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Synthesis and Anticancer Activity of Novel Bisindolylhydroxymaleimide Derivatives with Potent GSK-3 Kinase Inhibition

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ABCRF Analytical & Biological Chemistry Research Facility

UCC University College Cork, Ireland Coláiste na hOllscoile Corcaigh

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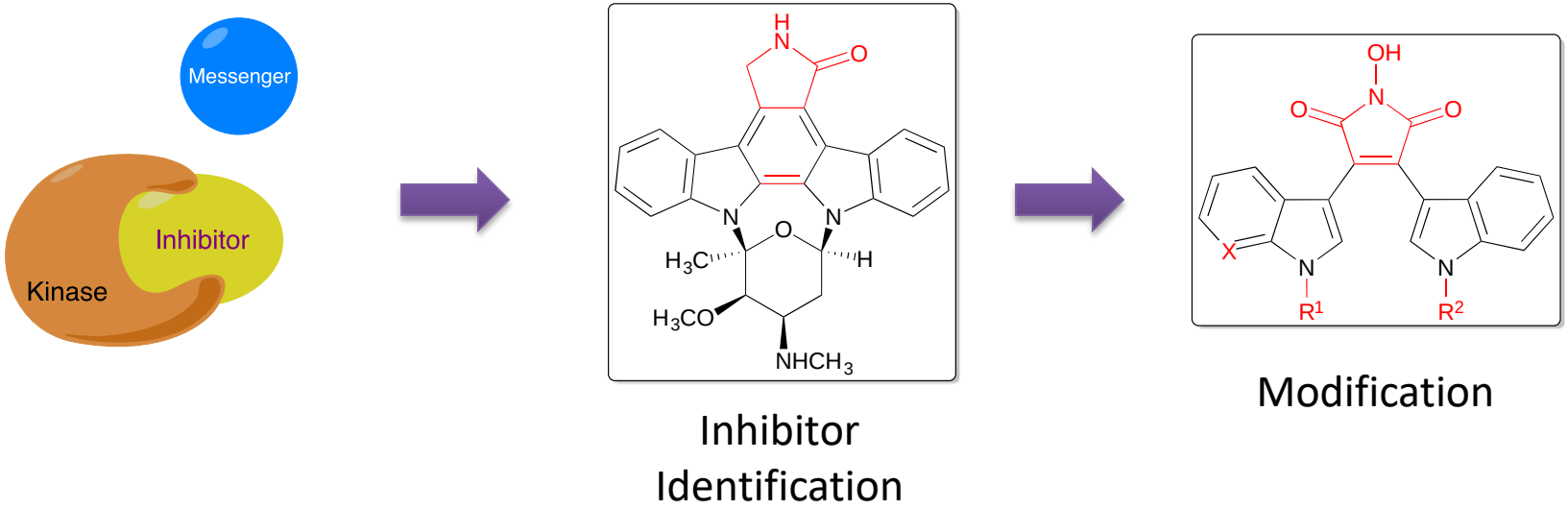
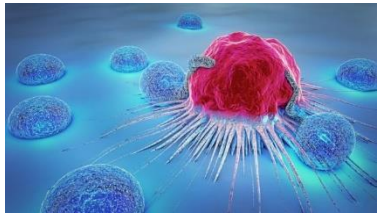
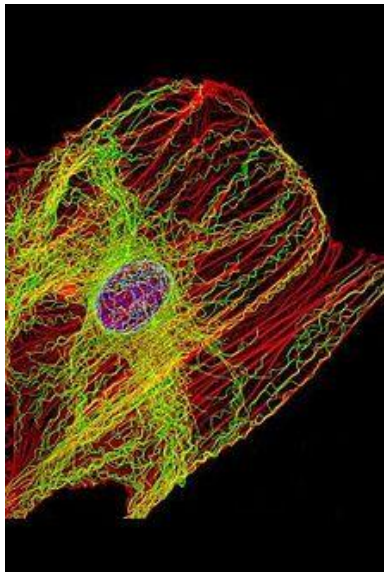
Graphical Abstract



Abstract: Glycogen synthase kinase-3 (GSK-3) refers to a group of multifaceted serine/threonine protein kinases that, in mammals, exist as two isoforms (GSK-3α and GSK-3β). Both isoforms share very similar homology but represent contrasting pharmacology. The quest for targeted GSK-3 inhibition has recently become a mainstay for pharmaceutical companies due to the enzymes' role in a multitude of under-addressed disease states including cancer, Alzheimer's and bipolar disorder. Herein, we describe the synthesis and evaluation of novel indole derivatives as anticancer agents. A bisindolyl template has been derived, starting from a substituted maleimide, through the introduction of an oxygen atom to the headgroup (hydroxymaleimide). Assessing the bioactivity of these derivatives through kinase assays allowed for the identification of substituent derived selectivity. Following on from this, indole substitution was completed and assessed with the identification of unique selectivity patterns in the GSK-3 and CDK kinase assays. Subsequent evaluation of anticancer activity utilising the NCI-60 cell screen showed growth inhibitory profiles towards a multitude of cell lines including: SNB-75 CNS cancer, A498 and UO-31 renal, MDA MB435 melanoma and a panel of leukemia cell lines. Achieving selective kinase inhibition through modulation of this bisindolyl template is evident and will inform future selective clinical candidates.
Keywords: Staurosporine; bisindolylhydroxymaleimide; cancer; kinase.

