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## ★ Abstract title:

Dose-dependent cytotoxicity of arfolitixorin, a direct-acting folate, versus leucovorin with 5-fluorouracil in patient-derived colorectal cancer tumoroids (PDTs)

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## Background

Folate enhances the anti-tumor effect of key chemotherapeutic agent 5-fluorouracil (5FU) in metastatic colorectal cancer (mCRC). Arfolitixorin ([6R]-5,10-methylene-tetrahydrofolate [ARF]), a direct-acting folate in development, may improve outcomes by bypassing the multi-step metabolic activation typically required by folate prodrugs such as leucovorin (LEU). In the Phase 3 study ISO-CC-007 (NCT03750786), ARF showed efficacy comparable to LEU, however, the primary endpoint of demonstrating superiority was not achieved, possibly due to the dose being suboptimal for a cellular response.

## Material and methods

The activity of 5FU, LEU, levoleucovorin (LLEU), ARF, and combinations thereof, were assessed in a panel of two CRC cell lines and 20 PDTs. In combination with 5FU, the folates were tested at a fixed concentration. Cell growth, cell death, and relative viability were assessed using metabolic readout and imaging. Dose-response was modelled using the Hill equation. Statistical significance was evaluated using area over the dose-response curve (AOC, higher values indicate higher activity) and Wilcoxon paired-sample tests.

## Results

For both cell lines and PDTs, 5FU was active at 0.5–5  $\mu\text{M}$ , a physiologically relevant dose range with a predominantly cytostatic effect. For PDTs, median IC<sub>50</sub> (half-maximal inhibitory concentration) for 5FU was 4.5  $\mu\text{M}$  (range: 1.8–NA) with viability ranging 19–69% at 10  $\mu\text{M}$ . In cell lines, the activity of LEU 20  $\mu\text{M}$  was indistinguishable from LLEU 10  $\mu\text{M}$ . The activity of ARF 6  $\mu\text{M}$  (ISO-CC-007 trial dose) was inferior to ARF 20  $\mu\text{M}$ . Increasing ARF doses led to increased activity, and ARF 20  $\mu\text{M}$  was superior to LEU 20  $\mu\text{M}$  in PDTs (**Table**). 5FU exhibited a partial cytostatic effect when combined with LEU 20  $\mu\text{M}$ , but cytotoxicity became evident when combined with higher doses of ARF.

## Conclusions

ARF displays a potent, dose-dependent cytotoxic effect and increased activity in 5FU-treated PDTs that is higher than with LEU at higher doses. These findings support that suboptimal dosage in the ISO-CC-007 trial contributed to insufficient efficacy. Further clinical development at higher doses could enhance the standard of care for patients, and a Phase 1/2 clinical trial based on this approach is currently being planned.

**Table: Potency of 5FU  $\pm$  LEU or ARF in PDTs.**

	Median IC <sub>50</sub> * (range) ( $\mu\text{M}$ , 5FU)	Median AOC <sup>†</sup> (range)	p-value <sup>‡</sup>
5FU	4.5 (1.8–NA)	0.41 (0.16–0.6)	reference
+LEU 20 $\mu\text{M}$	4.6 (1.7–NA)	0.42 (0.19–0.62)	<0.001
+ARF 10 $\mu\text{M}$	5.1 (1.4–NA)	0.41 (0.15–0.63)	0.01
+ARF 20 $\mu\text{M}$	3.8 (0.89–27)	0.47 (0.18–0.69)	<0.001
+ARF 30 $\mu\text{M}$	1.2 (0.18–10)	0.61 (0.35–0.81)	<0.001
+ARF 40 $\mu\text{M}$	0.45 (0.10–4.9)	0.75 (0.45–0.92)	<0.001

\*Half-maximal inhibitory concentration. †area over the dose-response curve. ‡P-values are from Wilcoxon paired-sample tests comparing against 5FU.

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