction in collective cell motility (Fig. 4.4A-B). However, different results were observed in chemotaxis assessed by the transwell assay using FBS as the chemoattractant (Fig. 4.4C-D). The incubation with MnTnHex-2-PyP (0.25 µM) significantly decreased the chemotactic migration to 61.5 % ± 5.4 ($p < 0.001$), when compared with non-treated control cells.

The random individual cell migration was also assessed and no significant decrease was observed for cells exposed to MnTnHex-2-PyP (0.25 µM), when compared with control cells (Fig. 4.4E).

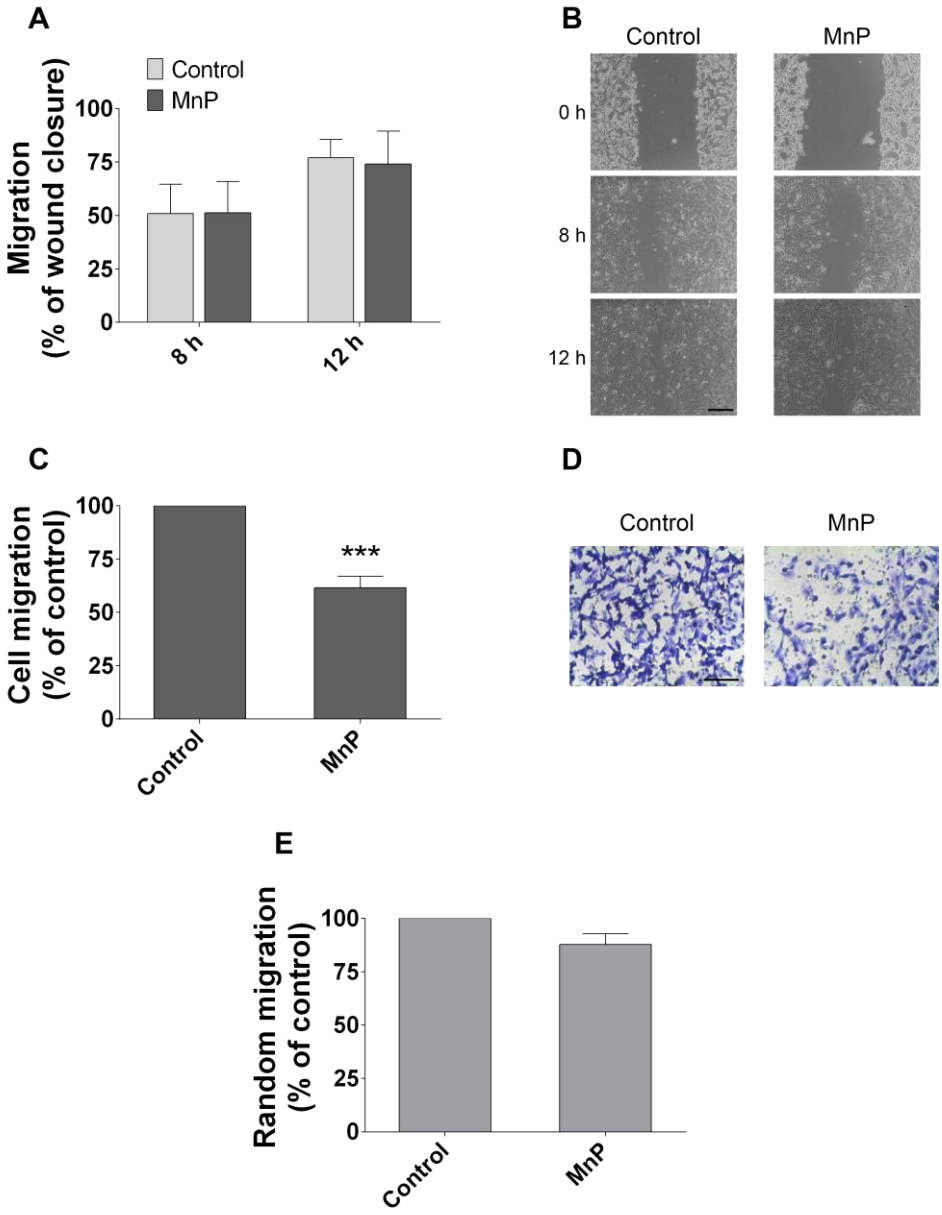

Fig. 4.4 – Effect of MnTnHex-2-PyP treatment (MnP; 0.25 µM) on 786-O cell motility. Collective cell migration was evaluated by the wound-healing assay (A-B) and chemotaxis was measured using a transwell assay (C-D; 12 h). The random individual migration was assessed by transwell assay with no chemoattraction (E; 12h). Representative microscopy images (B, D). Scale bar 200 µM. Values represent mean ± SD (n = 3); *** $p < 0.001$ (Student's t-test).

4.4. Discussion

Although many data have been published regarding the beneficial effects of MnPs in different pathologies, including cancer treatment, its influence in renal cancer was never addressed before.