Cancer and Chemotherapy



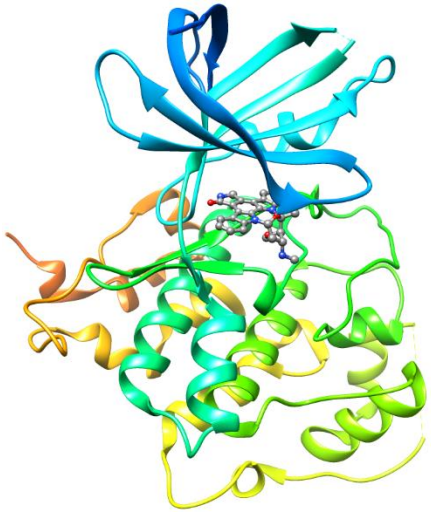


- Cancer refers multitude of disease states which share some common features such as that of uncontrolled, aggressive growth and invasion of other healthy tissues.
- In Ireland, it is estimated that someone gets a cancer diagnosis once every 3 minutes. There are over 40,000 new cancer diagnoses reported on an annual basis in Ireland alone.[1]
- It is projected that, by 2020, 1 in 2 Irish people will get a cancer diagnosis at some point in their lifetime.[1]
- Survival rates for cancer patients vary drastically between the different cancers. There are a multitude of treatments available but a lot of them are, unfortunately, not without their common and well known side effects.
- Many chemotherapeutic agents target all cells indiscriminately and this is a serious issue at the forefront of the clinic.
- There is an urgent need for new, targeted therapies.

1. https://www.cancer.ie/about-us/media-centre/cancer-statistics (accessed October 2018)

2. Manning, G. et al., *Science*, **2002**, 298, 1912
3. Kornev, A. P. et al., *Biochimica et Biophysica Acta*, **2010**, 1804, 440
4. Tamaoki, T. et al., *Biochem. Biophys. Res. Commun*, **1986**, 135, 397

Protein Kinases as Drug Targets

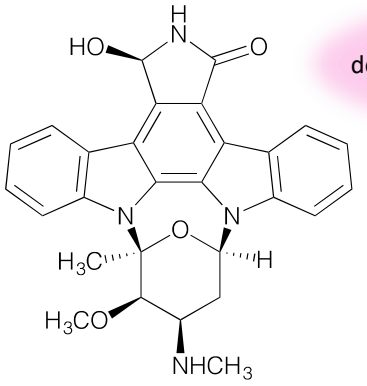


(Top) Staurosporine in complex with GSK-3β.
(Bottom) Structure of Staurosporine.

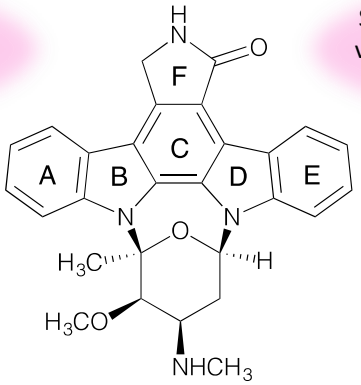
- Kinases are one of the largest family of proteins in humans with over 518 discovered to date. They play an intimate role in controlling many cellular functions and their deregulation has been implicated in oncogenesis and the progression of tumors.[2]
- Oncogenic kinases continuously activate signaling pathways that regulate cell cycle progression, proliferation and cell survival.
- Kinases commonly consist of an two lobes (an *N*-terminal and a *C*-terminal) with the cleft between these lobes forming the active site.[3]
- Staurosporine (STA) was found to be a potent but non selective inhibitor of a multitude of kinases (e.g. PKC IC_{50} = 2.7 nM).[4]
- Crystal structures of STA bound to kinases show that inhibition occurs in an ATP competitive manner.
- The ATP binding pocket is conserved across the kinome but exploitation of discreet differences in active site residues and conformations can help to confer selectivity.

The Indolo[2,3-*a*]carbazole (ICZ) Pharmacophore

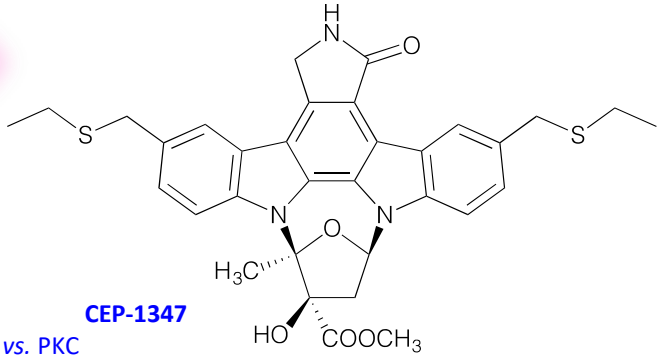
Staurosporine as a lead for kinase inhibition



Minor derivatisation of lactam F-ring



Substitution or variation of A/E rings



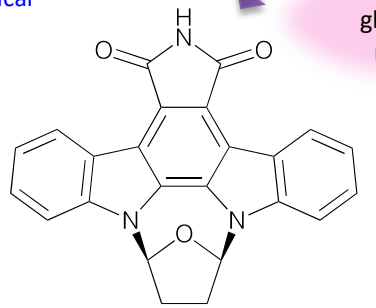
CEP-1347

- Inactive *vs.* PKC
- Potent JNK-1 inhibitor (IC_{50} = 30 nM)

