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 it's inhibition of DNA synthesis[1]. It is now used in its isotopic ^{18}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 100ug/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_2O 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.

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}FNMR 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}FNMR 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}FNMR 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.

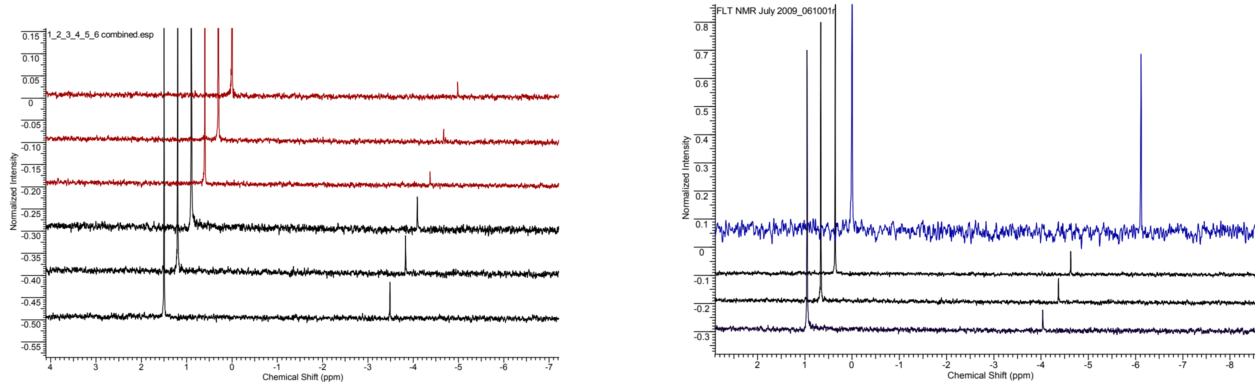

Figure 1 (left): ^{19}FNMR from cell extracts of RAF265 treated A375M cells (red spectra) and untreated cells (black spectra) - spectra are referenced to 5FU at 0 ppm and **Figure 2** (right): ^{19}FNMR spectra from untreated cell extracts (black spectra) and aqueous solution of ^{19}FLT (blue spectrum) – spectra are referenced to 5FU at 0 ppm

	G0/G1 (%)	S (%)	G2/M (%)	S+G2/M (%)	Cell count (×10^6)	Met active cells (×10^6)
Control	55.5	20.9	14.1	35	11.2	3.92
RAF265	63.6	18.7	8.8	27.5	10.8	2.97
Change	▲ 15%	▼ 11%	▼ 38%	▼ 22%	▼ 4%	▼24%

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

References

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