In order to characterize the influence of MnTnHex-2-PyP on the redox balance of 786-O cells, we used the DHR fluorescence probe. In the present study, a concentration-dependent increase in intracellular ROS levels was observed. While disproportionating superoxide anion, SODm generates H_2O_2 and it is known that in peroxidase-containing cells, H_2O_2 oxidizes DHR (Henderson and Chappell, 1993; Tarpey et al., 2004). Moreover, low activity of enzymes capable to detoxify H_2O_2, such as glutathione peroxidase (Sarto et al., 1999), catalase (Šverko et al., 2011) or both (Pljesa-Ercegovac et al., 2008), was reported in RCC. This evidence, along with the lack of catalase-like activity of MnPs (Tovmasyan et al., 2015a), can justify the ROS increase observed with this fluorescence probe.

MnSOD and MnPs were shown to reduce the cell viability or induce cell death in different *in vitro* cancer models, including in breast cancer (Weydert et al., 2006), skin cancer (Zhao et al., 2005), prostate carcinoma (Li et al., 1998) or colorectal cancer (Behrend et al., 2005). However, this cytotoxic effect in cancer cells is not always present as reported in other studies, including from our group (Fernandes et al., 2013) and may depend on several factors, namely the type of porphyrin, concentration, cancer cell model and cellular redox status. Nevertheless, in the present work, a concentration- and time-dependent cytotoxic effect in 786-O cells exposed to MnTnHex-2-PyP, evaluated by crystal violet and MTS assays was observed. Both assays were carried out using two different FBS concentrations (2 and 10 %). The highest FBS concentration was chosen to better mimic the *in vivo* conditions. Cell viability assays using 2 % FBS were performed in order to select a non-toxic concentration of MnTnHex-2-PyP to be

used in the subsequent experiments, since low FBS medium is more appropriate for cell migration assays. As dying cells poorly migrate, the use of non-cytotoxic concentrations is a requisite when testing cell migration (Fernandes et al., 2015).

Cytotoxicity is commonly accompanied by cell cycle alterations. Therefore, we aimed to explore the involvement of MnTnHex-2-PyP at this level. In our study, the MnTnHex-2-PyP (0.25 μM) showed a cell cycle distribution similar to that of control cells. However, the MnTnHex-2-PyP at the highest concentration increased significantly the sub-G1 population, which is related with DNA fragmentation and cell death. These results are in accordance with the cell viability assays previously discussed.

The MnPs have shown to be associated with a low toxicity potential in *in vitro* studies performed with non-tumor cell lines as well as in *in vivo* studies (Bandarra et al., 2013; Batinić-Haberle et al., 2014). This important feature, has already been reported for MnTnHex-2-PyP (up to 25 μM) in Vero cells, a non-human primate renal cell model (Costa et al., 2015). These differential effects could be attributed to the higher level of intracellular ROS, particularly H_2O_2 in cancer cells when compared with non-tumor cells. Conversely, the cellular antioxidant defenses in cancer cells are generally lower (Batinić-Haberle et al., 2014). Thus, the increase in intracellular levels of H_2O_2 induced by the inherent mechanism of MnPs, along with the inability of cells to detoxify this species, leads to cell death. Our data suggest that when 786-O cells were treated with the lowest concentration of MnTnHex-2-PyP, the increase in H_2O_2 was not high enough to trigger cell death mechanisms. On the other hand, with the concentration of 5 μM of MnTnHex-2-PyP the threshold of toxicity may be reached.

Given their capability to reduce the cellular reactive species levels, MnPs can block some of the RCC pathways related with oxidative stress. Interestingly, MnPs demonstrated also the ability to directly modulate some of the pathophysiological pathways of RCC. MnTnHex-2-PyP has the ability to inactivate the nuclear transcription factor kappa B (NF-κB) (Batinić-Haberle et al., 2014; Sheng et al., 2009),

which leads also to an indirect suppression of ROS or reactive nitrogen species (RNS) levels (Batinic-Haberle et al., 2015; Tovmasyan et al., 2013). Furthermore, it has been proposed that the resistance of RCC to chemotherapy and radiotherapy is due to increased levels of NF-κB activity and resistance to apoptosis (Qi and Ohh, 2003), which emphasize the potential role of MnPs in renal cancer therapy. Moreover, MnPs suppress the HIF-1α pathway and its downstream genes (e.g. VEGF) (Gauter-Fleckenstein et al., 2010) and down-regulate the mTOR signaling pathway. (Delmastro-Greenwood et al., 2013).

