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Heterocycles to block the cell cycle: novel ellipticines and their anticancer effects

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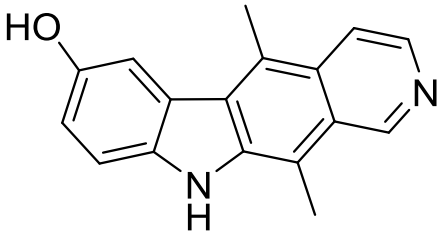
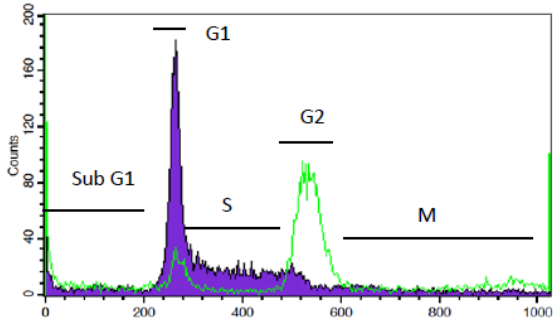
University College Cork, Ireland

Heterocycles to block the cell cycle: novel ellipticines and their anticancer effects

Graphical Abstract





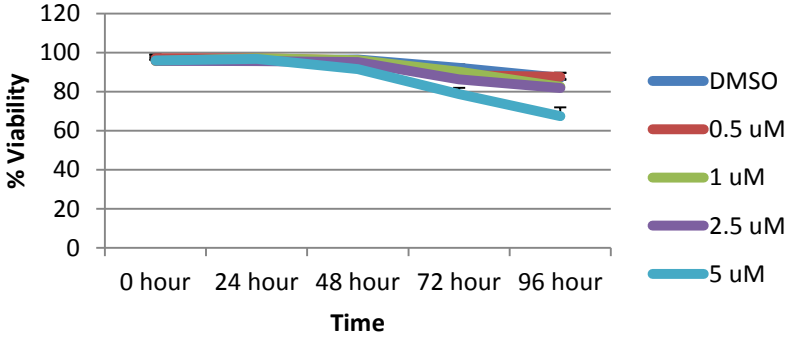


Figure 1: The effect of 7-hydroxyisoellipticine on MV4-11 viability.

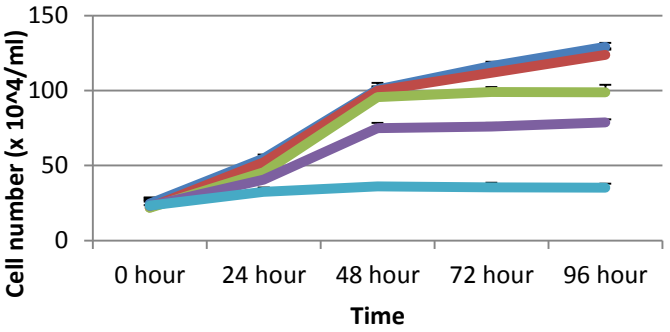


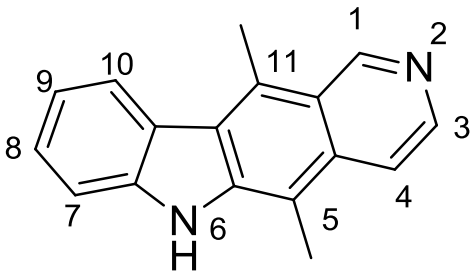
Figure 2: The effect of 7-hydroxyisoellipticine on MV4-11 proliferation.

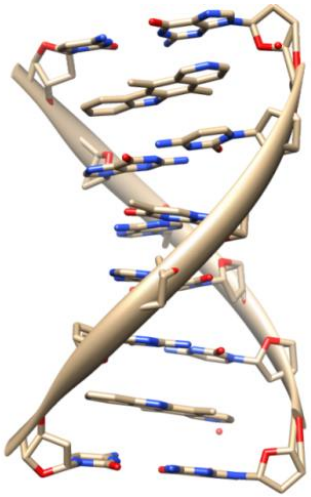
Abstract:
Ellipticine is a natural product possessing multimodal cytotoxic activity including DNA intercalation, topoisomerase II inhibition, c-Kit kinase inhibition and restoration of function to mutant p53 protein. While ellipticine itself is not a suitable candidate for therapeutic use, derivatives including 2-methyl-9-hydroxyellipticinium acetate and 2-(2-(diethylamino)ethyl)-9-hydroxyellipticinium chloride, have progressed to clinical trials. The effect of derivatisation on the isoellipticine is uncharted and structural diversification of isoellipticine could lead to drug candidates with a better clinical profile due to enhanced target specificity.
Our initial approach to this uses substitutent modification at positions 10, 7 and 2 (salt formation at the N2 position represents a favourable attribute for cytotoxic activity as illustrated by the two most successful ellipticines). A number of novel derivatives of isoellipticine have been synthesised and further derivatised.
Preliminary biological testing of novel compounds was performed using a topoisomerase II decatenation assay and via assessment of the anticancer profile using the National Cancer Institute 60 cell line screen for cellular activity.
We will present here the design, synthesis and anticancer properties and significant cell line selectivity of a series of novel ellipticine derivatives devised in our laboratory.

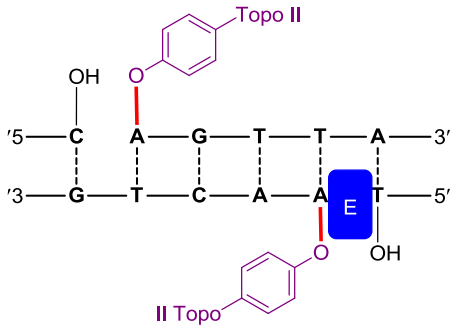
Keywords: Ellipticine; Isoellipticine; cancer; cell cycle.

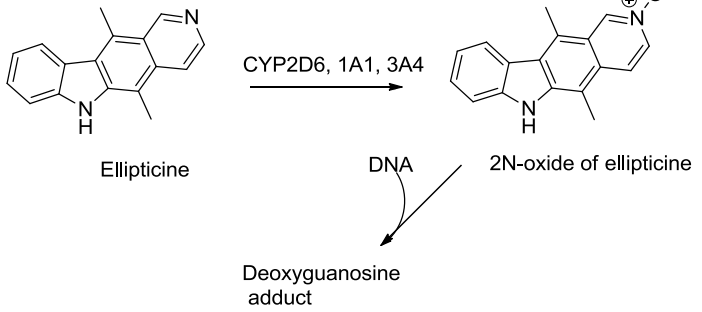
Ellipticine

- Isolated from leaves of *Oschrosia Elliptica* Labill in 1959
- Potent anti-tumour effects





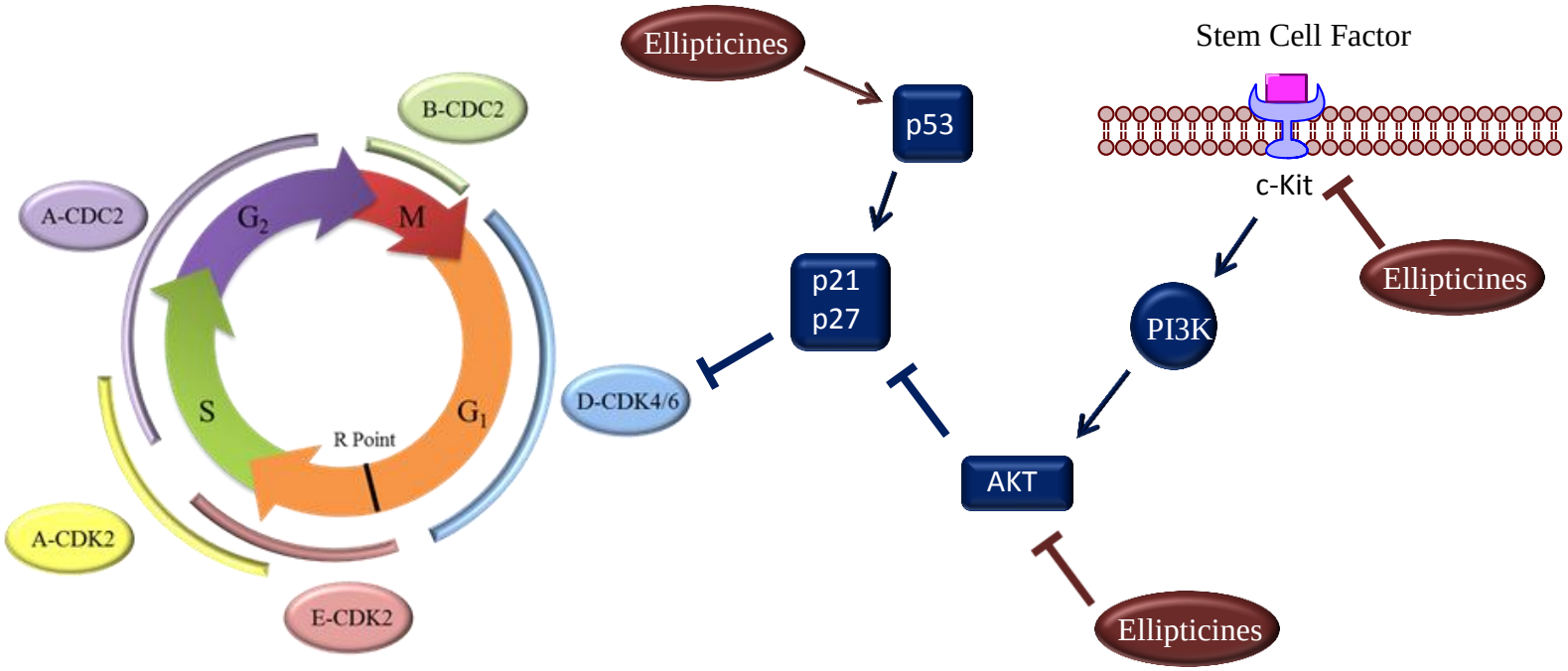




- First established modes of action were intercalation, topoisomerase II inhibition and formation of cytotoxic adducts with DNA

Goodwin, S.; Smith, A.F.; Horning E.C. *Journal of the American Chemical Society* **1959,** *81,* (8)1903-1908
O'Sullivan, E.C.; Miller, C.M.; Deane, F.M.; McCarthy F.O. *Studies in Natural Products Chemistry* **2013**, *39*, (6) 189-232