UCN-01

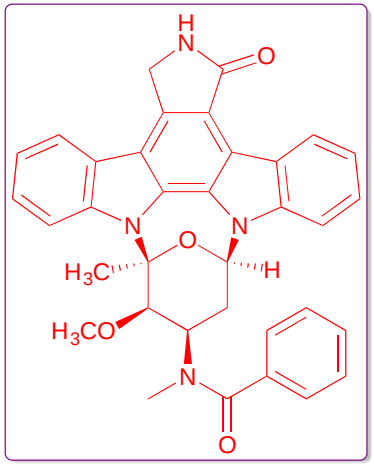
- Highly potent antiprolierative activity. Involved in clinical trials.
- PKC (IC_{50} – 10 nM)
- PDK1 (IC_{50} = 5 nM)

Modification of glycosidic moiety



SB-218078

- CHK1 selective (IC_{50} = 15 nM) *vs.* CDK1 (IC_{50} = 250 nM) and PKC (IC_{50} = 1000 nM)



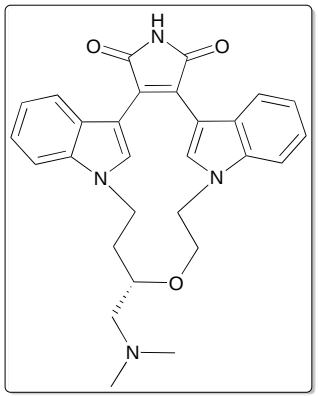
Midostaurin

- Clinically approved by the FDA in April of 2017 for the treatment of AML with an FLT-3 gene mutation

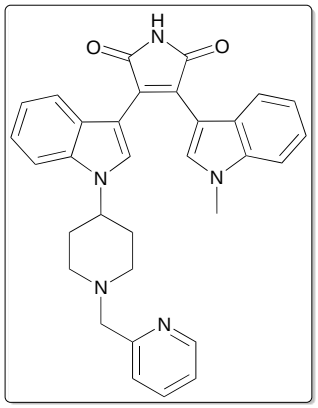
5. Frank, R. N. et al., *American Journal of Ophthalmology*, **2002**, 133, 693
6. Chen, Y. B. et al., *Expert Opinion on Investigational Drugs*, **2008**, 17, 939
7. Kuo, G. H., *J. Med. Chem*, **2003**, 46, 4021.

Bisindolylmaleimides (BIMs): Potent ICZ Precursors

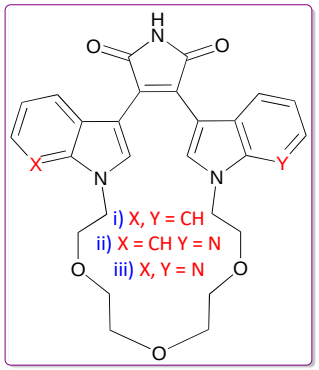
- Frequently used as synthetic precursors to ICZs. Disruption to ICZ planarity was found to confer remarkable selectivity against certain kinases.
- Ruboxistaurin has been reported to be a potent PKC specific inhibitor (below).[5]
- Enzastaurin is a highly potent inhibitor of PKCβ (IC_{50} = 6 nM) and the AKT/PI2 pathway.[6]
- Kuo *et al.* found that assimilation of 7-azaindole nucleus conferred notable activity profiles (below).[7]
- Evident that indole substitution and azaindole incorporation are important but elaboration of the maleimide pharmacophore in the SAR is unexplored.



Ruboxistaurin
PKC specific inhibitor:
$PKC\beta_1$ (IC_{50} = 4.7 nM)
PKCβ2 (IC_{50} = 5.9 nM)



Enzastaurin
Potent inhibitor of
PKCβ (IC_{50} = 6 nM) and
AKT/PI3 pathway

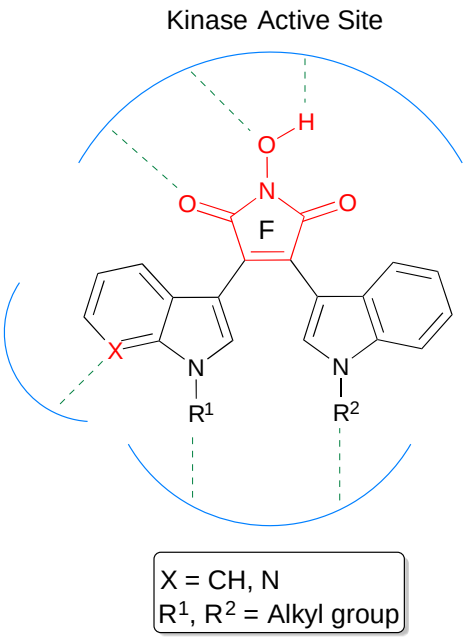


Aza BIMs by Kuo *et al.*
Azaindolyl assimilation
induced remarkable
GSK-3β selectivity.

GSK-3β
selectivity
achieved.

