Integrating the Hallmarks of Cancer to Radiation Biology
Molecular and Clinical Radiobiology Workshop
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ChemoRadiation

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Outline

- Chemoradiation background
- Therapeutic ratio
- Assessment of interaction
- Mechanisms of interactions
- Mechanisms of chemo-RT sensitization
- Clinical trials
- Preclinical example
- Conclusions
ChemoRadiation (CRT)

- RT a major treatment modality for local – locally advanced cancers
- Recurrence still impacts on cure rate and overall survival
- Advances in technology and biology (altered fx, IGRT, IMRT, molecular targeted Rx...) have led to improvement in tumor delineation and targeting resulting in improvement in tumor coverage and reduction in normal tissue (NT) exposure
- Both CRx (systemic) & RT (RT field) are cytotoxic to BOTH Tumor and NT
Therapeutic Ratio

• RT dose to produce maximal probability of tumor control with minimal frequency of complications

• Greater divergence between the curves, the more favorable the TR

• TR = LD50/ED50 or TD50/ED50
Principles

- Combination of Radiotherapy & Chemotherapy is a logical and reasonable approach

- Goal: improvement in locoregional control and/or distant metastasis:
  - local tumor control (RT) and metastatic tumor control (ChemoRx)
  - additive or supra-additive/synergy effect

- Steel & Peckham’ strategies:
  - Spatial cooperation: action of each modality is directed toward different anatomic sites.
  - Independent toxicity: careful selection of CRx to have minimal overlap with RT side effects
  - Enhancement of tumor response: RT sensitizers
  - Protection of normal tissue: Rt protector

- Other
  - NT protection: IGRT, IMRT; RT sensitizers or RT protectors

- Not a new concept
  - 1950’s Heidelberg et al, murine models for 5FU + RT

- Paradigm Shift:
  - 1970’s Nigro et al, neoadjuvant 5FU+MitC then RT, anal canal
  - 2months post, AbdominoPerinealResection (APR)
  - 2/3 complete pathological response
  - 1/3 decline surgery clinically disease free 14 moths post ChemoRx
  - ChemoRx-RT (CRT) clinical trials: CNS, H&N, GI & Gyne
Radiation Modifier

- To consider for CRT
- Understanding of mechanism(s) of action of each modality
- How they may overlap resulting in enhancement of cytotoxicity
- Effective timing to achieve enhancement
Ideal radiation modifier

- **Radiation Sensitizer**
  - acts selectively in Tumor vs NT
  - Adequate concentration within the tumor
  - Makes radiation dose more effective to tumor by:
    - Increase RT-induced damage
    - Increase cytotoxic pathways (Apoptosis)
    - Inhibits RT repair
    - Alter RT
  - Appropriate timing of drug delivery for maximal RT enhancement
- Noncytotoxic
- If cytotoxic: antitumor activity alone
  - L. Herscher et al, Cancer Network, 1999
Ideal radiation modifier

- Radiation Protector
  - Acts selectively in NT
  - Nontoxic
  - Concentration in NT enough to elicit RT modification
  - Renders RT dose less effective to NT
  - Appropriate timing of drug delivery & RT for maximal protection

- L. Herscher et al, Cancer Network, 1999
Assessment of interaction

- Preclinical, *in vitro* & *in vivo*, evaluation to assess antitumor effect and NT toxicity

- Cytotoxic assays: MTT & cell survival curve
  - MTT: colorimetric cell viability assay
  - Cell survival curve:
    - Relationship between cells retaining their reproductive integrity & RT absorbed dose
    - “After RT, cells may still intact and produce proteins, synthesize DNA and even go through 1 or 2 cells divisions. But it has lost the capability to reproduce indefinitely = DEAD” (E. Hall)
    - plotting survival fraction of cells [log. scale] & Rx or RT dose on linear scale
MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Live cells → Mitochondrial reductase present → converts

Absorbance read at 690 nm and subtract background at 570 nm.