RCC is associated with a high potential of metastasis (Raimondo et al., 2013). The formation of metastasis involves several biological mechanisms, including an increase in cell motility, which can be assessed by *in vitro* migration assays. Mechanistically, ROS may participate in various signaling pathways associated with cell migration (Fernandes et al., 2016). In this work, cell migration was evaluated by two distinct methodologies - the wound-healing assay and the chemotaxis assay. The wound-healing assay provides insights into collective cell motility, evaluating the movement of cells across a horizontal surface with the preservation of functional cell-cell junctions (Fernandes et al., 2015; Kramer et al., 2013). Conversely, the chemotaxis assay evaluates the migration of individual cells. This is characterized by an individual movement of cells through a membrane pore, toward a higher serum content (Kramer et al., 2013). In our study, the MnTnHex-2-PyP (0.25 μM) did not lead to a reduction in collective cell motility. Nevertheless, the MnTnHex-2-PyP significantly decreased the chemotactic migration. Despite this unequivocal result we used as a chemotaxis control experiment, an assay with the same percentage of FBS in both chambers. The migration of the MnTnHex-2-PyP treated cells was similar to the migration of the non-treated control cells. This demonstrates that the role of MnTnHex-2-PyP in the reduction of the individual migration is observed only in the presence of chemoattraction and excludes the interference of spontaneous migration in the results herein presented. The effect of the MnTnHex-2-PyP in cell migration could be related with the modulation of HIF and

the mTOR pathways, which influence cell migration (Finlay et al., 2012; Zong et al., 2014). The differences observed in both migration assays can be justified by the different molecular mechanisms, related pathways and extracellular stimulus that influence cell migration. The MnTnHex-2-PyP could act either as a direct migration modulator or indirectly through ROS, which can modulate several migration signaling pathways.

4.5. Conclusion

In conclusion, these very promising results suggest that MnTnHex-2-PyP can have a beneficial impact in reducing renal cancer cells viability and migration. Nevertheless, further studies regarding mechanistic insights into the MnPs mode of action and therapeutic strategies against human renal cancer should be performed.

4.6. References

Atilgan, D., Parlaktas, B.S., Uluocak, N., Kolukcu, E., Erdemir, F., Ozyurt, H., Erkorkmaz, U., 2014. The relationship between ALA16VAL single gene polymorphism and renal cell carcinoma. Adv. Urol. 2014.

Bandarra, S., Fernandes, A.S., Magro, I., Guerreiro, P.S., Pingarilho, M., Churchwell, M.I., Gil, O.M., Batinić-Haberle, I., Gonçalves, S., Rueff, J., *et al*., 2013. Mechanistic insights into the cytotoxicity and genotoxicity induced by glycidamide in human mammary cells. Mutagenesis 28, 721–729.

Batinić-Haberle, I., Spasojević, I., Stevens, R.D., Hambright, P., Fridovich, I., 2002. Manganese(iii) meso-tetrakis(ortho-N-alkylpyridyl)porphyrins. Synthesis, characterization, and catalysis of $O2^{\cdot -}$ dismutation. J. Chem. Soc. Dalt. Trans. 2689.

Batinić-Haberle, I., Tovmasyan, A., Roberts, E.R.H., Vujaskovic, Z., Leong, K.W., Spasojevic, I., 2014. SOD Therapeutics: Latest Insights into Their Structure-Activity Relationships and Impact on the Cellular Redox-Based Signaling Pathways. Antioxid. Redox Signal. 20, 2372–2415.

Batinic-Haberle, I., Tovmasyan, A., Spasojevic, I., 2015. An educational overview of the chemistry, biochemistry and therapeutic aspects of Mn porphyrins - From superoxide dismutation to H2O2-driven pathways. Redox Biol. 5, 43–65.

Behrend, L., Mohr, A., Dick, T., Zwacka, R.M., 2005. Manganese Superoxide Dismutase Induces p53-Dependent Senescence in Colorectal Cancer Cells. Mol. Cell. Biol. 25, 7758–7769.

Block, K., 2012. Oxidative Stress and Redox-Signaling in Renal Cell Cancer, in: Amato, R.J. (Ed.), Emerging Research and Treatments in Renal Cell Carcinoma. InTech.

Block, K., Gorin, Y., New, D.D., Eid, A., Chelmicki, T., Reed, A., Choudhury, G.G., Parekh, D.J., Abboud, H.E., 2010. The NADPH oxidase subunit p22phox inhibits the function of the tumor suppressor protein tuberin. Am. J. Pathol. 176, 2447–2455.

Costa, J.G., Saraiva, N., Guerreiro, P.S., Louro, H., Silva, M.J., Miranda, J.P., Castro, M., Batinic-Haberle, I., Fernandes, A.S., Oliveira, N.G., 2015. Ochratoxin A-induced cytotoxicity, genotoxicity and reactive oxygen species in kidney cells: an integrative approach of complementary endpoints. Food Chem. Toxicol. 87, 65–76.

Delmastro-Greenwood, M.M., Votyakova, T., Goetzman, E., Marre, M.L., Previte, D.M., Tovmasyan, A., Batinic-Haberle, I., Trucco, M.M., Piganelli, J.D., 2013. Mn porphyrin regulation of aerobic glycolysis: Implications on the activation of diabetogenic immune cells. Antioxidants Redox Signal. 19, 1902–1915.