Ellipticine 1990 – present: Old drug, new targets



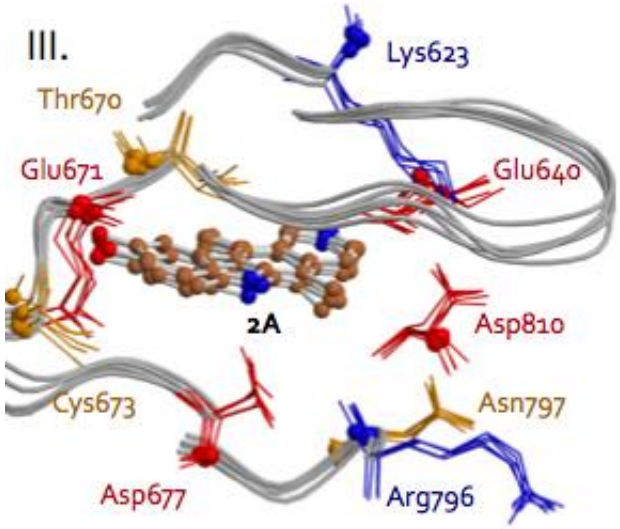
- Recently, Ellipticines shown to affect the regulation of the cell cycle, including restoration of function to mutant p53 tumour suppressor protein.
- Inhibition AKT kinase and wild-type and mutant c-Kit kinase is reported
  - Imatinib (Gleevec®) is used to treat cancers expressing wild type enzyme but is ineffective versus mutated forms which are a significant population
- Latterly, reported mechanisms of action include uncoupling of oxidative phosphorylation, activation of the mitochondrial pro-apoptotic pathway and inhibition of RNA polymerase 1

O'Sullivan, E.C.; Miller, C.M.; Deane, F.M.; McCarthy F.O. *Studies in Natural Products Chemistry* **2013**, *39*, (6) 189-232.
Andrews, W.J.; Panova, T.; Normand, C.; Gadal, O.; Tikhonova, I.G.; Panov, K.I. *Journal of Biological Chemistry* **2013**, (288) 4567-4582

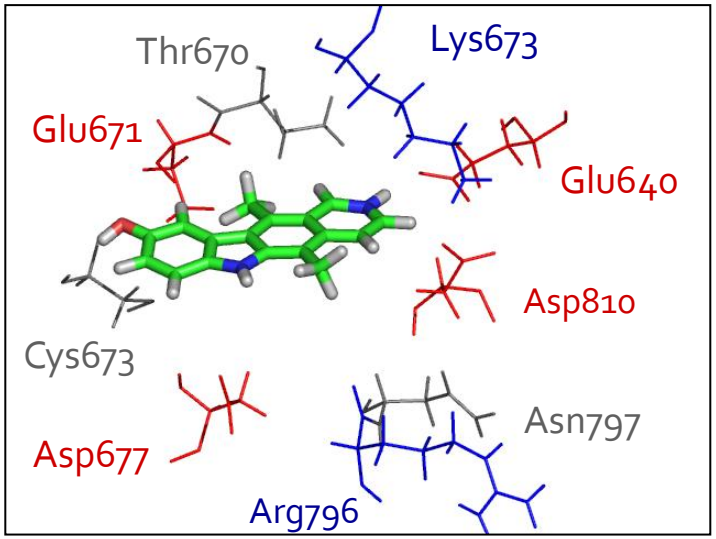
Molecular Modelling

Molecular Dynamics simulations of the binding mode of 9-hydroxyellipticine in the c-Kit kinase active site.

- Five different orientations of 9-hydroxyellipticine **2** were investigated.
- Two nanoseconds of molecular dynamics were performed for each complex.
- New binding mode proposed.



Five snapshots of the c-Kit active site at 20 ps intervals from the last 80 ps of the MD trajectory.



Major hydrogen bonding interactions:

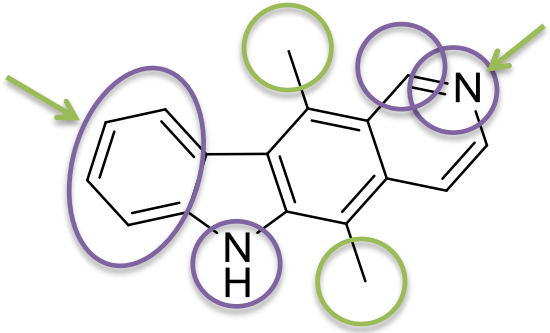
Protonated N-2:Glu640

9-Hydroxy group:Glu671

D. Thompson, C. Miller, F. O. McCarthy, *Biochemistry,* **2008**, 47, 10333-10344.

Novel Targets?

- Isoellipticine is a synthetic isomer of ellipticine which displays promising anti-cancer activity but has been subject to little investigation.
- Aim to increase potency and selectivity by substituent modification on the isoellipticine template, specifically at position 2, 7 and 10.

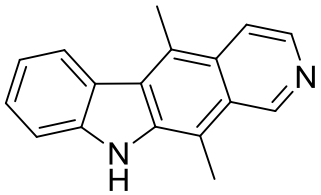


Previous work focused on:

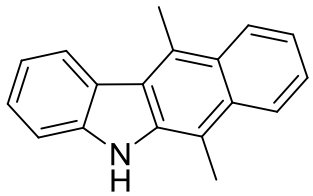
- A-ring substitution – particularly C-9
- *N*-2 Ellipticinium salts
- *N*-6 Substitution
- C-1 Substitution

New areas for investigation:

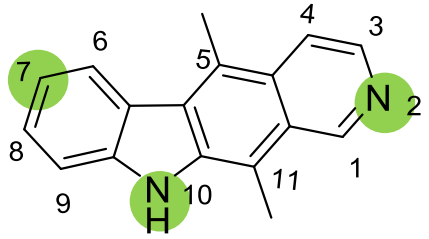
- Novel A/D-rings
- C-11 Substitution
- C-5 Substitution
- Ellipticine Analogues



Isoellipticine

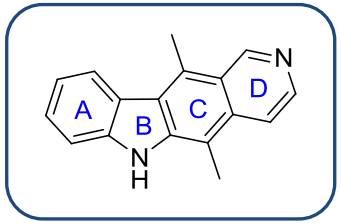


Deazaellipticine

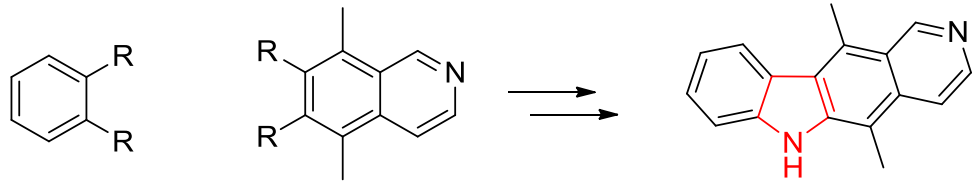


Isoellipticine

Synthesis of Ellipticines

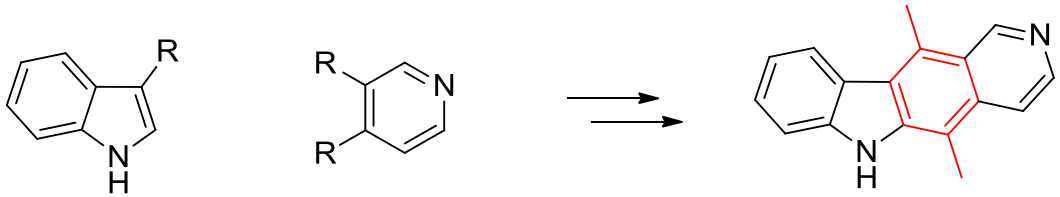


B-Type



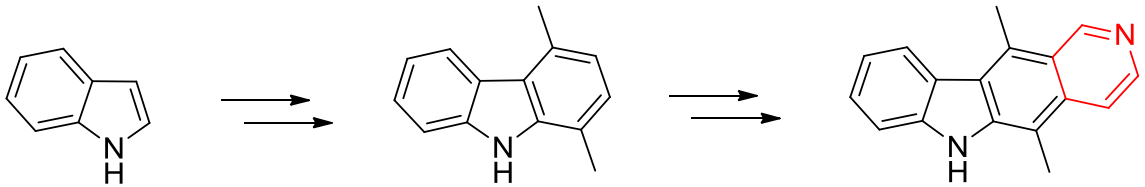
- Fischer indolisation
- Triazole intermediates
- Nitrene insertion

C-Type



- C-2 coupling
- C-3 coupling
- Cycloadditions

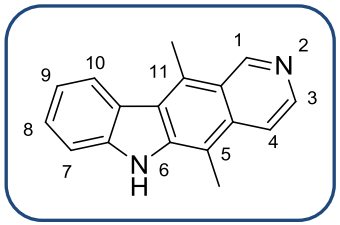
D-Type

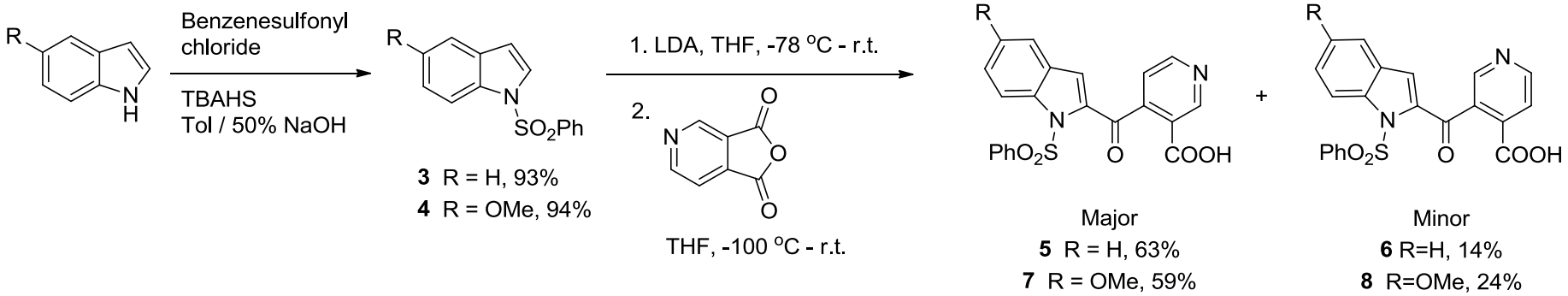


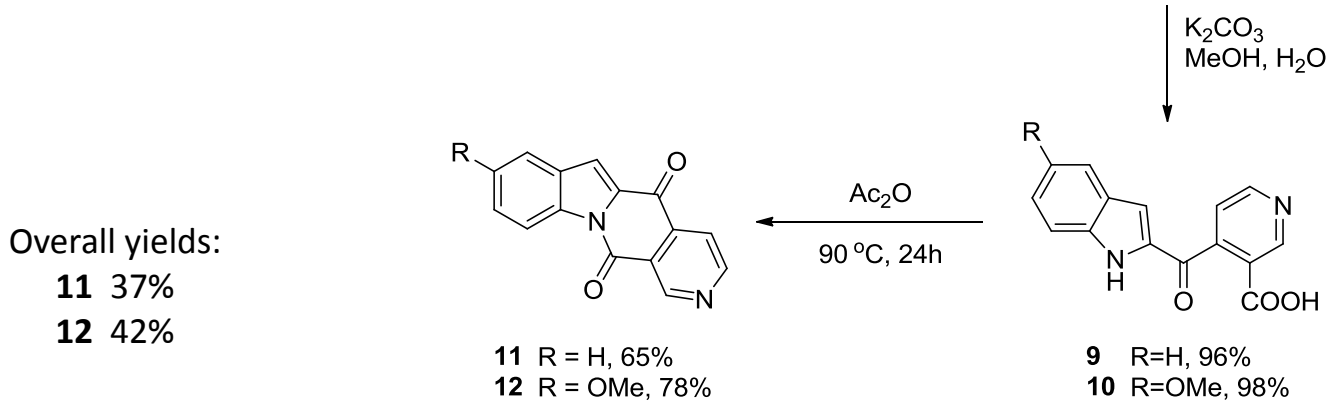
- Cranwell-Saxton

C. M. Miller and F. O. McCarthy. RSC Adv. 2012, **2**, 8883-8918
F.M. Deane, E.C. O'Sullivan, A.R. Maguire, J. Gilbert, J.A. Sakoff, A. McCluskey, F.O. McCarthy. *Org. Biomol. Chem*., 2013,**11**, 1334-1344
F.M. Deane, C.M. Miller, A.R. Maguire, F.O. McCarthy. *J. Het Chem.* 2011, **48 (4)**, 814–823