MTT test at different concentrations

The low non-cytotoxic dose can be determined.
Cell survival curve

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>No. of Cells Plated</th>
<th>No. of Colonies Counted</th>
<th>Plating Efficiency</th>
<th>Surviving Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>70</td>
<td>0.7</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>72</td>
<td>0.36</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
<td>70</td>
<td>0.12</td>
<td>0.16</td>
</tr>
<tr>
<td>6</td>
<td>1000</td>
<td>8</td>
<td>0.008</td>
<td>0.1</td>
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</table>

Incubate for 12-14 days
Cell survival curve

<table>
<thead>
<tr>
<th>Dose</th>
<th># Cells Plated</th>
<th># Colonies</th>
<th>Mean/X</th>
<th>% PE</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gy</td>
<td>100</td>
<td>82, 78, 80</td>
<td>80</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>2 Gy</td>
<td>200</td>
<td>55, 57, 48</td>
<td>53.3</td>
<td>26.6</td>
<td>33.3</td>
</tr>
<tr>
<td>4 Gy</td>
<td>600</td>
<td>48, 46, 41</td>
<td>45</td>
<td>7.5</td>
<td>9.4</td>
</tr>
<tr>
<td>6 Gy</td>
<td>1000</td>
<td>8, 12, 15</td>
<td>11.6</td>
<td>1.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\[ \text{PE} = \frac{\text{Number of colonies counted}}{\text{Number of cells plated}} \times 100 \]

\[ \text{SF} = \frac{\text{PE of treated sample}}{\text{PE of control}} \times 100 \]
Dose Enhancement Factor (DEF)

- DEF = Dose RT / Dose RT + Rx for same biological effect

- DEF > 1 sensitization

- DEF < 1 protection
Combination Index (CI)

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

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

- Increase of RT initial damage
- Inhibition of Repair
- Cell cycle Redistribution
- Reoxygenation
- Inhibition of Repopulation
Increase of RT initial damage

- DNA key target for RT damage
- Key damage cell death: DSB
- Tumor proliferation, Rx incorporate into DNA:
  - BrdURD/IdUrd
  - Replace normal nucleotide precursor
  - Substituted DNA is more easily broken
Inhibition of Repair

- Rx inhibit repair of SLD
- Halogenated pyrimidines
- Nucleoside analogs: Gemcitabine
Cell Cycle Redistribution

- CRx-RT more effective in proliferating cells
- Sensitive cell cycle position: G2 & M
- Selection of CRx base on their
- Elimination of S-phase cells with nucleoside analogs: Fludarabine, Gemcitabine
Reoxygenation Overcoming Hypoxia

- Sensitivity to radiation increases with oxygen
- Ineffective/defective vasculature resulting in areas of hypoxia
- Poorly or none proliferating
- Tumor < 1mm are fully oxic whereas larger ones develop areas of hypoxia
Reoxygenation
Reoxygenation: OER

OER

Oxygen Enhancement Ratio

Ratio of radiation doses in hypoxic and normoxic conditions to get the same biological effect.

X-rays/γ-Rays: maximum ~ 2.5-3.0

OER is less than 2 in G1 phase.

for High LET radiations (alpha particles)

X-Ray Efficacy: Hypoxic (x 2.5 - 3.0) = Normoxic
Reoxygenation

- Strong clinical correlation between hypoxia and both in-field treatment failure and overall survival

