NUC-3373 induces ER stress and the release of DAMPs in colorectal cancer cells

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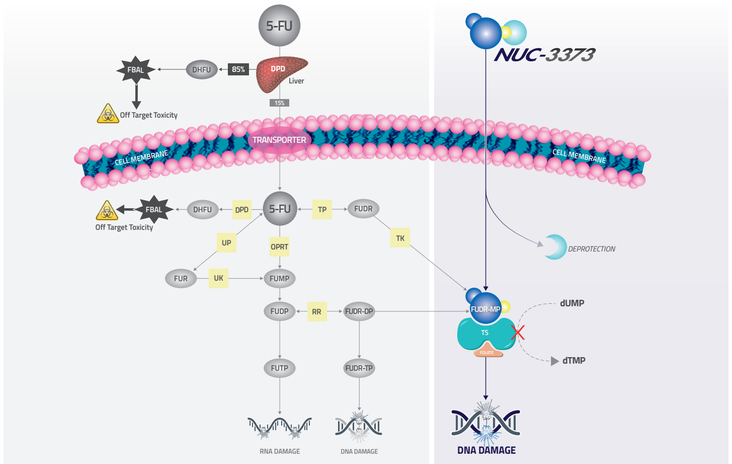
Email: fgmckissock@icloud.com

Fiona G McKissock, Oliver J Read, David J Harrison - University of St Andrews, St Andrews, UK & NuCana plc, Edinburgh, UK

Background

- 5-fluorouracil (5-FU) remains the cornerstone of treatment for patients with a broad range of tumors
- The active anti-cancer metabolite of 5-FU is FUDR-MP (FdUMP)
  - FUDR-MP binds and inhibits thymidylate synthase (TS)[1]
  - TS inhibition prevents conversion of dUMP to dTMP, leading to DNA damage and cell death
- 5-FU activity is limited by cancer resistance mechanisms

NUC-3373 bypasses the resistance mechanisms associated with 5-FU



NUC-3373: A targeted inhibitor of TS

- ProTide transformation of FUDR-MP, the active anti-cancer metabolite of 5-FU
- Designed to overcome the key 5-FU cancer resistance mechanisms [2,3]
- FUDR-MP generation is independent of membrane transporters and intracellular enzyme activation
- Causes an imbalance in the nucleotide pool (dUMP, dTMP) leading to DNA damage and cell death

	5-FU	NUC-3373
Plasma half-life	Short plasma half-life (8-14 minutes)[4]	Longer plasma half-life (6-10 hours)[5,6]
Administration	Prolonged infusion (≤46 hours)	Short infusion (1-4 hours)
DPD-mediated breakdown	Degraded by DPD[7]	Unaffected by DPD[8,9]
Hand-foot syndrome	Yes	No
Cardiotoxicity	Yes	No
Neurotoxicity	Yes	No
Toxic metabolite 5-FUTP	Activation required	Pre-activated

- Currently being investigated in clinical studies
  - NuTide:301 - Phase 1 dose-finding study in solid tumors
  - Nutide:302 - Phase 1b combination study in colorectal cancer (CRC)

Scientific Rationale

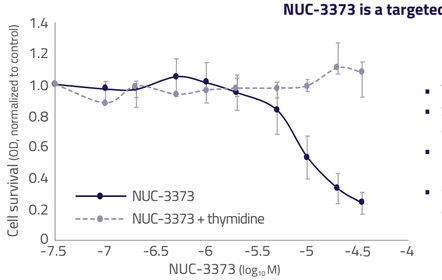
- In addition to being a targeted inhibitor of TS, NUC-3373 induces cancer cell death by triggering the ER stress response[10,11]
- ER stress response is
  - A consequence of TS ternary complex formation
  - Independent of DNA damage
- ER stress is known to stimulate the release of damage-associated molecular patterns (DAMPs) which have the potential to evoke immunogenic cell death (ICD)[12]
- We hypothesize that NUC-3373-induced ER stress will lead to the release of DAMPs

Methods

- Human CRC cells (HCT116) were treated with 10μM NUC-3373 (IC_{50}: 25μM)
- BiP and TS (free and ternary complex) protein expression were measured by Western blot (whole cell lysates)
- TS was knocked down using TYMS-targeting siRNA
- Cells were supplemented with 10μg/ml thymidine to prevent dTMP-depletion and subsequent DNA damage
- Calreticulin (CRT) was assessed by flow cytometry and fluorescence microscopy (24h exposure)
- Nuclear high mobility group box protein 1 (HMGB1) was assessed by fluorescence microscopy (24h & 48h exposure)

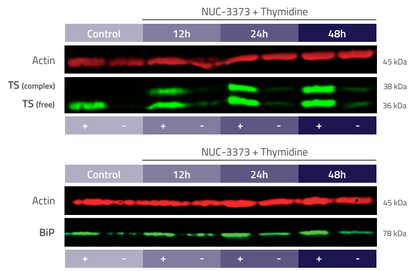
Results

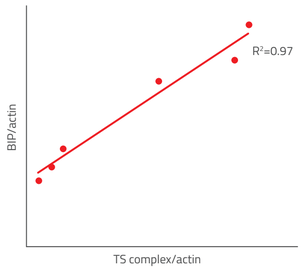
NUC-3373 is a targeted TS inhibitor



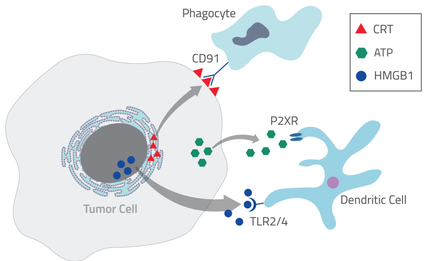
- Thymidine rescues cells from NUC-3373-induced death
- Supplementing nucleotide pool with exogenous thymidine counteracts the effects of TS inhibition
- Maintains pool of dTMP, allowing DNA replication and repair to continue
- Confirming that NUC-3373 targets the *de novo* pathway of dTMP synthesis