Ellipticine Series







Overall yields:

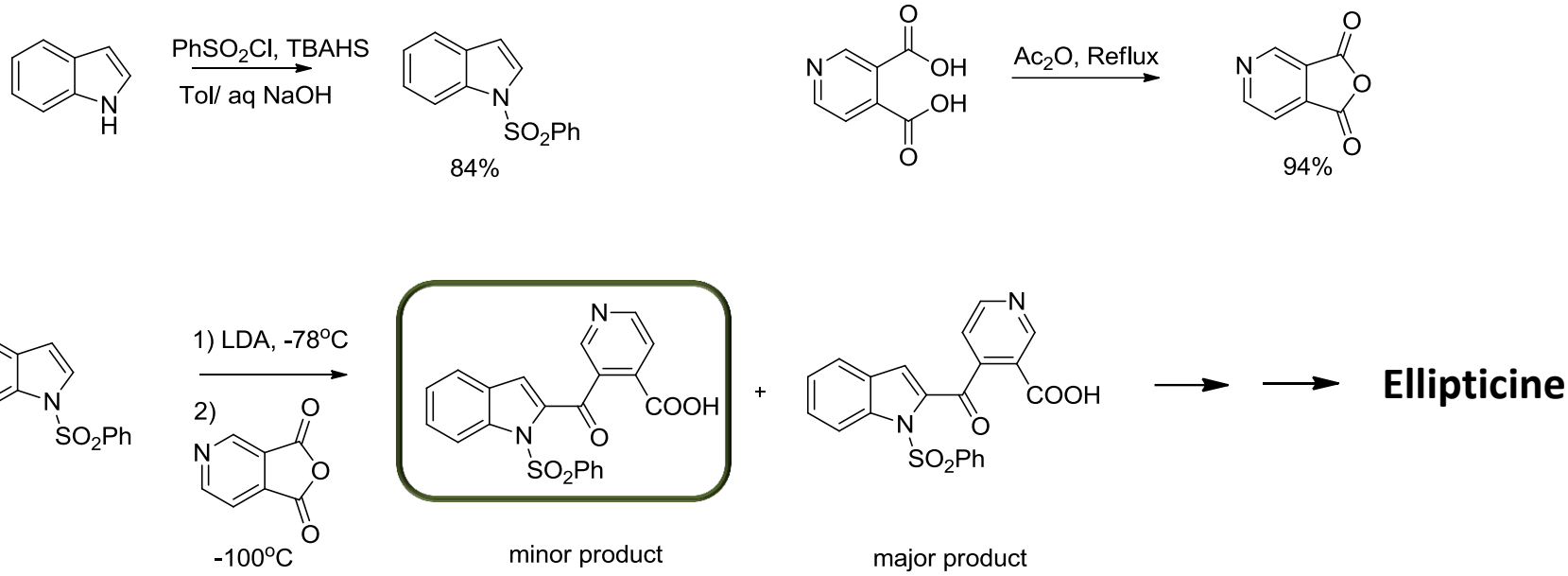
11 37%

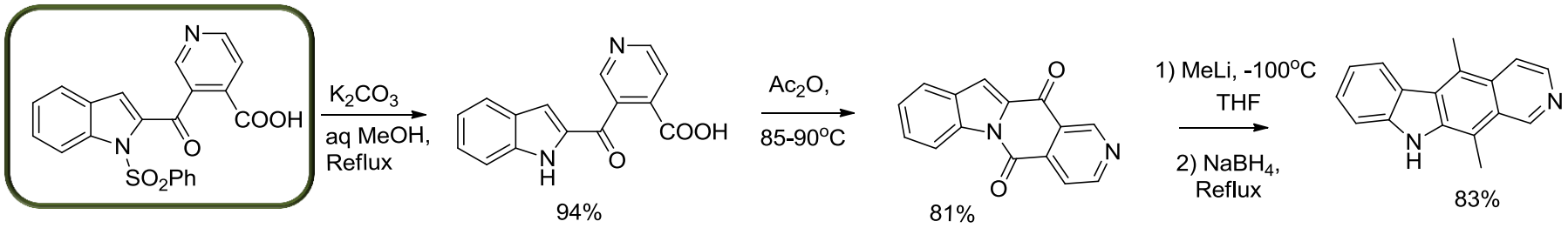
12 42%

G. W. Gribble *et al., J. Org. Chem.*, 1992, **57**, 5891-9.

C.M. Miller, E.C. O'Sullivan, K.J. Devine and F.O. McCarthy. *Org. Biomol. Chem.*, 2012,**10**, 7912-7921

Isoellipticine synthesis

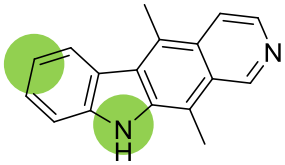




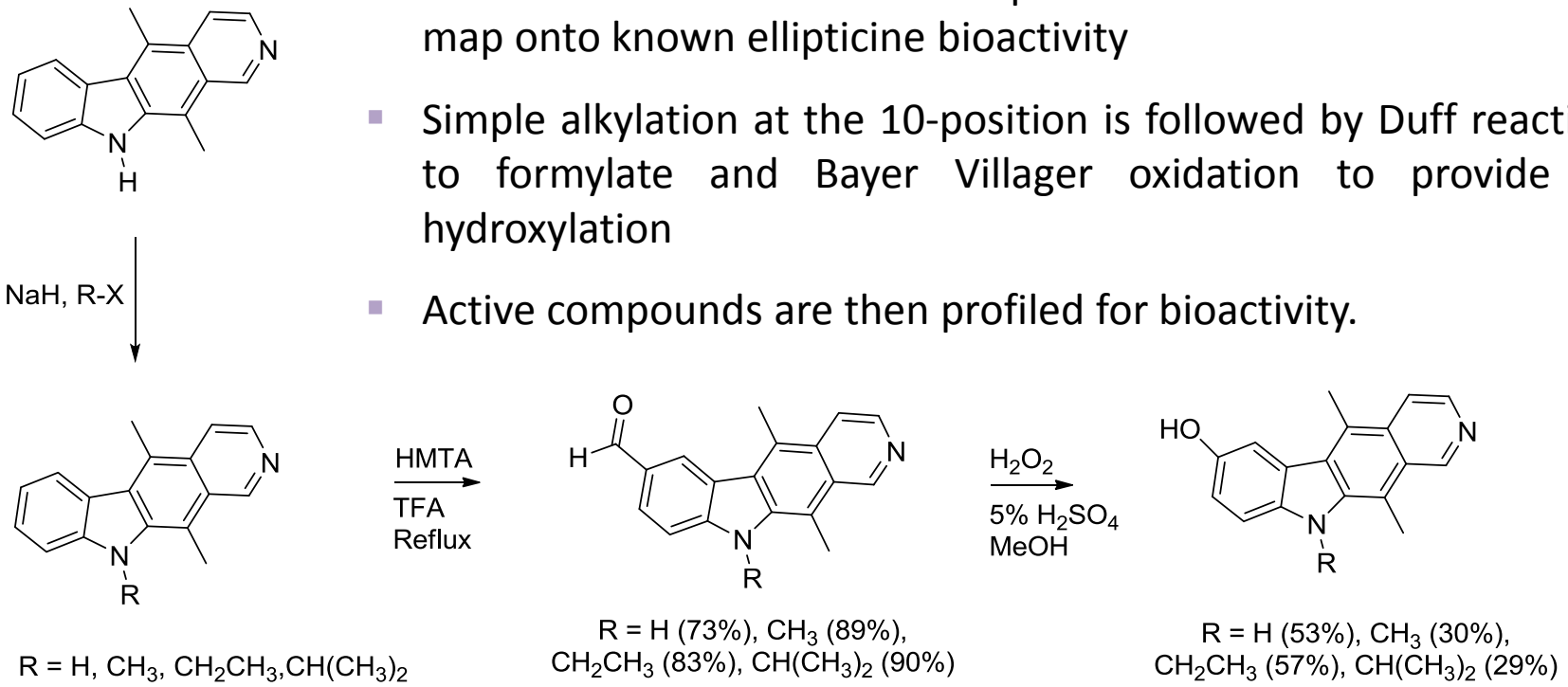
Isoellipticine

C.M. Miller, E.C. O'Sullivan, K.J. Devine and F.O. McCarthy. *Org. Biomol. Chem.,* 2012,**10**, 7912-7921

C7 & N10 Isoellipticine derivatisation

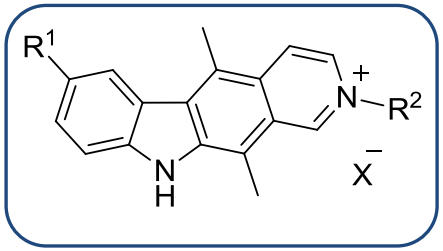


- Derivatisation of the 7-and 10-positions is essential in order to map onto known ellipticine bioactivity
- Simple alkylation at the 10-position is followed by Duff reaction to formylate and Bayer Villager oxidation to provide 7-hydroxylation
- Active compounds are then profiled for bioactivity.



C.M. Miller, E.C. O'Sullivan, K.J. Devine and F.O. McCarthy. *Org. Biomol. Chem.,* 2012,**10**, 7912-7921

Isoellipticinium salts



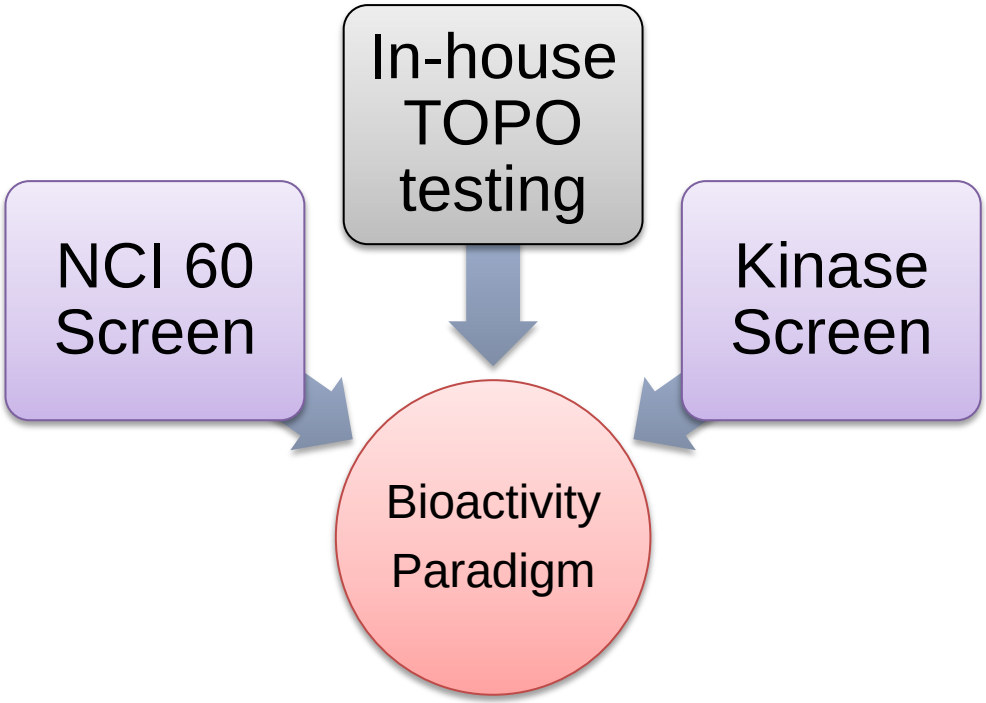
- Derivatisation of the 2-position will again map known ellipticine bioactivity
- Simple alkylation at the 2-position provides a series of isoellipticinium salts
- Full compound series is profiled for bioactivity.

