Effect of p53 or hMLH1 status on the mechanisms of cytotoxicity of 7-hydroxystaurosporine (UCN-01) or irinotecan (CPT-11) in colorectal cancer cell lines

Dissertation

zur
Erlangung des Doktorgrades des Fachbereichs Biologie, Chemie, Pharmazie der Freien Universität Berlin

Vorgelegt von Roberta Magrini geboren in Castel S.Pietro Terme (BO), Italien

2002
Reviewers:

Prof. Dr. G. Korge

Prof. Dr. C. Hanski

Date of thesis defence: 17.12.2002
Dedicated to the memory of Nonno Umberto, Nonna Eugenia, and Zia Luisa
Table of contents

1. ABBREVIATIONS ........................................................................ 1

2. ACKNOWLEDGMENTS ................................................................ 4

3. SUMMARY ............................................................................. 5

ZUSAMMENFASSUNG .................................................................. 8

4. INTRODUCTION ....................................................................... 11
   4.1 Colorectal cancer .................................................................. 11
   4.2 Cellular responses to chemotherapeutic agents: Apoptosis, cell cycle arrest, and mitotic catastrophe ... 11
      4.2.1 The intrinsic apoptotic pathway ........................................ 12
      4.2.2 Cell cycle arrest .............................................................. 13
      4.2.3 Mitotic catastrophe .......................................................... 17
   4.3 Role of p53 in the cellular response to chemotherapeutic agents ............................................. 17
   4.4 Role of hMLH1 in the cellular response to chemotherapeutic agents ....................................... 19
   4.5 7-Hydroxystaurosporine (UCN-01) ....................................................................................... 20
   4.6 Irinotecan (CPT-11) ................................................................ 24

5. OBJECTIVES OF THIS WORK ................................................. 26

6. MATERIALS AND METHODS .................................................. 27
   6.1 Chemicals, solutions, kits, and instruments ................................................................. 27
      6.1.1 Chemicals ................................................................. 27
      6.1.2 Solutions ................................................................. 29
      6.1.3 Kits .......................................................................... 30
      6.1.4 Instruments ............................................................. 30
   6.2 Molecular biology ................................................................ 33
      6.2.1 Isolation of total RNA ..................................................... 33
      6.2.2 Isolation of polyA⁺ RNA .................................................. 34
      6.2.3 Differential hybridization (Atlas™ Array) ................................................. 35
      6.2.4 Reverse-transcription polymerase chain-reaction (RT-PCR) ................................. 38
      6.2.4.1 cDNA synthesis ....................................................... 39
      6.2.4.2 Polymerase chain reaction (PCR) ........................................ 39
      6.2.4.3 PCR programs .......................................................... 40
      6.2.5 Agarose gel electrophoresis ............................................... 41
### Table of contents