- HNSCC, Cervix, Sarcoma
Inhibition of Repopulation

- In NT as in tumors balance between cell production and cell loss
- RT induces a compensatory cell regeneration mechanisms
  - Accelerated Repopulation
- In NT determines tissue tolerance
- In Tumors that balance in favor of cell production
- Influences local tumor control in SCCA (H7N, Cervix)
- Local control is reduced by ~ 0.5%/ day of overall treatment time prolongation
- Strategy that reduces or eliminates repopulation would improve RT.
<table>
<thead>
<tr>
<th>Strategy</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Sequential chemoradiation</td>
<td>- Least toxic</td>
<td>- Increased treatment time</td>
</tr>
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<td></td>
<td>- Maximizes systemic therapy</td>
<td>- Lack of local control</td>
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<td></td>
<td>- Smaller radiation fields if induction shrink tumor</td>
<td></td>
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<tr>
<td>Concurrent chemoradiation</td>
<td>- Shorter treatment time</td>
<td>- Compromised systemic therapy</td>
</tr>
<tr>
<td></td>
<td>- Radiation enhancement</td>
<td>- Increased toxicity</td>
</tr>
<tr>
<td>Concurrent chemoradiation and</td>
<td>- Maximizes systemic therapy</td>
<td>- No reinduction of tumor</td>
</tr>
<tr>
<td>adjuvant chemotherapy</td>
<td>- Radiation enhancement</td>
<td></td>
</tr>
<tr>
<td>Induction chemotherapy and</td>
<td>- Both local and distant therapy delivered up front</td>
<td>- Increased toxicity</td>
</tr>
<tr>
<td>concurrent chemoradiation</td>
<td>- Maximizes systemic therapy</td>
<td>- Increased treatment time</td>
</tr>
<tr>
<td></td>
<td>- Radiation enhancement</td>
<td>- Difficult to complete chemotherapy after chemoradiation</td>
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<td></td>
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<td></td>
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<tr>
<td>Class of Compound</td>
<td>Mechanism of Radiosensitization</td>
<td>References</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------------------------------------------------------------------------------------------</td>
<td>------------</td>
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<tr>
<td>Platinum-based compounds</td>
<td>Inhibition of DNA synthesis</td>
<td>48-51</td>
</tr>
<tr>
<td></td>
<td>Inhibition of transcription elongation by DNA interstrand cross-links</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibition of repair of radiation-induced DNA damage</td>
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<td>Taxanes</td>
<td>Cellular arrest in the G2/M phase of the cell cycle</td>
<td>20,21,52,53</td>
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<tr>
<td></td>
<td>Induction of apoptosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reoxygenation of tumor cells</td>
<td></td>
</tr>
<tr>
<td>Topoisomerase I inhibitors</td>
<td>Inhibition of repair of radiation-induced DNA strand breaks</td>
<td>54-56</td>
</tr>
<tr>
<td></td>
<td>Redistribution into G2 phase of the cell cycle</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Conversion of radiation-induced single-strand breaks into double-strand breaks</td>
<td></td>
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<tr>
<td>Hypoxic cell cytotoxins</td>
<td>Complementary cytotoxicity with radiation on euoxic and hypoxic tumor cells</td>
<td>58,59</td>
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<tr>
<td>Antimetabolites</td>
<td>Nucleotide pool perturbation</td>
<td>13,14,60,61</td>
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<tr>
<td></td>
<td>Lowering apoptotic threshold</td>
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<td></td>
<td>Cell cycle redistribution</td>
<td></td>
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<tr>
<td></td>
<td>Tumor cell reoxygenation</td>
<td></td>
</tr>
<tr>
<td>Temozolomide</td>
<td>DNA repair inhibition (radiosensitization effect may be subject to MGMT status)</td>
<td>62,63</td>
</tr>
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</table>
Clinical trials