No.	R^7	R^2	X	% Yield
77	H	CH_3	I	47
78	H	$C_5H_{10}COOH$	Br	77
79	H	$C_5H_{10}CN$	Br	82
80	H	$C_5H_{10}CONH_2$	Br	84
81	H	$C_5H_{10}CONHSO_2CH_3$	Br	76
82	OCH_3	CH_3	I	88
83	CHO	CH_3	I	54
84	CHO	$C_5H_{10}COOH$	Br	49
85	CHO	$C_5H_{10}CN$	Br	63
86	CHO	$C_5H_{10}CONH_2$	Br	60
87	CHO	$C_5H_{10}CONHSO_2CH_3$	Br	54
88	OH	$C_5H_{10}COOH$	Br	32
89	OH	$C_5H_{10}CN$	Br	31
90	OH	$C_5H_{10}CONH_2$	Br	46
91	OH	$C_5H_{10}CONHSO_2CH_3$	Br	52

C.M. Miller, E.C. O'Sullivan, K.J. Devine and F.O. McCarthy. *Org. Biomol. Chem.*, 2012,**10**, 7912-7921

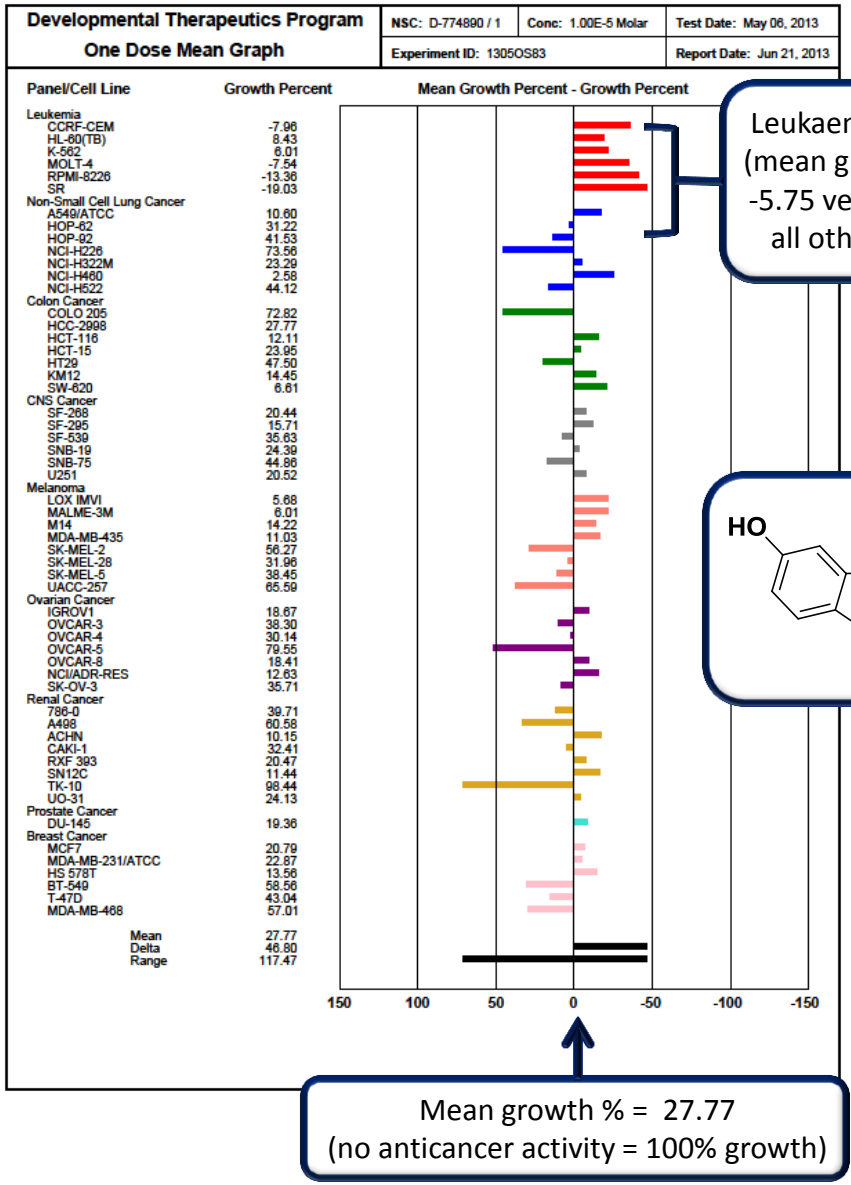
Biological evaluation of novel ellipticines

- Biological evaluation follows a predetermined programme beginning with cellular antiproliferative activity as measured at the NCI 60 cell line screen.
- Active compounds are then profiled for Topoisomerase I and II inhibition.
- Active compounds are also profiled for kinase inhibition in collaboration.



NCI screening – One dose

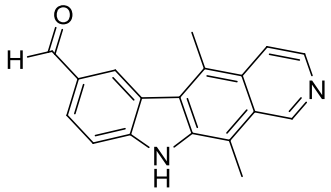
- Initial screening comprises testing *in vitro* against a panel of 60 cancer cell lines at a concentration of 10µM.
- Must satisfy inhibition threshold in a minimum number of cell lines to progress to 5 dose.
- Over 50 of our ellipticine derivatives have been brought forward for five-dose screen and tested against the cell line panel at concentrations ranging from 100 µM to 10 nM.
- Dose-response curves are generated for each cell line.Three characteristic *in vitro* parameters, GI_{50}, TGI and LC_{50}, are calculated for each cell line in response to the presence of the different drug candidates.



NCI 60-Cell Line Screen

1st Generation Compounds

- Single dose (10 µM)



7-Formylisoellipticine **62**

Mean Growth % = 51.47

Developmental Therapeutics Program — One Dose Mean Graph

NSC: D-754620 / 1 | Conc: 1.00E-5 Molar | Test Date: Oct 18, 2010

Experiment ID: 1010OS37 | Report Date: Nov 17, 2010

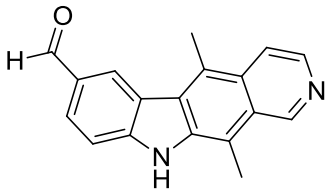
Panel/Cell Line	Growth Percent
Leukemia	
CCRF-CEM	11.07
HL-60(TB)	23.54
K-562	54.44
MOLT-4	-2.46
RPMI-8226	11.10
SR	-28.84
Non-Small Cell Lung Cancer	
A549/ATCC	81.69
EKVX	101.55
HOP-62	48.47
NCI-H226	58.38
NCI-H23	38.33
NCI-H322M	90.32
NCI-H460	45.56
NCI-H522	66.97
Colon Cancer	
COLO 205	80.78
HCC-2998	81.45
HCT-116	15.62
HCT-15	25.75
HT29	49.71
KM12	84.56
SW-620	31.91
CNS Cancer	
SF-268	35.01
SF-295	88.82
SF-539	45.83
SNB-19	32.07
SNB-75	66.00
U251	23.79
Melanoma	
LOX IMVI	10.80
MALME-3M	92.11
M14	39.85
MDA-MB-435	52.83
SK-MEL-2	64.50
SK-MEL-28	76.22
SK-MEL-5	58.08
UACC-257	96.84
UACC-62	34.89
Ovarian Cancer	
IGROV1	59.31
OVCAR-3	62.98
OVCAR-5	95.08
OVCAR-8	24.23
NCI/ADR-RES	44.95
SK-OV-3	61.00
Renal Cancer	
786-0	67.90
A498	62.12
ACHN	39.00
CAKI-1	92.70
RXF 393	44.71
SN12C	31.53
TK-10	83.95
UO-31	58.59
Prostate Cancer	
PC-3	39.66
DU-145	36.83
Breast Cancer	
MCF7	6.80
MDA-MB-231/ATCC	66.95
HS 578T	51.05
BT-549	64.37
T-47D	61.58
MDA-MB-468	42.73
Mean	51.47
Delta	80.31
Range	130.39