NUC-3373-induced ER stress is dependent on TS ternary complex formation but not dTMP depletion





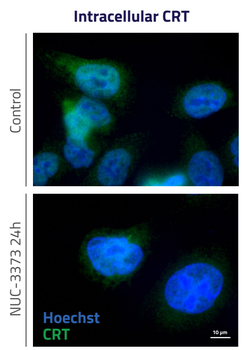
- Immunoglobulin-binding protein (BiP) was used as a marker of unfolded protein response (UPR) activation in CRC cells
- Supplementation with exogenous thymidine demonstrates that UPR occurs independently of DNA damage-related cell death
- TS knockdown studies confirmed that TS ternary complex formation is necessary for the induction of ER stress
- NUC-3373 causes rapid formation of TS ternary complexes, which correlate strongly with BiP upregulation (R^2=0.97)

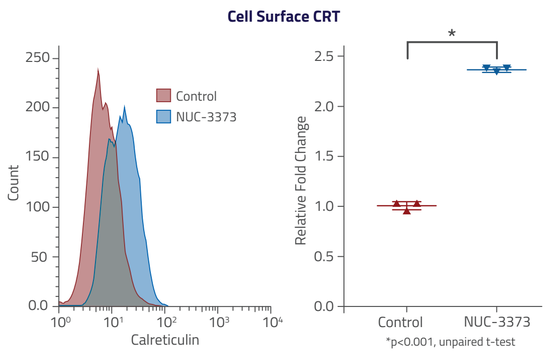


Damage associated molecular patterns (DAMPs)

- ER stress stimulates the release of DAMPs including
  - HMGB1 release from the nucleus to the extracellular environment
  - CRT release from the ER and exposure on the cell surface
  - Active secretion of ATP from cells
- Released DAMPs augment the interaction between cancer cells and the immune system, resulting in an immunogenic cell death (ICD)

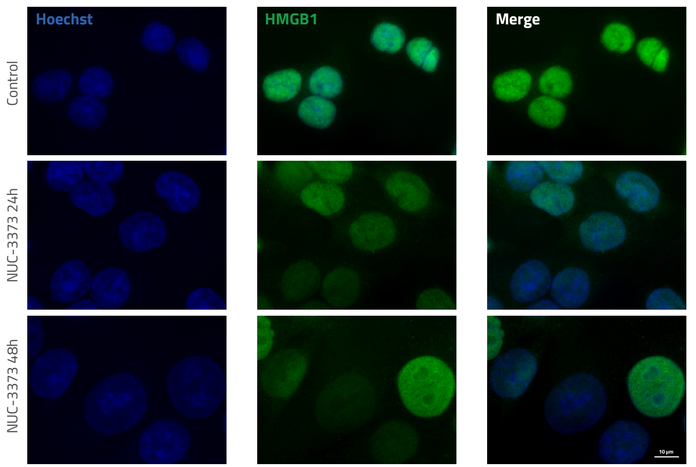
NUC-3373 increases cell surface CRT





- Under resting conditions, CRT is normally resident in the lumen of the rough ER, which is continuous with the nuclear envelope
- NUC-3373 causes CRT translocation from the ER to the cell surface

NUC-3373 induces a loss of nuclear HMGB1



- NUC-3373 causes reduction of nuclear HMGB1 (evident by loss of green fluorescence)

Conclusion

- NUC-3373 is a targeted TS inhibitor resulting in DNA damage and cancer cell death
- NUC-3373 also induces ER stress
  - Through formation of TS ternary complexes
  - Independent of the DNA damage pathway
- NUC-3373-induced ER stress causes release of DAMPs
  - Increases cell surface CRT
  - Loss of nuclear HMGB1
- NUC-3373 has the potential to evoke immunogenic cell death and may enhance the clinical utility of immunotherapy agents

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ABBREVIATIONS: 5-FU: 5-fluorouracil BiP: immunoglobulin binding protein CD91: alpha 2-macroglobulin receptor CRC: colorectal cancer CRT: calreticulin DAMPs: damage associated molecular patterns DPD: dihydropyrimidine dehydrogenase dTMP: deoxythymidine monophosphate dUMP: deoxyuridine monophosphate ER: endoplasmic reticulum FUDR-MP: fluorodeoxyuridine monophosphate HMGB1: high mobility group box protein 1 IC_{50}: half-maximal inhibitory concentration ICD: immunogenic cell death P2XR: P2X receptor siRNA: short interfering RNA TLR2/4: toll-like receptor 2/4 TS: thymidylate synthase (protein) TYMS: thymidylate synthase (gene) UPR: unfolded protein response