<table>
<thead>
<tr>
<th>Disease Site</th>
<th>Induction</th>
<th>Concomitant</th>
<th>Adjuvant</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain glioblastoma</td>
<td>± (level 1)</td>
<td>++ (level 1)</td>
<td>+ (level 2)</td>
<td>Scupp et al. (2005), Budach et al. (2006), Pignon et al. (2000), Forastière et al. (2003), Cooper et al. (2004), Bemiller et al. (2004)</td>
</tr>
<tr>
<td>Head and neck SCC</td>
<td>± (level 1)</td>
<td>++ (level 1)</td>
<td>- (level 2)</td>
<td>Powell and O’Rourke (2004)</td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>+++ (level 1)</td>
<td>++++ (level 1)</td>
<td>+ (level 1)</td>
<td>Vilong (2006), Bossert et al. (2005a, b), Wolmark et al. (2000), Bartelink et al. (1992)</td>
</tr>
<tr>
<td>Cancer of uterine cervix</td>
<td>± (level 1)</td>
<td>+++ (level 1)</td>
<td>-</td>
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<tr>
<td>Oesophageal carcinoma</td>
<td>-</td>
<td>+++ (level 1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rectal carcinoma</td>
<td>-</td>
<td>+++ (level 2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Anal carcinoma</td>
<td>-</td>
<td>+++ (level 2)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Level of evidence: Level 1, multiple randomized studies/meta-analysis; Level 2, one or two randomized studies, requiring further confirmation.
## Clinical trials

<table>
<thead>
<tr>
<th>Study</th>
<th>RT LCR</th>
<th>CRT LCR</th>
<th>RT OS</th>
<th>CRT OS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EORTC 22931</strong></td>
<td>69%</td>
<td>82%</td>
<td>40%</td>
<td>53%</td>
</tr>
<tr>
<td><strong>H&amp;N</strong></td>
<td>34%</td>
<td>47%</td>
<td>45%</td>
<td>55%</td>
</tr>
<tr>
<td><strong>EORTC-NCIC (RT+/- TMZ)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBM</td>
<td></td>
<td></td>
<td>10%</td>
<td>28%</td>
</tr>
<tr>
<td><strong>GOG109 (RT+/-CPPD-5FU)</strong></td>
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<tr>
<td>CERVIX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EORTC RT+/-MMC-5FU A.Canal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- **RT** represents Radiotherapy.
- **CRT** represents Chemoradiotherapy.
- **LCR** represents Local Control Rate.
- **OS** represents Overall Survival.
- **PFS** represents Progression-Free Survival.
Clinical trials

- DAHANCA trial: RT+/− Nimorazole
  - LRC: 33% vs 49%; P=.002
  - OS no benefit

- Vienna trial: (cf)RT vs (hfx)RT MMC
  - LRC: 31% vs 32% vs 48%; P=.05 and .03
  - OS: 24% vs 31% vs 41%; P=.03
  - Confluent Mucositis: 33% vs 90%

- HeadStart: RT +CPPD vs RT+CPPD/Tirapazamine
  - 2 y OS 65.7% vs 66.2%
  - No change FFS, TLRF, QOL
  - Follow-up trial closed early due to toxicity in exp. Arm

- Biological modifier: RT+/− Cetuximab
  - 5 y OS 36.4% vs 45.6%; P=.018
Limitations: late effects

<table>
<thead>
<tr>
<th>Agent</th>
<th>Toxicity</th>
<th>Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleomycin</td>
<td>Pneumonitis/pulmonary fibrosis</td>
<td>Undefined but related to total drug dose and effects on pulmonary macrophages, type 1 and 2 alveolar cells; effects/laterality exacerbated by the administration of radiation.</td>
<td>369-371</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>Hepatopathy</td>
<td>Altered liver function postirradiation leads to decreased metabolism of agents, including actinomycin, which in turn worsens the hepatopathy.</td>
<td>572,575</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Cardiomyopathy</td>
<td>There is an additive interaction between doxorubicin and radiation with potential; radiation effects occurring. Primary radiation effect is on the endothelial cell, whereas doxorubicin affects the connective tissue stroma of the myocardium.</td>
<td>374-377</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Leukemophthalopathy</td>
<td>Methotrexate may cause this syndrome on its own. Radiation effects on the blood-brain barrier and the choroid plexus can alter methotrexate clearance, leading to higher levels in the brain. Effects are increased when both modalities are used.</td>
<td>576-580</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Sensorineural hearing loss</td>
<td>While cisplatin alone can cause this, reports suggest radiation therapy concurrently with cisplatin may contribute to sensorineural hearing loss.</td>
<td>381-384</td>
</tr>
</tbody>
</table>
Preclinical development of CRT
Outline

- Introduction
  - Radiation therapy
  - MAPK/AKT pathway
  - Hypothesis

- Methods and results
  - *In vitro: (MDA-MB-468 and 4T1 cells) CA, FACS, IF, WB, Comet assay*
  - *In vivo: syngeneic mouse model*

- Conclusions
- Acknowledgments
Radiation Therapy

- 75% of cancer patients receive radiotherapy sometime during the course of their disease.