Mean Growth Percent - Growth Percent

150 100 50 0 -50 -100 -150

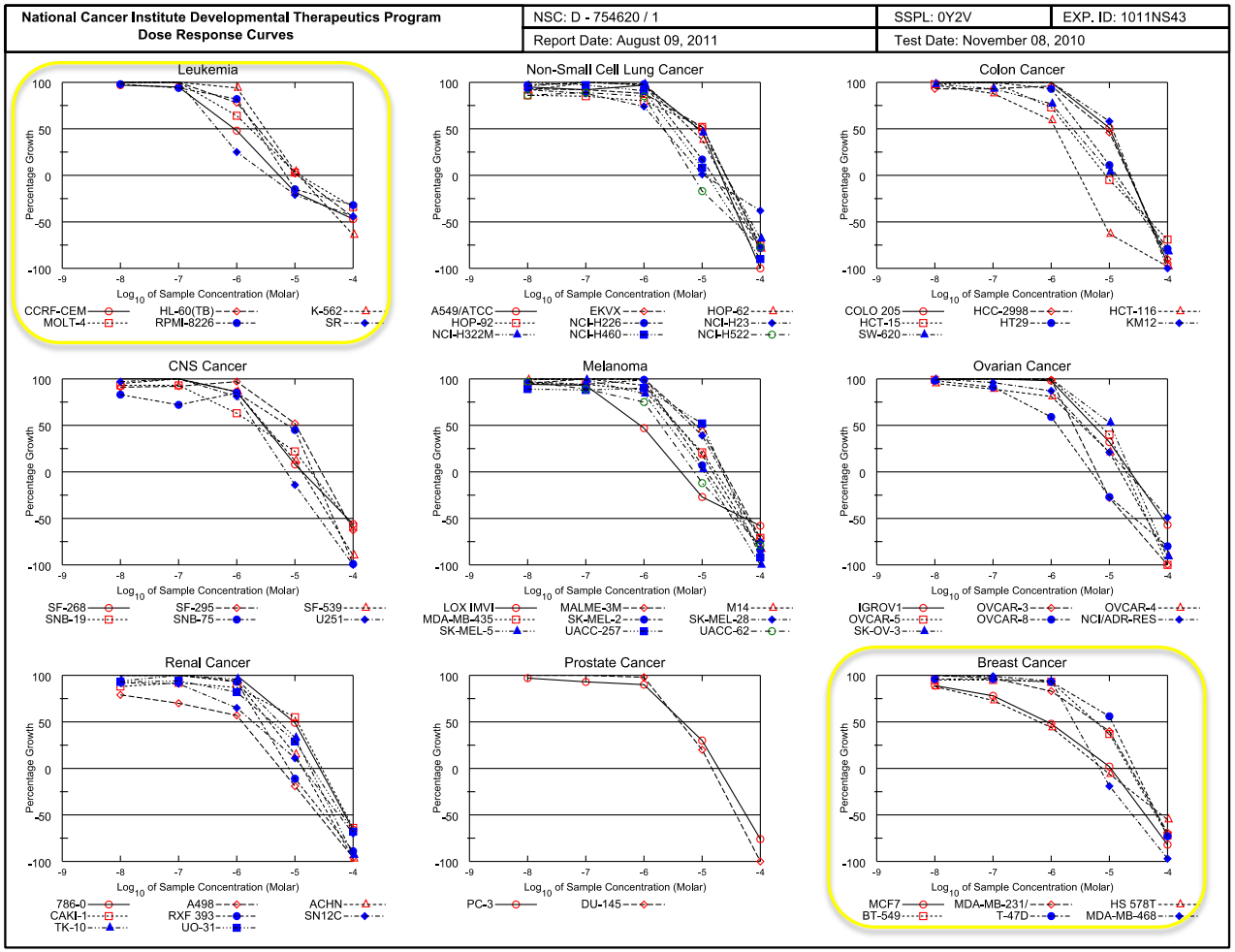
NCI 60-Cell Line Screen – Five dose

- Five dose results



7-Formylisoellipticine **62**

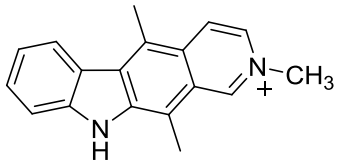
Type	Line	GI_{50} (μM)
Leukaemia	CCRF-CEM	0.89
	SR	0.49
Breast	MCF-7	0.88
	HS 578T	0.24



Five dose assay (10 nM, 100nM, 1 μM, 10 μM, 100 μM)

NCI 60-Cell Line Screen

- Single dose



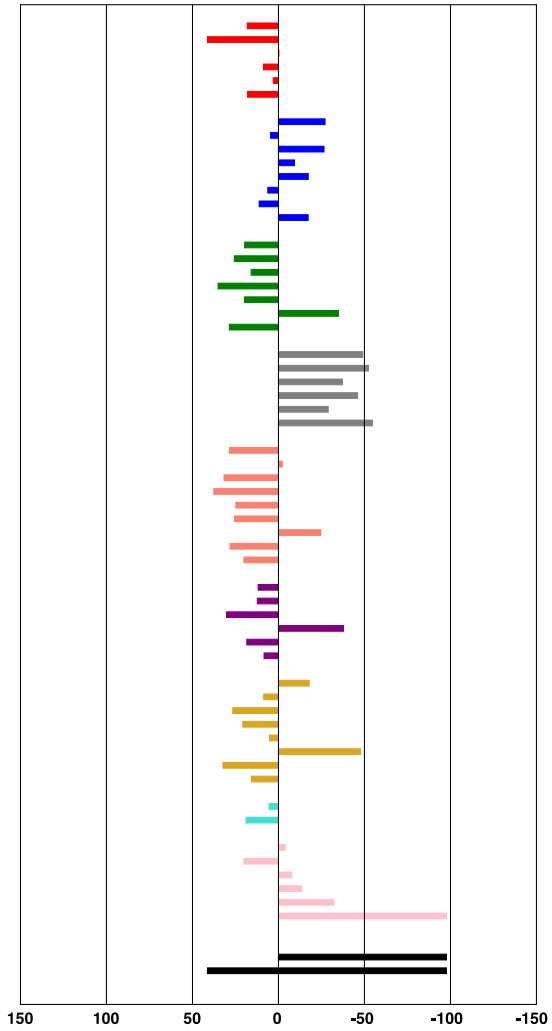
N-Methylisoellipticinium iodide **77**

Mean growth %: 65.75

Developmental Therapeutics Program
One Dose Mean Graph

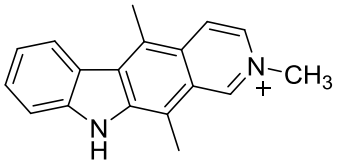
NSC: D-754621 / 1	Conc: 1.00E-5 Molar	Test Date: Oct 18, 2010
Experiment ID: 1010OS37		Report Date: Nov 17, 2010

Panel/Cell Line	Growth Percent
Leukemia	
CCRF-CEM	83.86
HL-60(TB)	107.04
K-562	65.48
MOLT-4	74.45
RPMI-8226	68.71
SR	83.67
Non-Small Cell Lung Cancer	
A549/ATCC	38.70
EKVX	70.36
HOP-62	39.29
NCI-H226	56.47
NCI-H23	48.40
NCI-H322M	71.92
NCI-H460	76.95
NCI-H522	48.47
Colon Cancer	
COLO 205	85.42
HCC-2998	91.35
HCT-116	81.64
HCT-15	100.88
HT29	85.46
KM12	30.90
SW-620	94.26
CNS Cancer	
SF-268	16.79
SF-295	13.44
SF-539	28.51
SNB-19	19.85
SNB-75	36.84
U251	11.15
Melanoma	
LOX IMVI	94.13
MALME-3M	63.47
M14	97.39
MDA-MB-435	103.30
SK-MEL-2	90.49
SK-MEL-28	91.22
SK-MEL-5	41.11
UACC-257	93.91
UACC-62	85.79
Ovarian Cancer	
IGROV1	77.57
OVCAR-3	78.05
OVCAR-5	95.92
OVCAR-8	27.89
NCI/ADR-RES	84.10
SK-OV-3	74.10
Renal Cancer	
786-0	47.89
A498	74.30
ACHN	92.27
CAKI-1	86.43
RXF 393	70.93
SN12C	18.03
TK-10	98.01
UO-31	81.47
Prostate Cancer	
PC-3	71.21
DU-145	84.61
Breast Cancer	
MCF7	61.88
MDA-MB-231/ATCC	85.82
HS 578T	57.99
BT-549	52.33
T-47D	33.59
MDA-MB-468	-31.95
Mean	65.75
Delta	97.70
Range	138.99



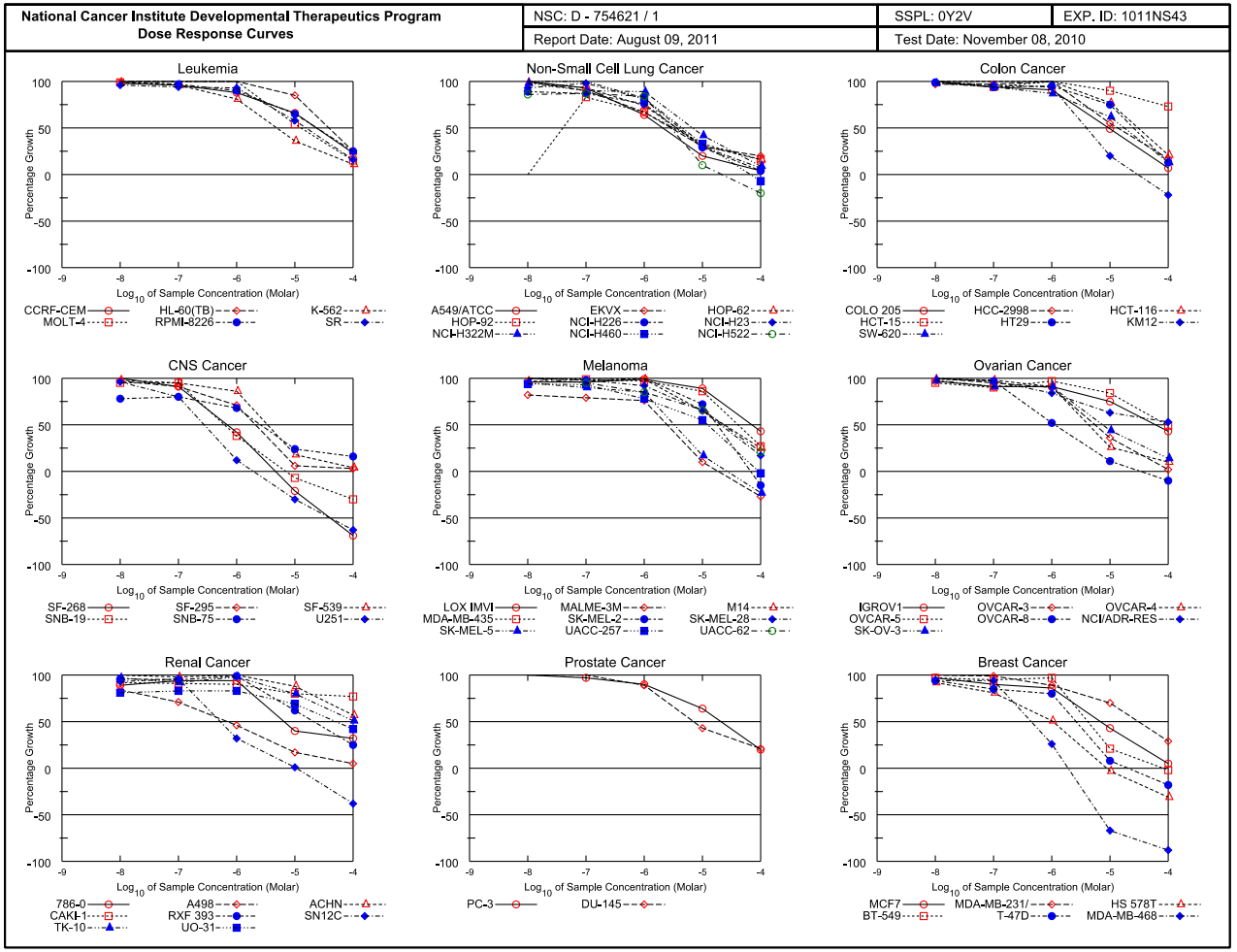
NCI 60-Cell Line Screen – Five dose

- Five dose



N-Methylisoellipticinium iodide **77**

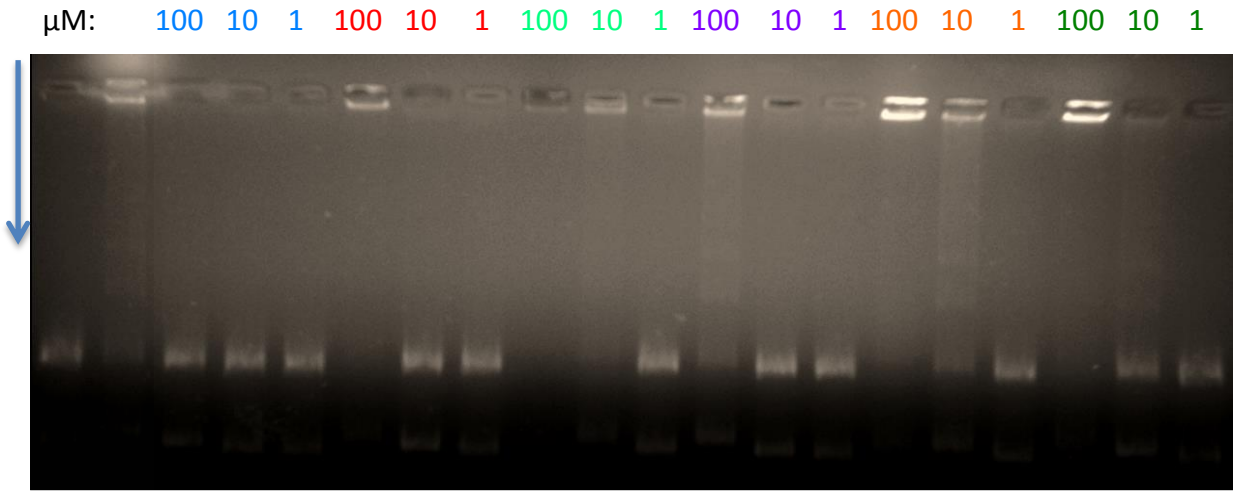
Type	Line	GI_{50} (μM)
CNS	SF-268	0.68
	SNB-19	0.61
	U251	0.28
Renal	A498	0.68
	SN12C	0.53
Breast	MDA-MB-468	0.44