Escudier, B., Porta, C., Schmidinger, M., Rioux-Leclercq, N., Bex, A., Khoo, V., Gruenvald, V., Horwich, A., on behalf of the ESMO Guidelines Committee, 2016. Renal cell carcinoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann. Oncol. 27, v58–v68.

Fernandes, A.S., Flórido, A., Cipriano, M., Batinic-Haberle, I., Miranda, J., Saraiva, N., Guerreiro, P.S., Castro, M., Oliveira, N.G., 2013. Combined effect of the SOD mimic MnTnHex-2-PyP5+ and doxorubicin on the migration and invasiveness of breast cancer cells. Toxicol. Lett. 221, Suppl, S70–S71.

Fernandes, A.S., Flórido, A., Saraiva, N., Cerqueira, S., Ramalhete, S., Cipriano, M., Cabral, M.F., Miranda, J.P., Castro, M., Costa, J., Oliveira, N.G., 2015. Role of the Copper(II) Complex Cu[15]pyN5 in Intracellular ROS and Breast Cancer Cell Motility and Invasion. Chem. Biol. Drug Des. 86, 578–588.

Fernandes, A.S., Gaspar, J., Cabral, M.F., Rueff, J., Castro, M., Batinic-Haberle, I., Costa, J., Oliveira, N.G., 2010. Protective role of ortho-substituted Mn(III) N-alkylpyridylporphyrins against the oxidative injury induced by tert-butylhydroperoxide. Free Radic. Res. 44, 430–440.

Fernandes, A.S., Saraiva, N., Oliveira, N.G., 2016. Redox Therapeutics in Breast Cancer: Role of SOD Mimics, in: Batinic-Haberle, I., Reboucas, J.S., Spasojevic, I. (Eds.), Redox-Active Therapeutics. pp. 451–467.

Fernandes, A.S., Serejo, J., Gaspar, J., Cabral, F., Bettencourt, A.F., Rueff, J., Castro, M., Costa, J., Oliveira, N.G., 2010. Oxidative injury in V79 Chinese hamster cells: Protective role of the superoxide dismutase mimetic MnTM-4-PyP. Cell Biol. Toxicol. 26, 91–101.

Finlay, D.K., Rosenzweig, E., Sinclair, L. V, Feijoo-Carnero, C., Hukelmann, J.L., Rolf, J., Panteleyev, A.A., Okkenhaug, K., Cantrell, D.A., 2012. PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8+ T cells. J. Exp. Med. 209, 2441–2453.

Gauter-Fleckenstein, B., Fleckenstein, K., Owzar, K., Jiang, C., Rebouças, J.S., Batinic-Haberle, I., Vujaskovic, Z., 2010. Early and late administration of MnTE-2-PyP5+ in mitigation and treatment of radiation-induced lung damage. Free Radic. Biol. Med. 48, 1034–1043.

Gius, D., Spitz, D.R., 2006. Redox Signaling in Cancer Biology. Antioxid. Redox Signal. 8, 1249–1252.

Guerreiro, P.S., Corvacho, E., Costa, J.G., Saraiva, N., Fernandes, A.S., Castro, M., Miranda, J.P., Oliveira, N.G., 2017. The APE1 redox inhibitor E3330 reduces collective cell migration of human breast cancer cells and decreases chemoinvasion and colony formation when combined with docetaxel. Chem. Biol. Drug Des, [In press].

Guerreiro, P.S., Estácio, S.G., Antunes, F., Fernandes, A.S., Pinheiro, P.F., Costa, J.G., Castro, M., Miranda, J.P., Guedes, R.C., Oliveira, N.G., 2016. Structure-based virtual screening toward the discovery of novel inhibitors of the DNA repair activity of the human apurinic/apyrimidinic endonuclease 1. Chem. Biol. Drug Des. 88, 915-925.

Henderson, L.M., Chappell, J.B., 1993. Dhydrorhodamine 123: a fluorescent probe for superoxide generation? Eur J Biochem 217, 973–980.

Holley, A.K., Miao, L., St Clair, D.K., St Clair, W.H., 2014. Redox-modulated phenomena and radiation therapy: the central role of superoxide dismutases. Antioxid. Redox Signal. 20, 1567–1589.

Keefe, S.M., Rathmell, W.K., Nathanson, K.L., 2015. Genomic Assessment of Renal Cancer, in: Renal Cell Carcinoma. Springer New York, New York, NY, pp. 39–56.

Kinnula, V.L., Crapo, J.D., 2004. Superoxide dismutases in malignant cells and human tumors. Free Radic. Biol. Med. 36, 718–744.

Koul, H., Huh, J.-S., Rove, K.O., Crompton, L., Koul, S., Meacham, R.B., Kim, F.J., 2011. Molecular aspects of renal cell carcinoma: a review. Am. J. Cancer Res. 1, 240–254.

Kramer, N., Walzl, A., Unger, C., Rosner, M., Krupitza, G., Hengstschläger, M., Dolznig, H., 2013. In vitro cell migration and invasion assays. Mutat. Res. 752, 10–24.