- Accelerated repopulation after exposure to ionizing radiation appears to be partly responsible for radio-resistance in cancer.
MAPK/PI3K/AKT Signaling Pathway

Ligand → TKR → PI3K → AKT

PI3K

RAS

RAF → MEK → ERK1/2

AKT

BAD

Caspase 9

Caspase 3

Apoptosis

Survival

Serine136

Serine112

P

P

P
EGFR Over-expression

- Known to be responsible for reduced sensitivity to chemotherapy and resistance to ionizing radiation.

- Often expressed at high levels in human malignancies such as breast (40%) and prostate carcinoma (32%)*.

*: Modern Pathology (2010) 23, 703–712; doi:10.1038/modpathol.2010.45; published online 5 March 2010
TK Inhibitors and Radiation Therapy

- Cytostatic agents can significantly increase the efficiency of radiation.

• Bonner et al, The new England Journal of Medicine, 2006
Combi-Molecule “ZRBA1”

- An EGFR TK inhibitor that targets specifically EGFR and HER2.

- ZRBA1 is a novel molecule that not only blocks EGFR (ATP site of TK) signaling but also induces cell killing by damaging DNA.
Our Hypothesis

ZRBA1 and radiation may increase the levels of DNA damage while the MAPK/PI3K pathway is downregulated through EGFR inhibition.

Increase the potency of treatment
Interaction of Radiation and ZRBA1
Colony survival

MDA-MB-468  4T1
Interaction of ionizing radiation and ZRBA1, a mixed EGFR/DNA-targeting molecule.

Heravi, Mitra; Rachid, Zakaria; Goudarzi, Atta; Schlisser, Ava; Jean-Claude, Bertrand; Radzioch, Danuta; Muanza, Thierry

Anti-Cancer Drugs. 20(8):659-667, September 2009. DOI: 10.1097/CAD.0b013e32832cb8bc

Fig. 3  Sequences of administration of ZRBA1 and radiation in the various combinations. (a) MDA-MB-468 cells were treated with 36 [μ]mol/l of ZRBA1 for 2 h and were irradiated 24 h later. They were further incubated for 72 h. (b) Cells were irradiated at 4 Gy of radiation. Twenty-four hours later they were treated with 36 [μ]mol/l of ZRBA1 for 2 h, and further incubated for 72 h. (c) Cells were treated with 36 [μ]mol/l of ZRBA1 for 2 h and were irradiated immediately after the drug had been washed out.
Interaction of ionizing radiation and ZRBA1, a mixed EGFR/DNA-targeting molecule.

Heravi, Mitra; Rachid, Zakaria; Goudarzi, Atta; Schlisser, Ava; Jean-Claude, Bertrand; Radzioch, Danuta; Muanza, Thierry

Anti-Cancer Drugs. 20(8):659-667, September 2009. DOI: 10.1097/CAD.0b013e32832cb8bc

Fig. 4 Comparison of cell survivals after exposure to ZRBA1 and radiation according to the sequences as determined by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Fig. 3a-c). MDA-MB-468 cells were treated with ZRBA1 (36 [μmol/l]) for 2 h with or without radiation (4 Gy). (Data are represented as mean and SEM of three independent experiments, *P<0.001).
Fig. 6