Five dose assay (10 nM, 100nM, 1 μM, 10 μM, 100 μM)

Topoisomerase II Decatenation Assay

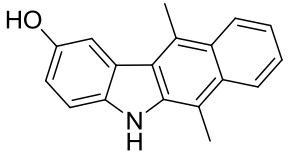
A-Ring Substituted Ellipticines and Analogues – 3-fold dilution



Lane	A	B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Activity	-	+	-	-	-	+	-	-	+	+	-	+	-	-	+	+	-	+	-	-

A, positive control = kDNA +ATP + Topo II
B, negative control = kDNA +ATP + Topo II + 100 µM ellipticine
C = dilution solvent / blank
Inhibitory activity: (-) = not active against topo II; (+) = topo II inhibition.

1 – 3



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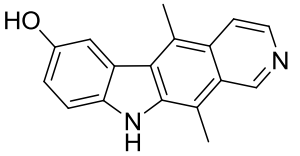

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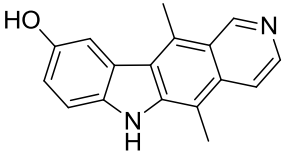

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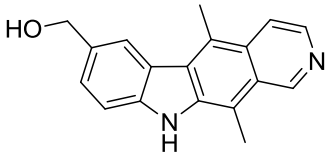

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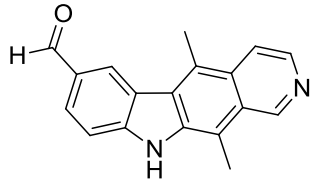

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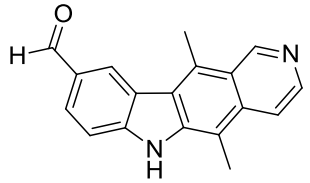
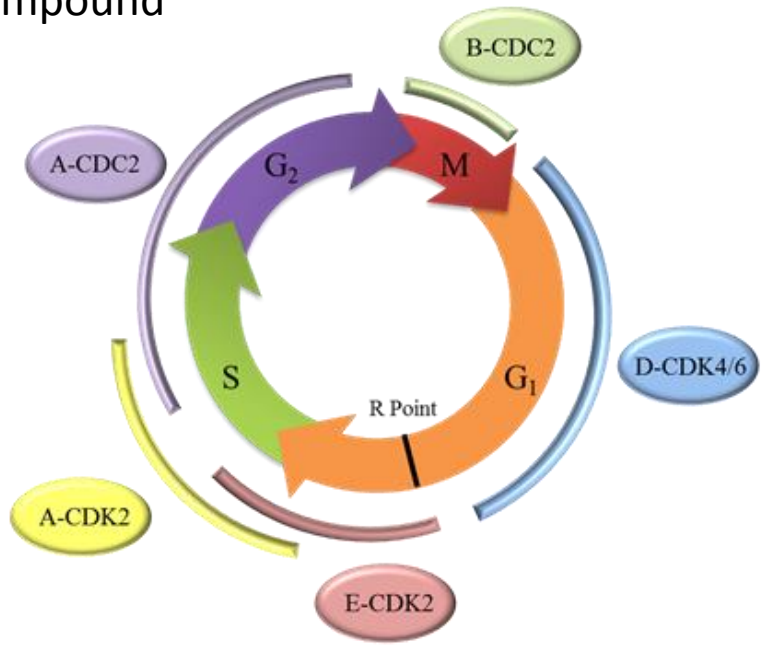


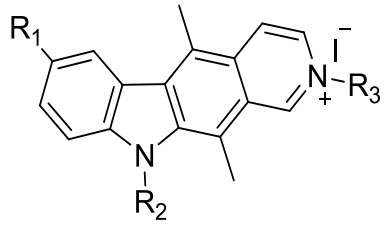
Structures for cell cycle study

- Eileen Russell and Prof. Thomas Cotter, UCC utilising acute myeloid leukaemia cell line MV4-11
- Flow cytometry enables determination of the proportion of cells in each stage of the cell cycle after exposure to the sample compound



- 7-Formyl and 7-hydroxyl derivatives chosen as parent compounds showed selectivity for leukaemia cell lines in NCI testing.

A

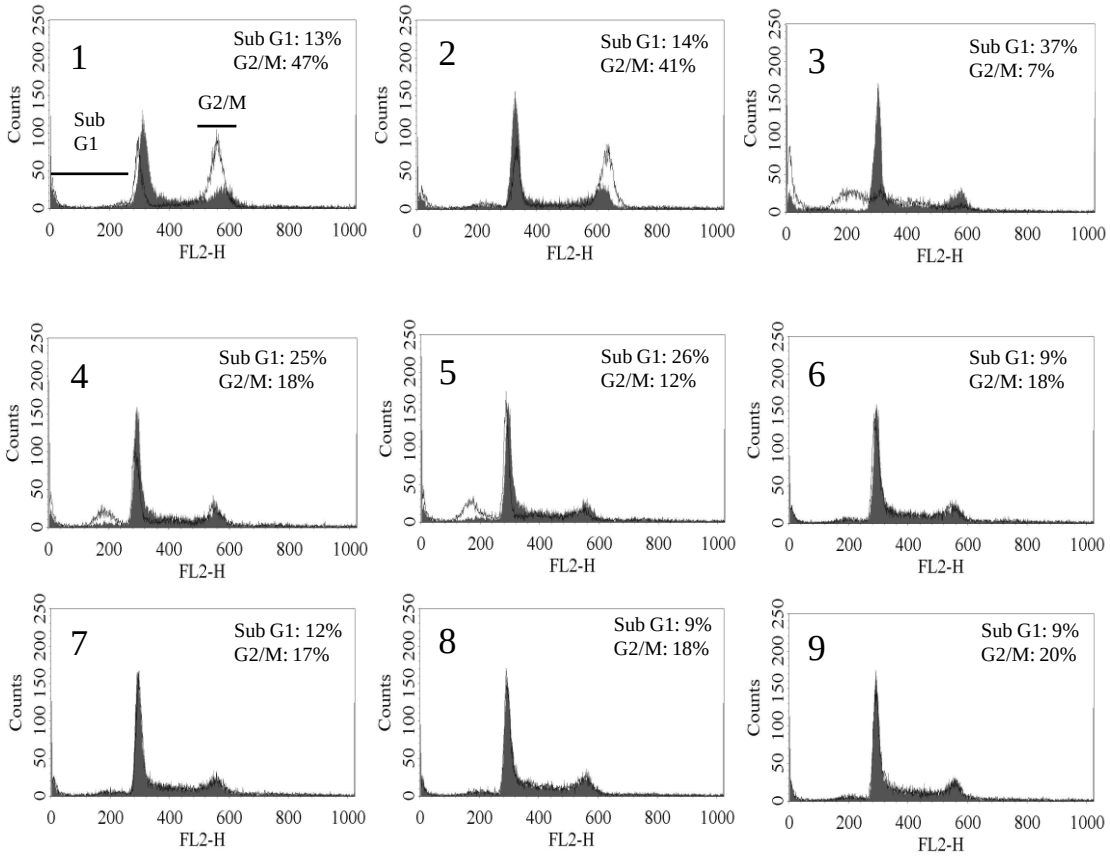


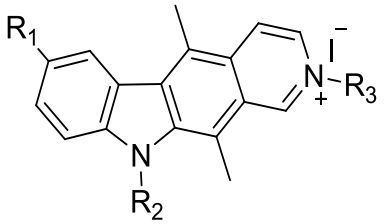
Compound	R_1	R_2	R_3
1	OH	H	-
2	OH	CH_3	-
3	CHO	CH_3	-
4	CHO	CH_2CH_3	-
5	CHO	$CH(CH_3)_2$	-
6	CHO	H	CH_2CH_3
7	OH	H	CH_3
8	OH	H	CH_2CH_3
9	CHO	H	CH_3

Figure 1. Structure of isoellipticine derivatives

E. G. Russell, E. C. O'Sullivan, C. M. Miller, J. Stanicka, F. O. McCarthy, T. G. Cotter. *Inv. New Drugs* (2014) **32 (6)**, 1113-1122

Flow cytometry for compounds 1-9





Compound	R_1	R_2	R_3
1	OH	H	-
2	OH	CH_3	-
3	CHO	CH_3	-
4	CHO	CH_2CH_3	-
5	CHO	$CH(CH_3)_2$	-
6	CHO	H	CH_2CH_3
7	OH	H	CH_3
8	OH	H	CH_2CH_3
9	CHO	H	CH_3

Figure 2. The panel of isoellipticine derivatives had contrasting effects on MV4-11 cell cycle

The cell cycle of MV4-11 cells incubated with 5 μM of each derivative for 24 hours was analyzed by propidium iodide staining. A representative profile is shown. Black line = 5 μM isoellipticine derivative, grey = 0.5 % DMSO control. Values represent the percentage (%) of cells in each cell cycle phase of the cells treated with isoellipticine.