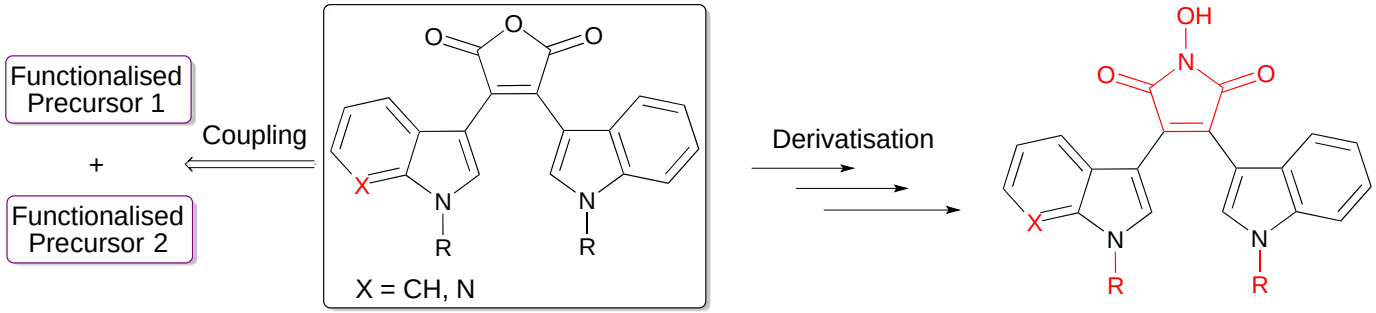
	IC_{50} (μM)		
Kinase Assay	i)	ii)	iii)
GSK3β	0.022	0.017	**0.034**
CDK1	1.251	4.428	>10
CDK2	0.922	2.439	>10
VEGFR	1.450	3.560	>10
PKCα	0.086	0.673	>10
PKCβII	0.006	0.046	1.163
PKCθ	0.065	0.835	>10
PKCγ	2.73	1.34	>10
PKA	>10	>10	>10

Aims and Objectives



- Primary objective is the exploration of the F-ring paradigm. Oxygen insertion into the maleimide N-H bond forms a central tenet of this in order to probe the effect of atomic incorporation.
- It is envisaged that such a modification would open up the potential to exploit new H-bonding contacts within the kinase active site.
- Indole *N*-substitution will seek to explore binding modes within the ribose pocket.
- 7-Azaindole incorporation will seek to ameliorate bioactivities and, in turn, achieve more selective kinase inhibition.
- Initial evaluation of antiproliferative activity is followed by further investigation of discrete biological mechanism of action through a kinase screen in collaboration with KISSf, Nantes, France.

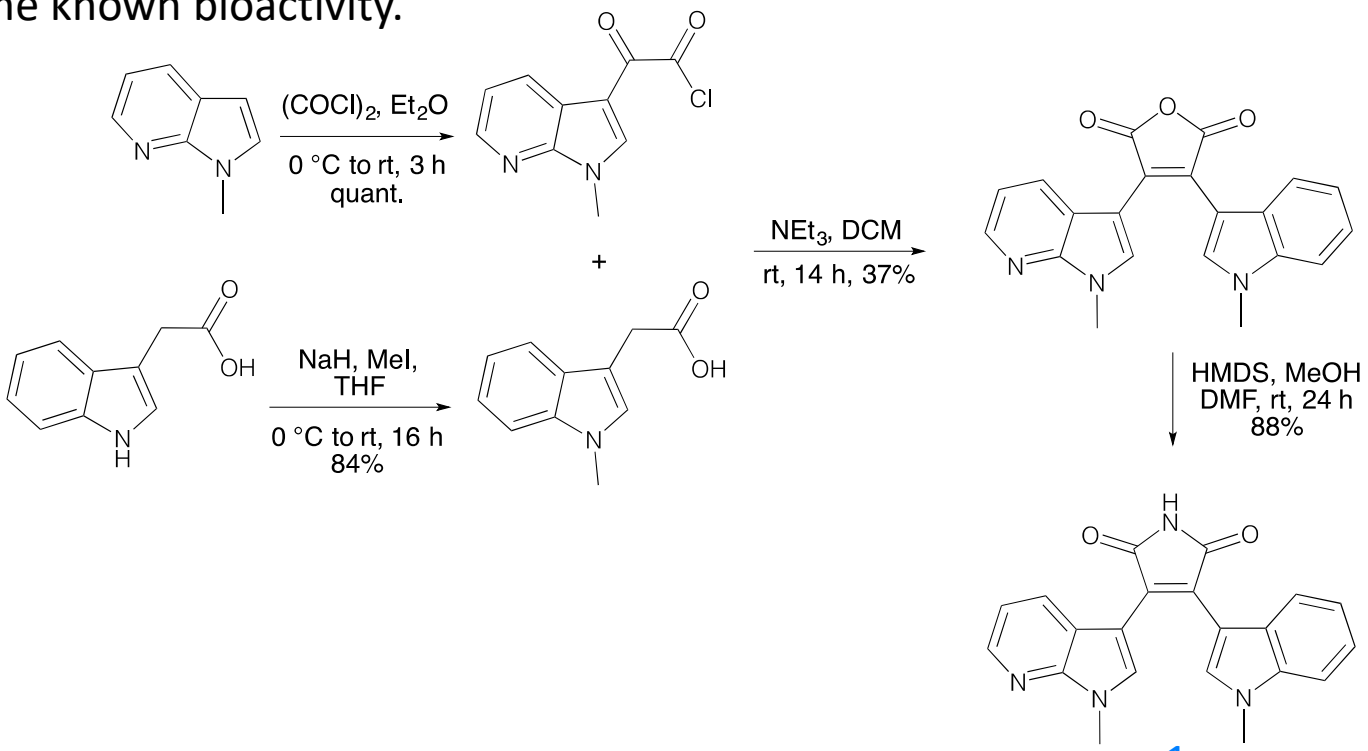
Synthetic Overview



- Synthesis is centred around a key, versatile, maleic anhydride intermediate.
- Application of a Perkin-type condensation will furnish these intermediates.
- Subsequent derivatisation and modification can be effectuated on these key intermediates in order to obtain a panel of novel BIM hydroxymaleimides.
- Starting from functionalised indole or 7-azaindole precursors will give rise to bisindolylmaleimides or bisazaindolylmaleimides.