Li, N., Oberley, T.D., Oberley, L.W., Zhong, W., 1998. Overexpression of manganese superoxide dismutase in DU145 human prostate carcinoma cells has multiple effects on cell phenotype. Prostate 35, 221–233.

Liang, C.-C., Park, A.Y., Guan, J.-L., 2007. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat. Protoc. 2, 329–333.

Linehan, W.M., Ricketts, C.J., 2016. Kidney cancer in 2016: RCC — advances in targeted therapeutics and genomics. Nat. Rev. Urol.

Linehan, W.M., Ricketts, C.J., 2014. Decade in review-kidney cancer: discoveries, therapies and opportunities. Nat. Rev. Urol. 11, 614–616.

Linehan, W.M., Rubin, J.S., Bottaro, D.P., 2009. VHL loss of function and its impact on oncogenic signaling networks in clear cell renal cell carcinoma. Int. J. Biochem. Cell Biol. 41, 753–756.

Oberley, L.W., Buettner, G.R., 1979. Role of superoxide dismutase in cancer: a review. Cancer Res 39, 1141–1149.

Oberley, T.D., Sempf, J.M., Oberley, L.W., 1996. Histology and lmmunogold analysis of antioxidant enzymes in common renal cancers. Histol. Histopathol.

Ozcan, A., Krishnan, B., Truong, L., 2014. Renal Tumors, in: McManus, L.M., Mitchell, R.N. (Eds.), Pathobiology of Human Disease. Elsevier, pp. 2869–2899.

Pani, G., Galeotti, T., Chiarugi, P., 2010. Metastasis: Cancer cell's escape from oxidative stress. Cancer Metastasis Rev. 29, 351–378.

Pljesa-Ercegovac, M., Mimic-Oka, J., Dragicevic, D., Savic-Radojevic, A., Opacic, M., Pljesa, S., Radosavljevic, R., Simic, T., 2008. Altered antioxidant capacity in human renal cell carcinoma: role of glutathione associated enzymes. Urol. Oncol. 26, 175–181.

Qi, H., Ohh, M., 2003. The von Hippel-Lindau tumor suppressor protein sensitizes renal cell carcinoma cells to tumor necrosis factor-induced cytotoxicity by suppressing the nuclear factor-kB-dependent antiapoptotic pathway. Cancer Res 63, 7076–7080.

Raimondo, F., Morosi, L., Corbetta, S., Chinello, C., Brambilla, P., Della Mina, P., Villa, A, Albo, G., Battaglia, C., *et al*., 2013. Differential protein profiling of renal cell carcinoma urinary exosomes. Mol. Biosyst. 9, 1220–1233.

Robbins, D., Zhao, Y., 2014. Manganese superoxide dismutase in cancer prevention. Antioxid. Redox Signal. 20, 1628–1645.

Sarto, C., Frutiger, S., Cappellano, F., Sanchez, J.-C., Doro, G., Catanzaro, F., Hughes, G.J., Hochstrasser, D.F., Mocarelli, P., 1999. Modified expression of plasma glutathione peroxidase and manganese superoxide dismutase in human renal cell carcinoma. Electrophoresis 20, 3458–3466.

Sheng, H., Yang, W., Fukuda, S., Tse, H.M., Paschen, W., Johnson, K., Batinic-Haberle, I., Crapo, J.D., Pearlstein, R.D., Piganelli, J., *et al*., 2009. Long-term neuroprotection from a potent redox-modulating metalloporphyrin in the rat. Free Radic. Biol. Med. 47, 917–923.

Shuch, B., Amin, A., Armstrong, A.J., Eble, J.N., Ficarra, V., Lopez-Beltran, A., Martignoni, G., Rini, B.I., Kutikov, A., 2015. Understanding pathologic variants of renal cell carcinoma: Distilling therapeutic opportunities from biologic complexity. Eur. Urol. 67, 85–97.

Silva, C.O., Molpeceres, J., Batanero, B., Fernandes, A.S., Saraiva, N., Costa, J.G., Rijo, P., Figueiredo, I.V., Faísca, P., Reis, C.P., 2016. Functionalized diterpene parvifloron D-loaded hybrid nanoparticles for targeted delivery in melanoma therapy. Ther. Deliv. 7, 521–544.

Šverko, A., Sobočanec, S., Kušić, B., Mačak-Šafranko, Ž., Šarić, A., Leniček, T., Kraus, O., Andrišić, L., Korolija, M., Balog, T., *et al*., 2011. Superoxide dismutase and cytochrome P450 isoenzymes might be associated with higher risk of renal cell carcinoma in male patients. Int. Immunopharmacol. 11, 639–645.

Szatrowski, T.P., Nathan, C.F., 1991. Production of Large Amounts of Hydrogen Peroxide by Human Tumor Cells. Cancer Res. 51, 794–799.

Tarpey, M.M.M., Wink, D. a, Grisham, M.B., 2004. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R431-444.

