Fluorothymidine as a therapeutic response marker of the investigational anticancer agent RAF265: Insights from $^{19}$F-NMR and flow cytometry

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Introduction: The purine analogue 3'-deoxy-3'-fluorothymidine (FLT) was originally developed as an antiviral agent because of its inhibition of DNA synthesis[1]. It is now used in its isotopic $^{19}$F form as a PET imaging marker of cellular proliferation and is advocated as an early indicator of therapeutic response[2]. The relationship between imaging FLT signal changes and underlying cellular processes however, remains limited. The aim of this study was to correlate changes in the cellular content of $^{19}$FLT in A375M cells treated with the investigational anticancer agent RAF265 (Novartis Pharmaceutical Corporation, NJ, USA) using $^{19}$F-NMR and flow cytometry (FC).

Materials and Methods

Cell culture and flow cytometry A375M cells, (melanoma cell line) were grown in Eagle’s Minimal Essential Media (ATCC) with 10% HI FCS (Hyclone), penicillin 100U/ml and streptomycin 100μg/ml (Invitrogen). Cells were grown in either 1:1000 DMSO (control) or 1μM solution of the experimental drug RAF265. All experiments were performed in triplicate including one parallel experiment under identical conditions for Annexin V and cell cycle analysis. After 24 hours, 1.5 mM FLT (Sigma Aldrich, St.Louis, MO) was added to the culture for 2 hours following which an immediate perchloric acid extraction was performed on trypsinized cells. The lysate was collected, neutralized with KOH and frozen at -80°C for subsequent NMR analysis. FC measurements were performed on a B-D Canto II (BD Biosciences, San Jose, CA) with Diva software (BD Biosciences) and analyzed with FlowJo (TreeStar Inc., Ashland, OR) Analysis software with Dean-Jett-Fox cell cycle modeling.

$^{19}$F-NMR- All experiments were performed at room temperature on a Bruker 300 MHz DPX spectrometer equipped with a QNP probe. All samples were doped with D$_2$O to provide a spin-lock and 5-fluorouracil (Sigma Aldrich, St. Louis MO) as the internal $^{19}$F reference. $^{1}$H-decoupled experiments were run using 4000 transients and a TD of 64k. The pH of the solutions remained constant. $^{1}$H-decoupled spectra of $^{19}$FLT in aqueous solution were obtained under identical experimental conditions. LC-MS experiments were performed on samples using a Waters LC-MS system with an ACQUITY UPLC and a LCT Premier TOF mass spectrometer. MS experiments were performed on samples using a Waters LC-MS system with an ACQUITY UPLC and a LCT Premier TOF mass spectrometer.

Results: Flow cytometry (table 1) indicated an 11% and 38% decrease in the proportion of cells in the S, G2/M phase and a 22% decrease in the proportion of cells in combined S+G2/M phase. There was a 15% increase in the proportion of cells in the G0/G1 phase and a 4% lower cell count in the treated samples. There was a 24% drop in the proportion of metabolically active cells as measured by the product of cell count and proportion of cells in the G2/M phase. Samples from the treated group returned $^{19}$F-NMR spectra with a distinctly lower amplitude resonance than controls (fig.1) suggesting drug treated cells have a lower content of NMR detectable $^{19}$F-nuclei over controls. In addition, comparison of $^{19}$F-spectra from the lysate against $^{19}$FLT in aqueous solution (fig.2) suggests a chemically distinct $^{19}$F-species in the cell extract. LC-MS data of the cell extract shows FLT is absent, but a component with MW of 324 that is, 80 D higher than FLT was observed. On the basis of mass-spectroscopy and NMR the $^{19}$F resonance of the cell extract is tentatively assigned to a phosphate metabolite of FLT.

Discussion and conclusion: To the authors’ best knowledge this is the first description via $^{19}$F-NMR of the metabolism of $^{19}$FLT in cellular culture. $^{19}$FNMRR demonstrated a substantially diminished $^{19}$F resonance in cells treated with RAF265 over the control group and coincided with a change in the FC cell count and cell cycle profile indicating cellular modulation by RAF265. The results suggest that the $^{19}$FNMRR reflects a change in the proportion of cells in the cell-cycle but without a substantial change in cell number. The $^{19}$F resonance in the cell extract was clearly distinct to FLT in aqueous solution and is tentatively assigned to a phosphate metabolite of FLT on the basis of our initial MS results and previous literature[3, 4]. In conclusion, we have demonstrated that $^{19}$FNMRR and FC can be combined to understand the behavior of $^{19}$FLT in the presence of an anticancer agent. This could help interpret signals detected in the clinic.

Table 1 (above): Representing changes in the % of cells throughout the cell cycle and actual cell count with and without treatment

<table>
<thead>
<tr>
<th></th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>S+G2/M (%)</th>
<th>Cell count ($\times 10^6$)</th>
<th>Met active cells ($\times 10^6$)</th>
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<tr>
<td>Control</td>
<td>55.5</td>
<td>20.9</td>
<td>14.1</td>
<td>35</td>
<td>11.2</td>
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<td>RAF265</td>
<td>63.6</td>
<td>18.7</td>
<td>8.8</td>
<td>27.5</td>
<td>10.8</td>
<td>2.97</td>
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<tr>
<td>Change</td>
<td>▲ 15%</td>
<td>▼ 11%</td>
<td>▼ 38%</td>
<td>▼ 22%</td>
<td>▼ 4%</td>
<td>▼ 24%</td>
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References