Synthesis of Monoaza Maleimide

Immediate focus envisaged a bisindolylmaleimide and hydroxymaleimide in order to probe atomic incorporation. Our starting point incorporated an indole, a 7-azaindole and a maleimide component. Hence, the initial maleimide was formulated as a reference with some known bioactivity.

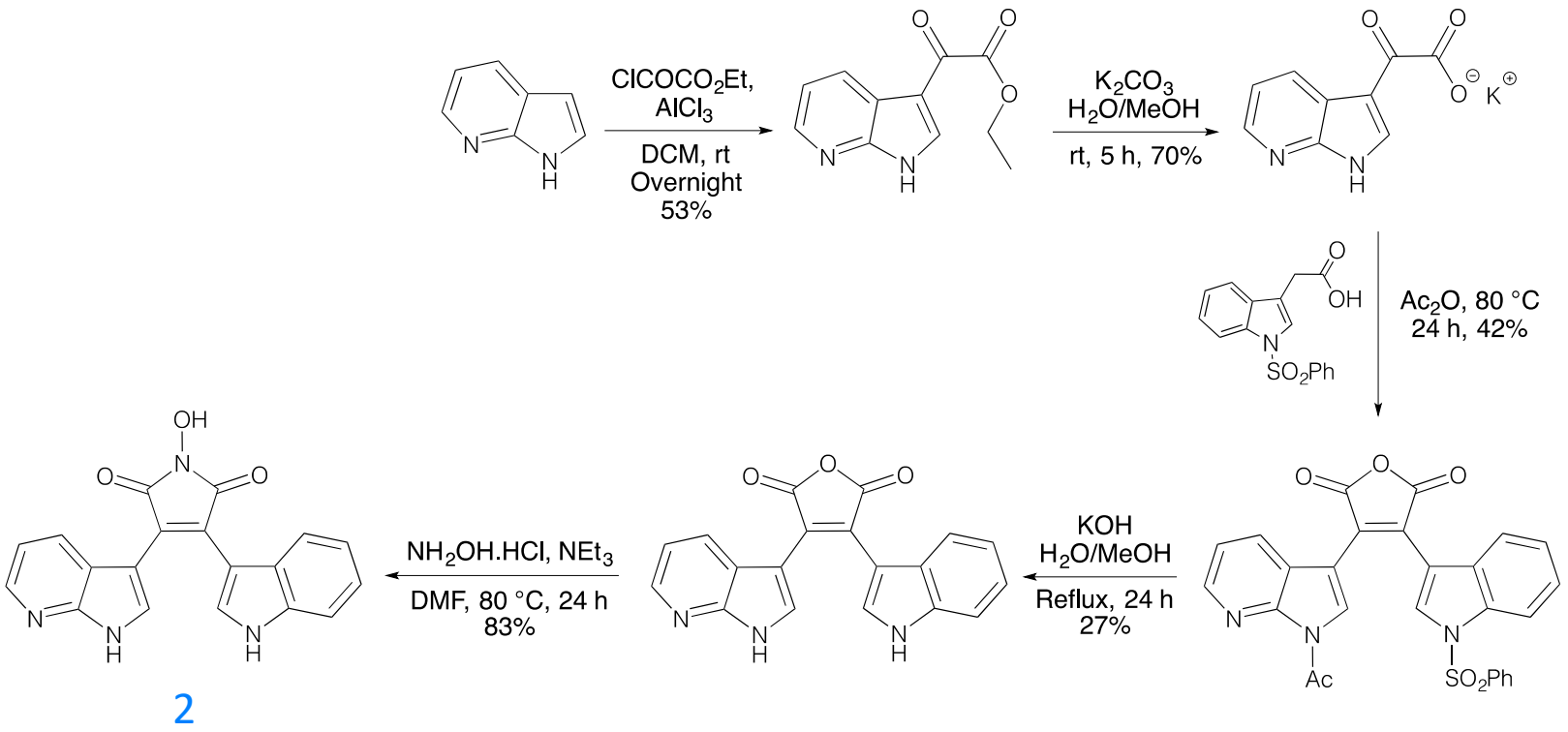


Winfield, H. J. et al, *Bioorg. and Med. Chem*, **2018**, 26, 4209

1

Synthesis of Monoaza Hydroxymaleimide

Synthesis of a related hydroxymaleimide was undertaken in order to probe the effect of oxygen insertion into the headgroup and the effect of indole substitution.



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Winfield, H. J. et al, *Bioorg. and Med. Chem*, **2018**, 26, 4209

Evaluation of Kinase Inhibitory Activity of Leads 1 and 2

- Preliminary evaluation of 1 and 2 was undertaken considering the common reports of BIMs as kinase inhibitors and the reported pharmacology of this class.
- GSK-3β has been shown to reduce apoptosis signals and may be useful for the treatment of Alzheimer's disease and for protection against cell death.
- In addition to this, there are also reports of BIM involvement with GSK-3β in the regulation of murine embryonic stem cell self-renewal so it is an obvious starting point.
- The evaluation of derivatives for their inhibition of cancer-related protein kinases is also of relevance: Haspin, Aurora kinase B, RIPK3, CDK2, CDK5, CDK9, DYRK1A, PIM1 and CK1δ.
- Results from one dose assay at 10 μM concentration confirmed that maleimide 1 was significantly more active than hydroxymaleimide 2.
- Significant inhibition was seen for 1 *vs.* CDKs in addition to GSK-3α/β and PIM1.
- By contrast, converting to 2 reduced CDK activity completely with significant inhibition only evident in PIM1 kinase and GSK-3α/β .
- This intriguing disparity and potential selectivity led to an exploration of the molecular space occupied by the headgroup and the indole substituents.