Tovmasyan, A., Maia, C.G.C., Weitner, T., Carballal, S., Sampaio, R.S., Lieb, D., Ghazaryan, R., Ivanovic-Burmazovic, I., Ferrer-Sueta, G., Radi, R., *et al.*, 2015a. A comprehensive evaluation of catalase-like activity of different classes of redox-active therapeutics. Free Radic. Biol. Med. 86, 308–321.

Tovmasyan, A., Sampaio, R.S., Boss, M.K., Bueno-Janice, J.C., Bader, B.H., Thomas, M., Reboucas, J.S., Orr, M., Chandler, J.D., Go, Y.M., *et al.*, 2015b. Anticancer therapeutic potential of Mn porphyrin/ascorbate system. Free Radic. Biol. Med. 89, 1231–1247.

Tovmasyan, A., Sheng, H., Weitner, T., Arulpragasam, A., Lu, M., Warner, D.S., Vujaskovic, Z., Spasojevic, I., Batinic-Haberle, I., 2013. Design, mechanism of action, bioavailability and therapeutic effects of Mn porphyrin-based redox modulators. Med. Princ. Pract. 22, 103–130.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C.J.C.J., Telser, J., 2004. Role of oxygen radicals in DNA damage and cancer incidence. Mol. Cell. Biochem. 266, 37–56.

Waris, G., Ahsan, H., 2006. Reactive oxygen species: role in the development of cancer and various chronic conditions. J. Carcinog. 5, 14.

Weydert, C.J., Waugh, T.A., Ritchie, J.M., Iyer, K.S., Smith, J.L., Li, L., Spitz, D.R., Oberley, L.W., 2006. Overexpression of manganese or copper-zinc superoxide dismutase inhibits breast cancer growth. Free Radic. Biol. Med. 41, 226–237.

Zhao, Y., Chaiswing, L., Oberley, T.D., Batinic-Haberle, I., St. Clair, W., Epstein, C.J., St. Clair, D., 2005. A mechanism-based antioxidant approach for the reduction of skin carcinogenesis. Cancer Res. 65, 1401–1405.

Zhao, Z., Azadzoi, K., Choi, H.-P., Jing, R., Lu, X., Li, C., Wang, F., Lu, J., Yang, J.-H., 2017. LC-MS/MS Analysis Unravels Deep Oxidation of Manganese Superoxide Dismutase in Kidney Cancer. Int. J. Mol. Sci. 18, 319.

Zong, H., Yin, B., Zhou, H., Cai, D., Ma, B., Xiang, Y., 2014. Inhibition of mTOR pathway attenuates migration and invasion of gallbladder cancer via EMT inhibition. Mol. Biol. Rep. 41, 4507–4512.

Chapter 5

Concluding Remarks

Oxidative stress plays important roles in different disorders and pathologies. Reactive species are present and contribute directly and indirectly to all tumorigenesis stages. The radical $O_2^{\bullet-}$ is one of the most important ROS, which is associated with several pathological conditions, including cancer. Moreover, SOD enzymes are crucial antioxidant defenses and are decreased in some disorders. Therefore, it is essential to comprehend the redox mechanisms of diseases in order to mitigate the deleterious effects associated with oxidative stress in biological systems.

SODm can be useful in the treatment of pathologies related with oxidative stress, including cancer either, alone or combined with other treatments. It is important to note that SODm albeit being polyfunctional antioxidants can also promote pro-oxidant actions. SODm have the ability to protect normal tissue from anticancer treatments, which in general are known to produce ROS. Nonetheless, SODm can act as pro-oxidative agents as well, due to their capacity to generate higher amounts of H_2O_2 in tumor cells, where the basal ROS levels are highest and the antioxidant defenses are severally lower.

In the present work, the selection of a MnP was primarily based on the very high SOD-like activity presented the SODm from this class along with their high stability in physiological conditions. As described in Chapter 1, MnPs have been extensively used with success in various experimental models, as well as in clinical trials. MnPs can be useful mechanistic tools to assess the involvement of oxidative stress in pathological and toxicological conditions. In addition, MnPs are also promising drug candidates for redox-based therapeutic approaches against different pathologies, including cancer. Among the MnPs, the choice of MnTnHex-2-PyP was fully justified by its unique characteristics. This compound is one of the most potent SODm ever synthesized, allowing its use in low concentrations. This MnP is a ROS and RNS scavenger and modulates key redox-based cellular transcription factors. Moreover, MnTnHex-2-PyP is one of the most lipophilic cationic MnPs being preferentially distributed to tumor relatively to non-tumor tissues. Moreover, it accumulates predominantly in mitochondria thus mimicking the mitochondrial SOD (MnSOD). MnTnHex-2-PyP had already demonstrated beneficial

effects in different pathologies and disorders, namely stroke, cerebral palsy or kidney ischemia injuries. In addition, it was used in cancer models as radioprotector and as anticancer agent *per se*, as previously described. Despite all of the preexisting knowledge on this MnP, its effects in kidney cancer have never been explored before. The pharmacokinetic studies of MnTnHex-2-PyP in *in vivo* models showed an oral availability of approximately 20% and a good tissue penetration and retention due to its high lipophilicity. This MnP has a higher bioavailability in the liver, followed by the kidney. The administration of MnTnHex-2-PyP through different routes in mice led to concentrations in the kidney in the micromolar range, which is in accordance with the concentrations generally used in *in vitro* studies, including those herein evaluated. Moreover, this distribution profile suggests that the oral administration of MnTnHex-2-PyP could be a feasible therapeutic approach to treat kidney injuries.