E. G. Russell, E. C. O'Sullivan, C. M. Miller, J. Stanicka, F. O. McCarthy, T. G. Cotter. *Inv. New Drugs* (2014) **32 (6)**, 1113-1122

Flow cytometry of 7OH-IsoE at different concentrations

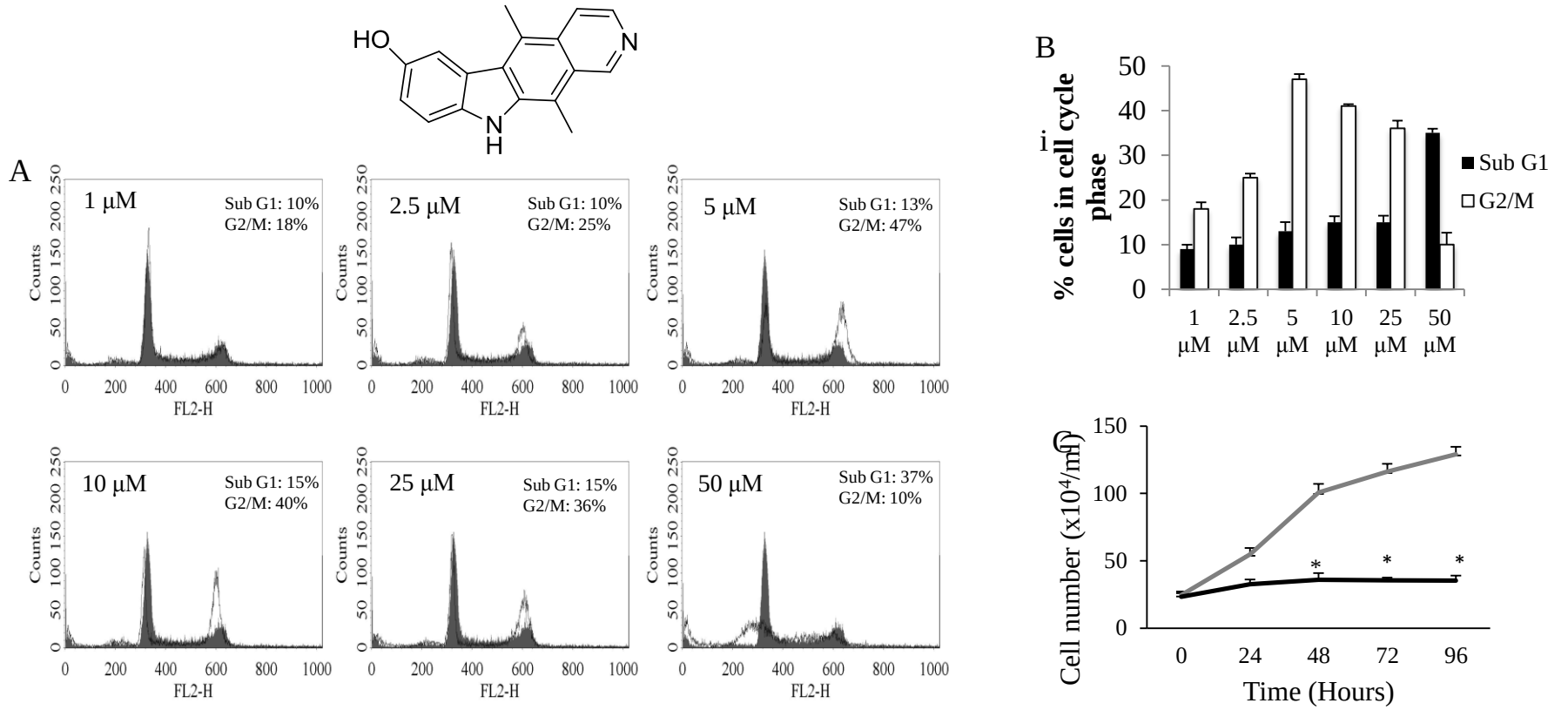


Figure 3. 5 μM 7-hydroxyisoelliptine causes G2/M cell cycle arrest in MV4-11 cells

A. The cell cycle of MV4-11 cells incubated with different doses of 7-hydroxyisoelliptine for 24 hours was analyzed by propidium iodide staining. A representative profile is shown. Values represent the percentage (%) of cells in each cell cycle phase of the cells treated with 7-hydroxyisoellipticine. **B.** Quantification of cell cycle analysis. **C.** The effects of 5 μM 7-hydroxyisoellipticine on cell number measured by trypan blue exclusion. Black line = 5 μM 7-hydroxyisoellipticine, grey = 0.5 % DMSO control. * = p-value < 0.01. The error bars represent ±SD.

Flow cytometry against other leukaemia cell lines

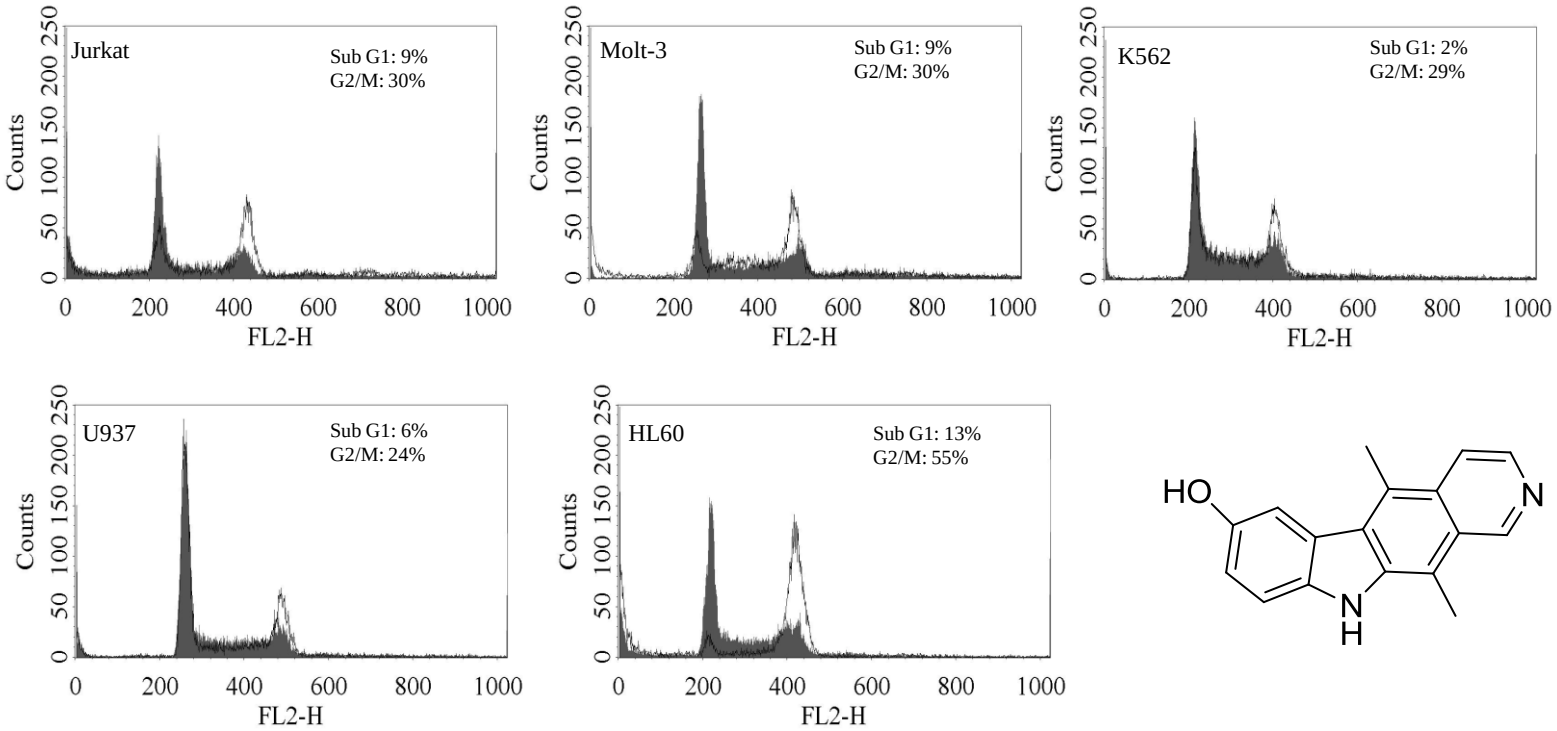


Figure 4. 5 µM 7-hydroxyisoellipticine causes a G2/M cell cycle arrest in a number of leukaemia cell lines
The cell cycle of leukaemia cells incubated with 5 µM 7-hydroxyisoellipticine or 0.5% DMSO control for 24 hours was analyzed by propidium iodide staining of nuclei and flow cytometry. Values represent the percentage (%) of cells in each cell cycle phase of the cells treated with 7-hydroxyisoellipticine. Black line = 5 µM 7-hydroxyisoellipticine, grey = 0.5 % DMSO control.

E. G. Russell, E. C. O'Sullivan, C. M. Miller, J. Stanicka, F. O. McCarthy, T. G. Cotter. *Inv. New Drugs* (2014) **32 (6)**, 1113-1122

Distribution and mechanism of action studies

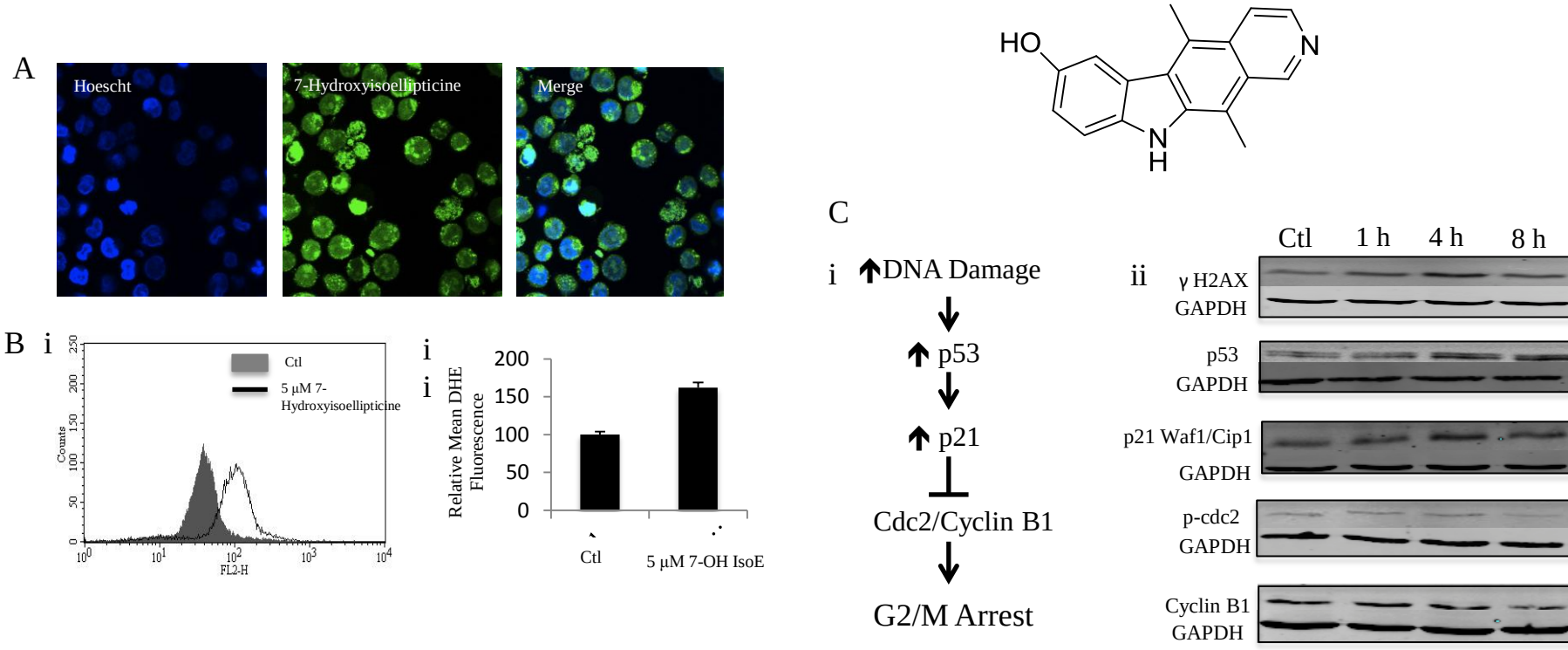


Figure 5. 7-Hydroxyisoellipticine activates the p53 pathway and increases ROS levels
A. Cells were treated with 5 μM 7-hydroxyisoellipticine for 4 hours and imaged using Olympus FluoView FV1000-ASW confocal microscope. **B. i.** ROS levels were measured by flow cytometry using dihydtroethidium. **ii.** Quantification of ROS levels. Grey = Control, black = 5 μM 7-hydroxyisoellipticine. The error bars represent ±SD. **C. i.** Schematic of the DNA damage pathway. **ii.** Western blot of γH2AX, p53, p21 Waf1/Cip1, phospho-cdc2 and cyclin B1 protein expression in MV4-11 cell treated with 0.5% DMSO control (Ctl) and with 5 μM 7-hydroxyisoellipticine at 1, 4, 8 and 24 hours respectively using GAPDH as a loading control.