Winfield, H. J. et al, *Bioorg. and Med. Chem*, **2018**, 26, 4209

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The line colour in the table represents the kinase source:
Blue = Human
Green = Porcine

Kinase Single Dose Screen

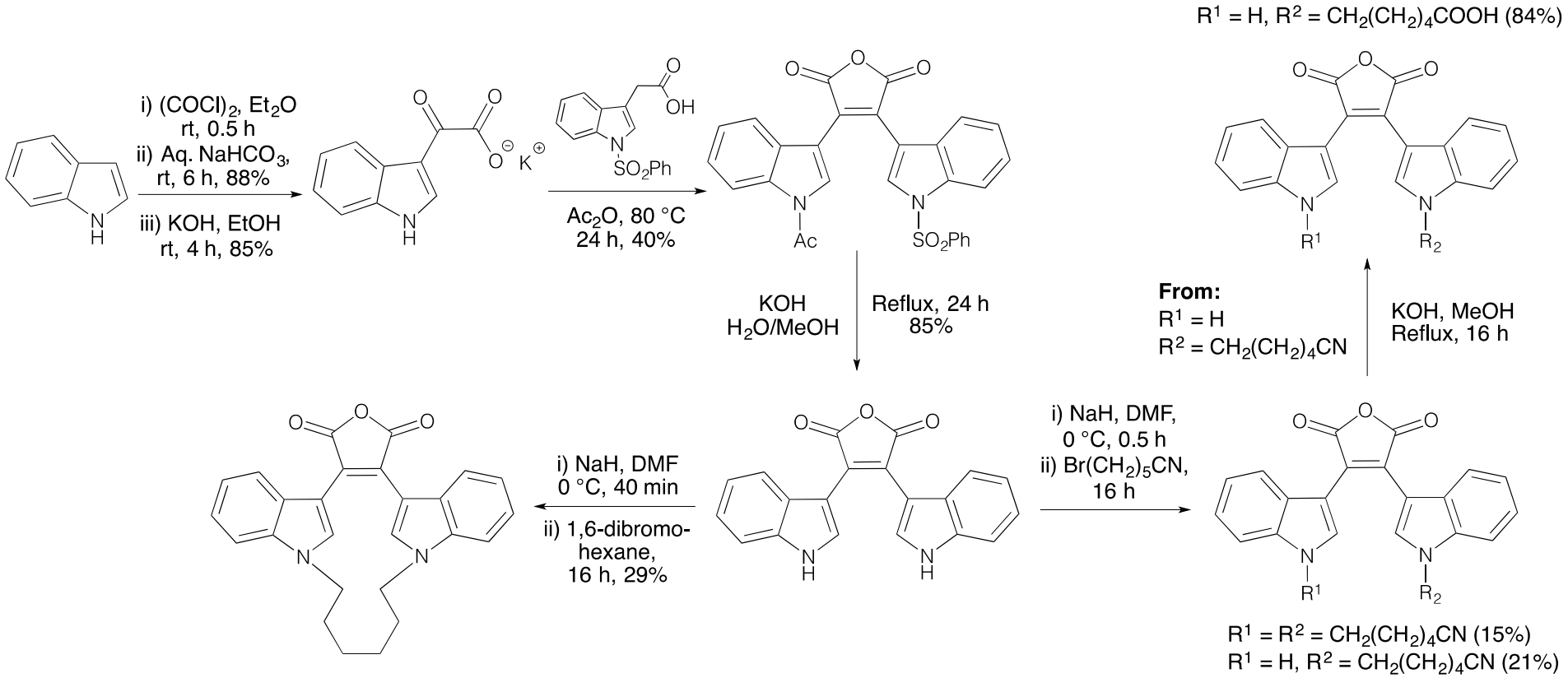
Kinase screen at 10 µM (values quoted are the residual % activity).

Compound	HASPIN	AURKB	RIPK3	CDK2/ CyclinA	CDK5/p25	CDK9/ CyclinT	DYRK1A	GSK-3α/β	PIM1	CK1δ/ε
1	101	84	79	-3	-1	-10	30	-21	1	76
2	98	61	102	85	87	89	73	45	16	41

- Significant inhibition was seen for 1 *vs.* CDKs in addition to GSK-3α/β and PIM1.
- By contrast, converting to 2 reduced CDK activity completely with significant inhibition only evident in PIM1 kinase and GSK-3α/β .
- This intriguing disparity and potential selectivity led to an exploration of the molecular space occupied by the headgroup and the indole substituents.