The main objectives of this thesis were focused on the role of SODm in the kidney cancer initiation and progression stages. To achieve this purpose, different experimental models and complementary methodologies to characterize and better understand the mode of action (MOA) of SODm were performed.

The *in vitro* model used to study the initiation stage was the Vero cell line. Vero cells derived from epithelial cells of the African green monkey (non-diseased) that were established in the 1960's by Japanese scientists. Some clones of this cell line, such as Vero E6 cells were then obtained. Vero cells are one of the most common mammalian continuous cell lines used in research, mostly in virology studies, but also in toxicology and pharmacology areas. The *in vitro* assessment of kidney injuries presents some complexity related with the different cell types present in this organ, which have different characteristics and, consequently, distinct toxicological responses. Nevertheless, renal epithelial cell lines present some advantages, such as the preservation of the original functions from kidney *in vivo*. Moreover, the specific location of epithelial cells in the kidney enhances their sensitivity to toxic compounds. Vero-E6 cells in particular, have been used as model for different toxicological assays and proved to be one of the most

sensitive model used in those studies. In the present work, Vero cells also showed to be an efficient cell model to study the protective effects of MnPs, as well as the toxicity induced by OTA.

To mimic the kidney cancer progression stage, 786-O cells were used. 786-O cells are human epithelial cells derived from a primary clear-cell adenocarcinoma from a male patient that have been used as an *in vitro* model of RCC. The clear-cell RCC (ccRCC) is the most common type of RCC and it incidence is higher in men than women. The selection of 786-O cells in this study was therefore based on the clinical and epidemiological data of RCC and also on specific cellular characteristics presented by these cells, namely their inherent invasive characteristics. By using both cellular models Vero and 786-O it was therefore possible to study and compare the effects of the SODm in different stages of kidney cancer.

The range of concentrations of OTA used in this work is in accordance with the concentrations previously evaluated in several toxicological studies. Nevertheless, these concentrations can be considered above those usually quantified in humans, namely in healthy individuals. However, it should be noted that *in vivo* values within the micromolar range have already been detected in some human serum samples and that OTA levels might be affected by different factors (e.g. chronic kidney diseases). The use of higher concentrations than those generally found in a scenario of human exposure is often needed in toxicological mechanistic studies, since in many cases a short term treatment with very low concentrations would not be enough to clearly disturb cell functions. In this regard, our data is relevant fundamentally in a proof of concept basis. Additionally, it is also important to highlight that most experiments carried out herein were performed in culture medium containing 10 % of FBS, which better mimics the *in vivo* conditions. Particularly, the presence of FBS renders the free fraction of OTA in the nanomolar range, much below the total OTA concentration, since toxicokinetics data on OTA indicate a very high percentage of binding to serum proteins ($\geq$ 99.9 %).

To assess the role of SODm in renal cancer initiation and development, different and complementary methodologies were used throughout this thesis. Due to the inherent limitations of each cytotoxicity method and since the assessment of an effect on cell viability may depend on the assay chosen, the use of different endpoints for cytotoxicity evaluation is usually recommended. Therefore, this approach included a battery of four mechanistically different and complementary methodologies: crystal violet (CV), neutral red (NR), LDH leakage and MTS reduction assays. The CV stains adherent cells nuclei, providing information on total cell biomass. The NR is based on the uptake and subsequent lysosomal accumulation of the supravital dye NR by viable cells. The LDH leakage assay is based on the measurement of lactate dehydrogenase activity in the extracellular medium, which is an indicator of cell membrane damage and cell death. Finally, the MTS reduction assay measures the mitochondrial reduction of MTS tetrazolium compound by metabolically active cells. Importantly, all the viability assays showed somehow similar results in each experiment.

The genotoxicity induced by OTA was also assessed through complementary methodologies: the cytokinesis-block micronucleus (CBMN) and the comet assays. The comet assay allows the evaluation of primary DNA lesions, such as single-and double-strand breaks. Additionally, with the FPG modified comet assay, it is possible identify oxidative DNA damage. The micronuclei may be generated through clastogenic or aneugenic events that are markers of irreversible and persistent damage. In the present study, the results of CBMN assay were coherent with the induction of DNA breaks detected by the comet assay.

The evaluation of intracellular ROS was performed using fluorescence microscopy and flow cytometry techniques. Two fluorescence probes were used, DHE and DHR, which have different reactivity towards reactive species. The fluorescence microscopy provides information on cell morphology and viability. Through this methodology it is possible to identify the cellular structures or compartments more responsible for the higher levels of ROS and also to obtain representative cellular images.