G2/M cell cycle arrest of 7-OH IsoE

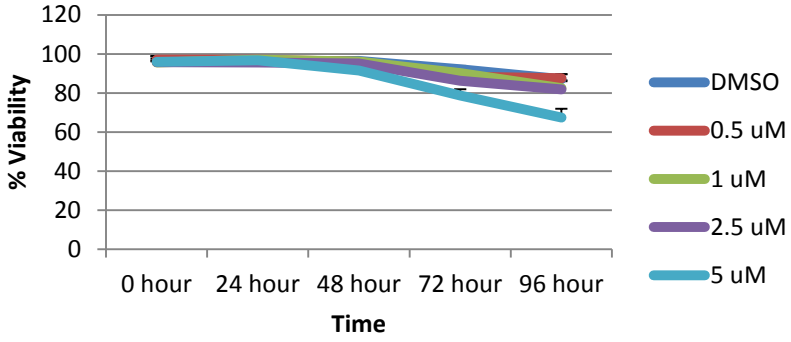


Figure 1: The effect of 7-hydroxyisoellipticine on MV4-11 viability.

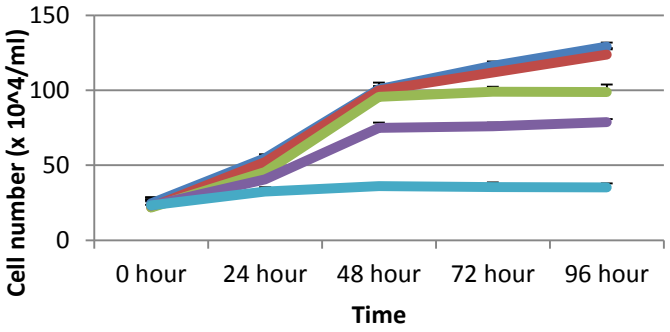
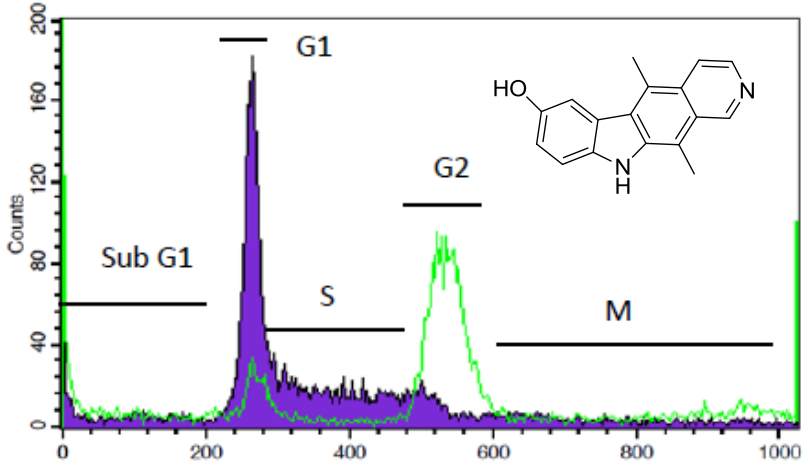


Figure 2: The effect of 7-hydroxyisoellipticine on MV4-11 proliferation.

- After 96 hours 70% of cells are still viable after treatment with 5 µM 7-hydroxyisoellipticine however cell numbers are not increasing.
- G2/M arrest also evident in five other leukaemia cell lines tested



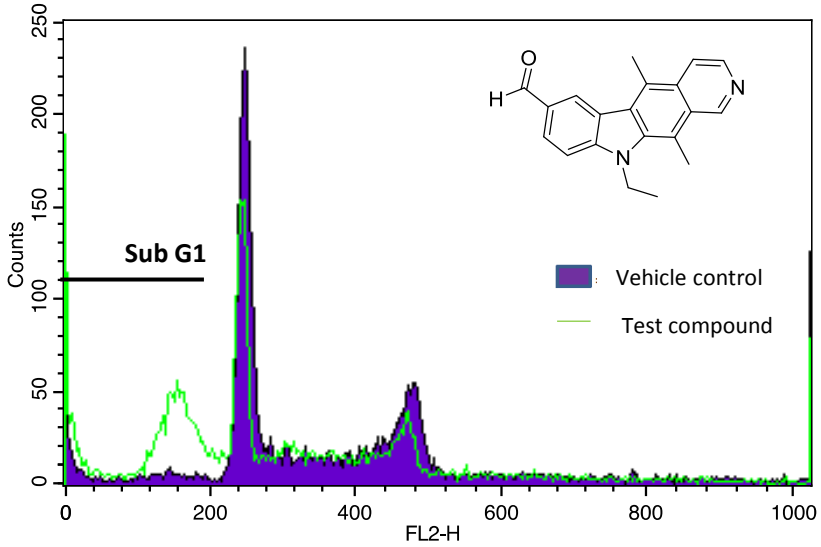
Vehicle control

7-Hydroxyisoellipticine

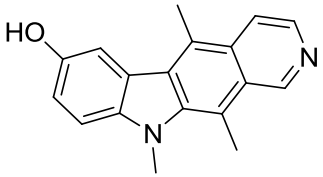
- 7-Hydroxyisoellipticine causes G2/M arrest
- Indication that it acts as a cytostatic rather than cytotoxic agent
- Cytostatic compounds possess attractive features for inclusion in chemotherapy

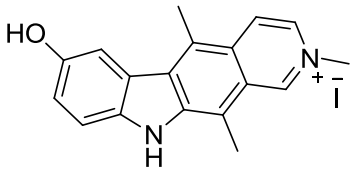
Cytotoxic effect of other of IsoE's

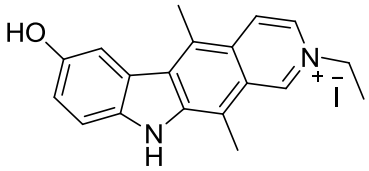
- Isoellipticine derivatives also synthesised to explore whether this cytostatic effect could be replicated with increased potency
- Sub G1 peak observed when cells treated with 7-formyl derivatives and N2/N10 7-hydroxyl derivatives indicating they act as cytotoxic agents

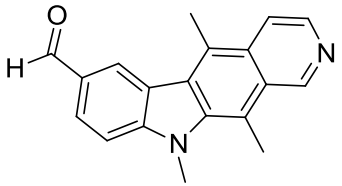


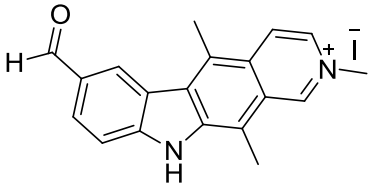
- Adaptability of template allows for additional cytotoxic activity
- Very exiting area of future research





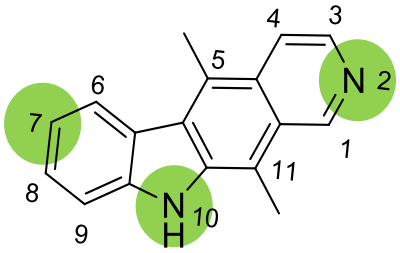




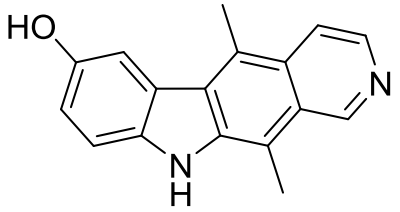


Conclusions

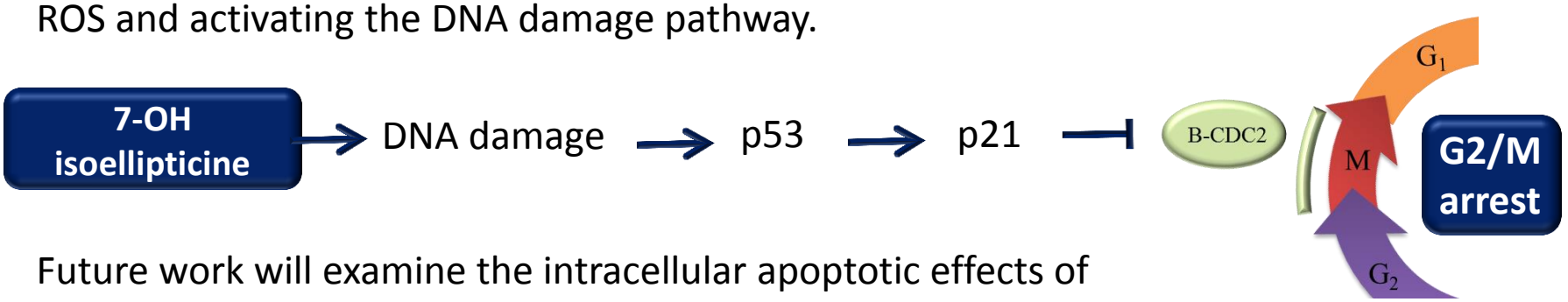
- A range of novel isoellipticines have been synthesised with varying substitutions at positions 2, 7 and 10.



- Over 100 novel isoellipticines have been submitted to the NCI to date with >40 taken forward to 5 dose testing
  - Mean growth inhibitions as low as 0% observed
  - Bulkier substitutions at N10 were found to be generally less active but could result in enhanced selectivity
  - COMPARE analysis has been performed to identify intracellular targets and led to flow cytometry studies



- 7-Hydroxyisoellipticine induces G2/M cell cycle arrest by inducing ROS and activating the DNA damage pathway.



- Future work will examine the intracellular apoptotic effects of isoellipticine derivatives

Acknowledgments

- Funding
  - Irish Research Council
  - PRTLI
- Ellipticine team
  - Fiona Deane PhD
  - Charlotte Miller PhD
  - Elaine O'Sullivan
  - Mary McKee
  - Dr. Eileen Russell
  - Professor Thomas Cotter
  - Dr. Ken Devine
  - National Cancer Institute