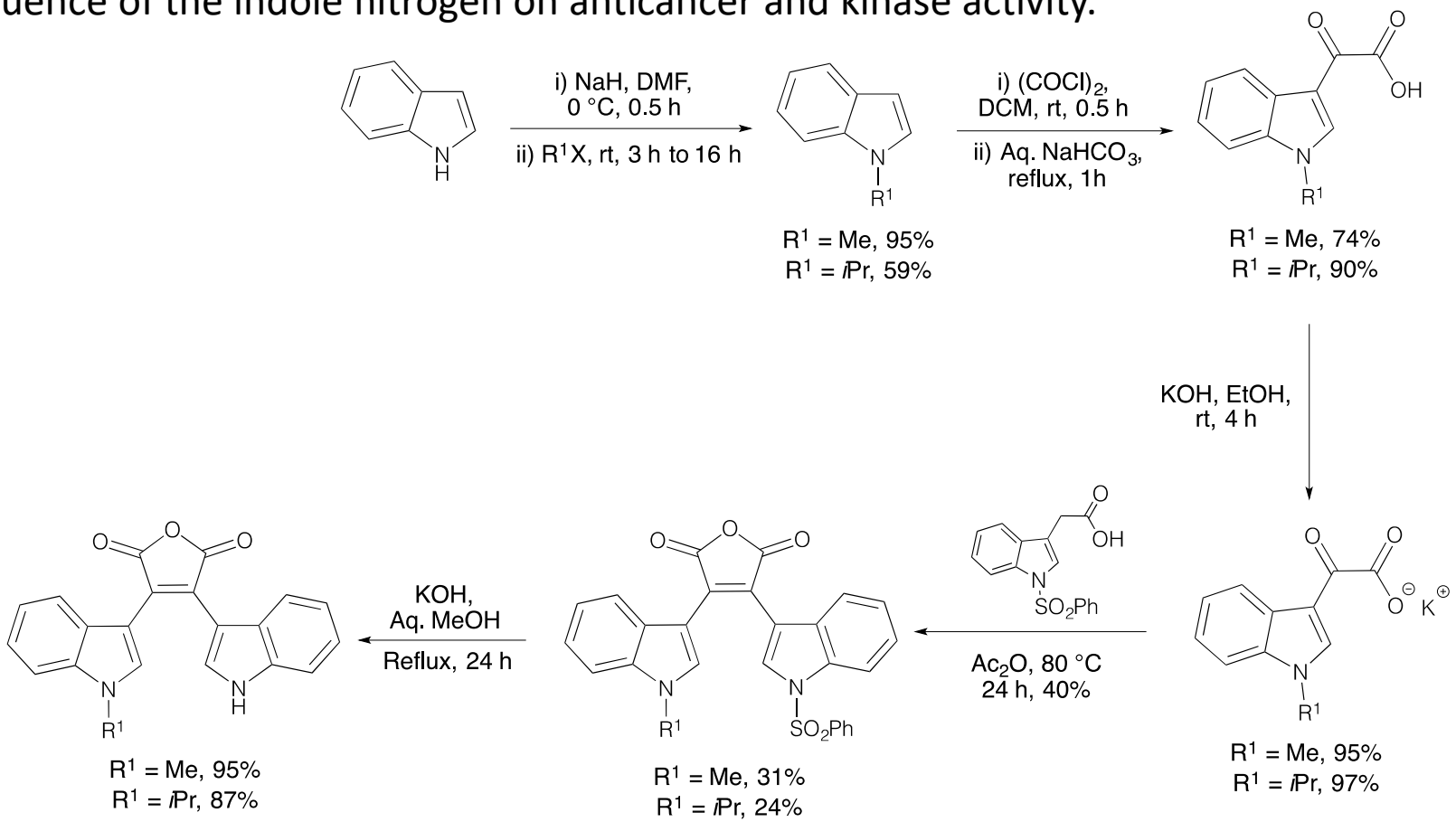
Synthesis of Novel Symmetrically Substituted Anhydrides

Subsequent focus aimed towards BIM series in order to probe *N*-substitution effects without any conflicting/competing effects from the nitrogen of 7-azaindole.

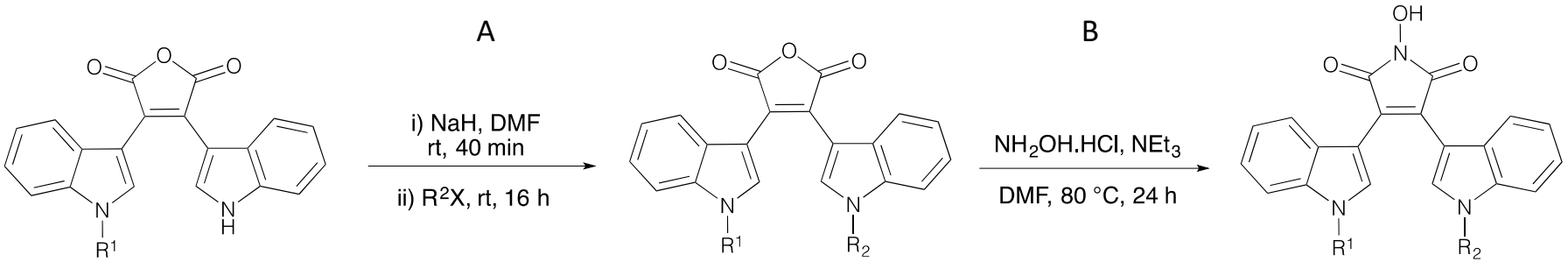


Synthesis of Novel Unsymmetrically Substituted Anhydrides

Route to incorporate individual indole functionality was devised in order to probe the influence of the indole nitrogen on anticancer and kinase activity.



Synthesis of Novel Bisindolylhydroxymaleimides



Formation of the final hydroxymaleimides was achieved upon treatment of the precursor maleic anhydrides with hydroxylamine hydrochloride in the presence of triethylamine and were generally furnished in excellent yields.