Interaction of ionizing radiation and ZRBA1, a mixed EGFR/DNA-targeting molecule.
Heravi, Mitra; Rachid, Zakaria; Goudarzi, Atta; Schlisser, Ava; Jean-Claude, Bertrand; Radzioch, Danuta; Muanza, Thierry
DOI: 10.1097/CAD.0b013e32832cb8bc

Fig. 6  Cell cycle analysis of MDA-MB-468 cells after exposure to ZRBA1 or radiation and corresponding combination. (a) Cell distribution in G1, S, and G2/M. Cells were treated with ZRBA1 (25 [μmol/l]) alone and in combination with radiation (4 Gy), and cell cycle was analyzed by flow cytometry 24 h later. Data are represented as means and SEM of three independent experiments. (b) A representative histogram showing the G2/M arrest in combined treatment.
**Fig. 7**

Interaction of ionizing radiation and ZRBA1, a mixed EGFR/DNA-targeting molecule.
Heravi, Mitra; Rachid, Zakaria; Goudarzi, Atta; Schlisser, Ava; Jean-Claude, Bertrand; Radzioch, Danuta; Muanza, Thierry

DOI: 10.1097/CAD.0b013e32832cb8bc

Fig. 7 Double-strand breaks induced by FD105, Iressa, ZRBA1 or radiation, and corresponding combinations as determined by a neutral comet assay. Cells were treated with drugs (36 [μmol/l]) for 2 h, irradiated (4 Gy), and analyzed by microelectrophoresis as described in the Materials and methods. Data are represented as means and SEM of three independent experiments.
Fig. 8

Interaction of ionizing radiation and ZRBA1, a mixed EGFR/DNA-targeting molecule.
Heravi, Mitra; Rachid, Zakaria; Goudarzi, Atta; Schlisser, Ava; Jean-Claude, Bertrand; Radzioch, Danuta; Muanza, Thierry

DOI: 10.1097/CAD.0b013e32832cb8bc

Fig. 8 Time course analysis of apoptosis induced by ZRBA1 or radiation and corresponding combination in MDA-MB-468. Cells were treated with ZRBA1 (50 μmol/l) alone and in combination with radiation (4 Gy) and were harvested at the indicated time points. There are two peaks of apoptosis at 6 and 48 h post-treatment. Presented data are means and SEM of three independent experiments (at 6 h post-treatment: control vs. ZRBA1, PP>0.05; control vs. radiation and ZRBA1, PP>0.05. At 48 h post-treatment: control vs. ZRBA1, PPPP>0.05).
<table>
<thead>
<tr>
<th></th>
<th>RT</th>
<th>+</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
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<tbody>
<tr>
<td>ZRBA1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5 µM</td>
<td>10 µM</td>
<td>-</td>
<td>-</td>
<td>5 µM</td>
<td>10 µM</td>
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**EGFR Inhibitory Activity**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>ZRBA1</td>
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<td>-</td>
<td>-</td>
<td>5 µM</td>
<td>10 µM</td>
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<td>-</td>
<td>5 µM</td>
<td>10 µM</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Referenced Proteins and Phosphorylation Levels:**

- **p-Tyr EGFR**
- **p-Erk1/2**
- **Erk1/2**
- **p-Bad (112)**
- **T-Bad**
- **Tubulin**
- **p-Akt**
- **T-Akt**
- **Tubulin**
Fig. 3. Analysis of DNA damage induced by ZRBA1, radiation (XRT), or both. Cells were treated and analyzed by microelectrophoresis as described in Methods and Materials. Double strand breaks induction or repair determined by neutral comet assays in MDA-MB-468 (a) and 4T1 (b) cells. (C, D) Detection of single strand breaks and alkali labile sites determined by alkaline comet assays in MDA-MB-468 (c) and 4T1 (d) cells. Data are means and standard deviations of 3 independent experiments.
DNA Damage & Repair

γH2AX-IF staining

Control  γH2AX-IF staining  ZRBA1

Radiation (0.5 Gy)  Combination

γH2AX- FACS analysis
Cell Cycle Analysis

- The left graph shows the percent of G2/M cells across different treatments: control, ZRRA1, radiation, and ZRRA1 and radiation. The significance levels are indicated as p=0.0251 and p=0.046.