The flow cytometry technique is more robust, since it allows to analyze a much higher number of cells than the fluorescence microscopy. Moreover, flow cytometry has more accuracy and has also higher detection sensitivity. In addition, the data acquisition with flow cytometry is faster, avoiding some problems related with the oxidation of the fluorescence probes and, the analysis of the samples is less time-consuming. The background fluorescence is mostly avoided with flow cytometry, while with fluorescence microscopy it is necessary remove it manually for each sample. The use of probes with different reactivities and the wavelengths filters used in the fluorescence microscopy assays provided an indication of RS possible generated by the treatments under study. However, to properly identify the RS involved, more specific techniques should be used such as the identification of the DHE oxidation products by HPLC or the assessment of H_2O_2 using HyPer probe.

The impact of MnP in the cell cycle and cell death was performed through flow cytometry, using PI staining and double staining with PI and Annexin V, respectively. Since cytotoxicity and genotoxicity are frequently accompanied by cell cycle arrest and apoptosis, this approach reinforced the results obtained before.

To study the kidney cancer progression, the cell migration was evaluated through two different migration assays: the wound-healing and the transwell assays. The wound-healing assay evaluates collective cell motility and the chemotaxis assay evaluates the migration of individual cells. The results obtained suggest that the MnP influences the individual cell migration, but not collective migration. These different types of cell migration are not necessarily coupled due to the different molecular mechanisms that are involved in each case. Therefore, pharmacological interventions may show differential impact in the two assays. The chemotaxis of tumour cells is a well-known characteristic that is essential to tumour dissemination during progression and metastasis. The impact of MnP at this level is therefore a significant finding that deserves to be further explored, for example, using an *in vivo* model. Overall, the general approach herein adopted consisting of different and complementary methodologies to properly study each

characteristic, enable us to compare each endpoint and to analyze the global results in order to draw more consistent and robust conclusions.

Cancer is a complex disease associated with multiple factors with an interaction between genetic and environmental factors. The family history of the disease is relevant, and the genetic background of the individual can be determinant, although many other risk factors are crucial to cancer etiology. Environmental factors include tobacco smoke, alcohol consumption, sunlight and ionizing radiation exposure, organic and inorganic chemicals, viruses and bacteria, hormonal factors and diet. Some of these factors may lead to the accumulation of mutations in key genes, such as in tumor suppressor genes, oncogenes, apoptosis genes or DNA repair genes, which contribute to alterations in cell division and proliferation. Humans are daily exposed to various combinations of different carcinogens. Those compounds can be present in water, food, air, houses and personal care products. Taking into account the particular physiological functions of the kidney, as previously described in Chapter 1, this organ has a higher susceptibility to carcinogenic compounds. Among such compounds, the contamination of food and feed by mycotoxins is an important public health problem worldwide. The OTA is one of the most common mycotoxins detected in food that is associated with different disorders in humans and animals. The exposure to OTA has been associated with kidney cancer and also with other kidney dysfunctions, such as chronic kidney disease. The choice of OTA as a renal cancer initiation model was based in its common and global human exposure. Moreover, it was also important to clarify the mode of action of OTA in renal cancer initiation, namely the involvement of oxidative stress. With the present work, was possible to show the presence of oxidative genotoxic damage induced by OTA. However, regarding the global results, the RS do not seem to play a central role in OTA toxicity to Vero kidney cells.

The RCC has different etiological factors that can contribute individually or in combination to carcinogenesis. It is fundamental to continue the study and the evaluation of other potential kidney carcinogen compounds, as well as the existence of other risk factors associated with RCC. In this regard, SODm can constitute a valuable tool since

many of the kidney carcinogens induce oxidative stress that can contribute to their mechanism of toxicity. In addition, the role of SODm in the mitigation of renal genotoxic lesions should also be considered. It is also important to mention that many compounds can act as cancer promotors and influence the cancer progression. Therefore, after the initiation process, the continuous exposure to such compounds can negatively affect the prognosis of the disease. Accordingly, it is vital to perform risk-assessment studies directed to more potential carcinogens and also to different exposure combinations.

The cellular models and the methodologies developed and optimized in the present work can be useful for further studies of other potential nephrocarcinogens. Such experiments could be focused on renal cancer initiation, as well as on renal cancer migration and invasion processes.

SODm can be useful as protective drugs by their antioxidant action in scenarios of occupational or continuous exposure to toxic compounds. Moreover, as already demonstrated in several studies, SODm can be useful in the treatment of oxidative stress-related pathologies, as well as in cancer treatment. More experiments with special focus on molecular and cellular redox pathways (e.g. NF-κB) are needed to understand the mechanisms of action of SODm. In addition, studies evaluating the use of SODm in combination with standard chemotherapy drugs may shed light on the potential benefits of these compounds and will contribute to drive SODm into clinical practice.