6.3 Cellular biology

6.3.1 Culture of established human colorectal cell lines

6.3.1.1 Maintenance and passaging of the cultured cell lines

6.3.2 Treatment of cultured cells

6.3.3 Assays of cell growth and cytotoxicity

6.3.3.1 Measurement of viable cells by trypan blue exclusion

6.3.3.2 MTT assay

6.3.3.3 Clonogenic assay

6.3.4 Analyses of cell death and apoptosis

6.3.4.1 Measurement of dead cells by trypan blue exclusion

6.3.4.2 Detection of apoptosis: Cell Death Detection ELISA

6.3.4.3 Detection of apoptosis: Flow cytometry

6.3.4.4 Detection of apoptosis: PARP cleavage

6.3.4.5 Detection of apoptosis: DAPI nuclear staining

6.3.4.6 Detection of apoptosis: Immunocytochemistry with M30 CytoDeath antibody

6.3.5 Cell cycle analyses

6.3.5.1 Synchronization of cells in S-phase of the cell cycle

6.3.5.2 Synchronization of cells in mitotic-phase of the cell cycle

6.3.5.3 Mitotic index measurement

6.3.5.4 Abrogation of G2/M-phase arrest

6.3.5.5 Flow cytometric analysis (FACS)

6.4 Protein biochemistry

6.4.1 SDS-polyacrylamide gel electrophoresis of proteins

6.4.2 Western blot

6.4.3 Immunoprecipitation of cdk2 and cdc2/cyclin B1 complexes and determination of their kinase activities

6.4.3.1 Detection of cdk2, p27KIP1, p21CIP1, cyclin B1, and cdc2 in the immunoprecipitates

6.4.4 Inhibition of the MAP kinase pathway

7. RESULTS

7.1 Effect of hMLH1 or p53 status on the mechanism of cytotoxicity of UCN-01 in colorectal cancer cell lines

7.1.1 Effect of hMLH1 status on the cellular response to UCN-01

7.1.1.1 The cytotoxic effect of UCN-01 is different in hMLH1+ or hMLH1- cells

7.1.1.2 UCN-01 induces immediate apoptosis in hMLH1- cells

7.1.1.3 UCN-01 induces a delayed and prolonged apoptosis in hMLH1+ cells

7.1.1.4 UCN-01 induces a stronger G1-phase arrest in hMLH1+ cells than in hMLH1- cells

7.1.1.5 G1-phase arrest in hMLH1+ cells is due to hypophosphorylation of the Rb protein and inhibition of cdk2 kinase activity

7.1.1.6 hMLH1- cells partly escape from G1-phase arrest induced by UCN-01

7.1.1.7 hMLH1+ cells, but not hMLH1- cells, entering the cell cycle, undergo apoptosis

7.1.1.8 Apoptosis induced by UCN-01 is not correlated to changes of expression of Bax and Bcl-2 proteins
7.1.1.9 UCN-01 is inducing MEK2 upregulation in hMLH1+ cell line at the mRNA level. 77
7.1.1.9.1 Inhibition of MEK1/2 kinase activities enhances UCN-01-induced short-term apoptosis in hMLH1- cells. 78
7.1.2 Effect of p53 status on the cellular response to UCN-01. 81
7.1.2.1 p53−/− cells are less susceptible to UCN-01 than p53+/+ cells. 81
7.1.2.2 UCN-01 induces apoptosis in p53+/+ cells but not in p53−/− cells. 81
7.2 Effect of p53 or hMLH1 status on the mechanism of cytotoxicity of CPT-11 in colorectal cancer cell lines. 83
7.2.1 p53 loss or hMLH1 defect affect sensitivity to CPT-11 measured in MTT assay but not in clonogenic assay. 83
7.2.2 Induction of apoptosis occurs mainly in p53−/− cells. 84
7.2.3 p53 protein is necessary for the induction, but not for the maintenance, of CPT-11-induced G2/M-phase arrest. 85
7.2.4 CPT-11-induced G2/M-phase arrest prevents apoptosis in p53+/+ cells. 89
7.2.5 Induction of G2/M-phase arrest is associated with phosphorylation of cdc2 at Tyr-15. 90
7.2.6 Maintenance of G2/M-phase arrest is associated with binding of p21^{CIP1} protein to cdc2/cyclin B1 complex and inhibition of cdc2 kinase activity. 91
7.2.7 CPT-11-induced apoptosis in p53−/− cells is correlated with inhibition of expression of the anti-apoptotic protein Bcl-XL. 92

8. DISCUSSION. 95
8.1 Effect of hMLH1 or p53 status on the mechanism of cytotoxicity of UCN-01 in colorectal cancer cell lines. 95
8.1.1 Mechanism of cytotoxicity of UCN-01 in colon carcinoma cells: Effect of hMLH1 status. 95
8.1.2 Mechanism of cytotoxicity of UCN-01 in colon carcinoma cells: Effect of p53 status. 101
8.2 Effect of p53 or hMLH1 status on the mechanism of cytotoxicity of CPT-11 in colorectal cancer cell lines. 102

9. CONCLUSIONS. 108

10. REFERENCES. 109

11. CURRICULUM VITAE. 129