- The right graph depicts the percent of G1 cells under the same treatments, with no significant differences indicated.
Fig. 4. DNA double-strand breaks repair analysis. (a) Flow cytometric analysis of level of phosphorylated ATM (Ser1981), H2AX (Ser193), and DNA-PKcs (ser2056) in MDA-MB-468 cells. Fluorescence intensity indicates the relative amount of phosphorylation of proteins 1 hour and 24 hours after treatment. (b) Distribution of ATM (Ser1981), H2AX (Ser193), and DNA-PKcs (ser2056) throughout the cell cycle 24 hours after treatment. (c) Analysis of the same cells by Western blot to determine levels of BRCA1, BRCA2, and Rad51 proteins.
Tumour Growth Delay Assay

4T1 cells
BALB/c mice

*: P = 0.0131
+: P = 0.0461
Tumor Response

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Growth delay (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.5</td>
</tr>
<tr>
<td>Iressa</td>
<td>13</td>
</tr>
<tr>
<td>ZRBA1</td>
<td>14</td>
</tr>
<tr>
<td>Radiation</td>
<td>26</td>
</tr>
<tr>
<td>Radiation + Iressa</td>
<td>27.5</td>
</tr>
<tr>
<td>Radiation + ZRBA1</td>
<td>47</td>
</tr>
</tbody>
</table>

- Tumour growth delay is expressed as number of days that tumour required to grow triple in volume.

- Tumour-free rate is expressed as the fraction of the number of the mice without the tumor at 84 days after the treatment out of the total number of mice with the tumor before the treatment in each group.
Conclusions

- ZRBA1 potentiates the radiation response in a breast cancer model *In vitro* and *In vivo*.

- The higher potency of this combination is due to increased DNA damage, delayed DNA damage repair process and cell cycle arrest as well as to down-regulated MAPK pathway through EGFR inhibition and inducing cell death.
**Purpose:** ZRBA1 is a combi-molecule designed to induce DNA alkylation lesions and to block epidermal growth factor receptor (EGFR) TK domain. Inasmuch as ZRBA1 downregulates the EGFR TK-mediated antisurvival signaling and induces DNA damage, we postulated that it might be a radiosensitizer. The aim of this study was to further investigate the potentiating effect of ZRBA1 in combination with radiation and to elucidate the possible mechanisms of interaction between these 2 treatment modalities.

**Methods and Materials:** The triple negative human breast MDA-MB-468 cancer cell line and mouse mammary cancer 4T1 cell line were used in this study. Clonogenic assay, Western blot analysis, and DNA damage analysis were performed at multiple time points after treatment. To confirm our in vitro findings, in vivo tumor growth delay assay was performed.

**Results:** Our results show that a combination of ZRBA1 and radiation increases the radiation sensitivity of both cell lines significantly with a dose enhancement factor of 1.56, induces significant numbers of DNA strand breaks, prolongs higher DNA damage up to 24 hours after treatment, and significantly increases tumor growth delay in a syngeneic mouse model.

**Conclusions:** Our data suggest that the higher efficacy of this combination could be partially due to increased DNA damage and delayed DNA repair process and to the inhibition of EGFR. The encouraging results of this combination demonstrated a significant improvement in treatment efficiency and therefore could be applicable in early clinical trial settings. © 2015 Elsevier Inc. All rights reserved.
Conclusions

- The combination of chemotherapy and radiotherapy has become a standard of care for many locally advanced solid tumors.

- It improved local control and in some settings overall survival.

- ... still room for improvement.

- New agents, molecular targeted have potential to further improve the TR.

- Preclinical studies: biological rational, better design of trials: patients selections, biomarkers of tumor and normal tissue response.
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Thank you!

Questions?