Compound (B)	R^1	R^2	Yield (A)	Yield (B)
3	Me	H	–	83%
4	Me	Me	80%	84%
5	Me	Et	72%	69%
6	Me	$(CH_2)_5CN$	40%	82%
7	Me	$(CH_2)_5COOH$	76%	79%
8	*i*Pr	H	–	77%
9	*i*Pr	Me	46%	93%
10	*i*Pr	Et	51%	80%
11	$-CH_2(CH_2)_4CH_2-$		–	82%
12	$(CH_2)_5CN$	$(CH_2)_5CN$	–	87%
13	$(CH_2)_5COOH$	H	–	86%
14	$(CH_2)_5CN$	$(CH_2)_5CN$	–	90%**

**Using HMDS rather than hydroxylamine to form the corresponding maleimide for reference purposes.

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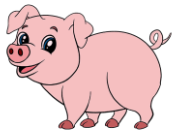
Kinase Assay

The line colour in the table represents the kinase source:
Blue = Human
Green = Porcine



IC_{50} values from the kinase inhibition assay (values quoted in µM).

Compound	HASPIN	AURKB	RIPK3	CDK2/CyclinA	CDK5/p25	CDK9/CyclinT	DYRK1A	GSK-3α/β	PIM1	CK1δ/ε
1	>10	>10	>10	0.10	0.80	0.08	>10	0.03	2.0	>10
2	>10	>10	>10	>10	>10	>10	>10	4.0	4.5	>10
3	>10	>10	>10	>10	>10	>10	>10	0.20	5.0	>10
4	>10	>10	>10	>10	>10	>10	>10	3.0	>10	>10
5	>10	>10	>10	>10	>10	>10	>10	0.30	>10	>10
6	>10	>10	>10	>10	>10	>10	5.00	0.27	>10	>10
7	>10	>10	>10	4.0	>10	0.70	1.00	3.20	>10	>10
8	>10	>10	>10	>10	>10	>10	>10	1.5	>10	>10
9	>10	>10	>10	7.0	>10	1.5	>10	0.20	>10	>10
10	>10	>10	>10	>10	>10	0.70	>10	0.40	>10	>10
11	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
12	>10	>10	>10	>10	>10	>10	>10	0.75	>10	>10
13	>10	>10	>10	>10	>10	2.00	2.00	0.75	>10	>10
14	>10	>10	>10	2.50	>10	0.42	0.62	0.12	>10	>10

Values shaded in orange represent activity <10 µM; values which are shaded in pink are <1 µM.

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Kinase Assay

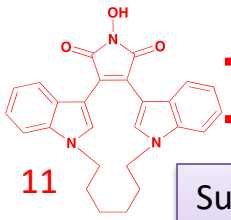
Most potent derivative. IC_{50}:
- 30 nM *vs.* GSK-3
- 80 – 800 nM *vs.* CDKs
- 2 µM *vs.* PIM1.

Kinases substantially affected by the panel

IC_{50} values from the kinase inhibition assay (values quoted in µM).

Compound	HASPIN	AURKB	RIPK3	CDK2/CyclinA	CDK5/p25	CDK9/CyclinT	DYRK1A	GSK-3α/β	PIM1	CK1δ/ε
1	>10	>10	>10	0.10	0.80	0.08	>10	0.03	2.0	>10
2	>10	>10	>10	>10	>10	>10	>10	4.0	4.5	>10
			>10	>10	>10	>10	>10	0.20	5.0	>10
			>10	>10	>10	>10	>10	3.0	>10	>10
			>10	>10	>10	>10	>10	0.30	>10	>10
			>10	>10	>10	>10	5.00	0.27	>10	>10
7	>10	>10	>10	4.0	>10	0.70	1.00	3.20	>10	>10
8	>10	>10	>10	>10	>10	>10	>10	1.5	>10	>10
9	>10	>10	>10	7.0	>10	1.5	>10	0.20	>10	>10
	>10	>10	>10	>10	>10	0.70	>10	0.40	>10	>10
	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
	>10	>10	>10	>10	>10	>10	>10	0.75	>10	>10
				>10	>10	2.00	2.00	0.75	>10	>10
14				2.50	>10	0.42	0.62	0.12	>10	>10

Small changes in the substitution patterns affect the ability to modulate phosphorylation in a discreet fashion.



11

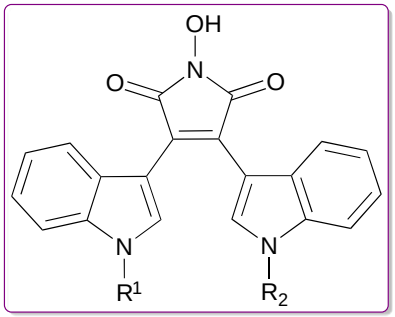
Surprisingly, no activity for macrocyclic derivative 11. (Highlighted in red lines).

Kinase Assay: Further Results of Note

- Hydroxymaleimides are active *vs*. GSK-3 irrespective of the indole substituent, suggesting the incorporation of anchoring substituents may be fruitful.
- Considering 1 *vs*. 2, the removal of *N*-substitution and hydroxymaleimide incorporation induced selective inhibition of GSK-3 and PIM1 (albeit at 4 - 4.5 µM).
- Comparison of 3 and 4 illustrates how the presence of one N-H gives rise to more potency against GSK-3 (IC_{50} = 200 nM).
- This observation is not constant. Alkyl extension as per 5, 6, 9 and 10 induces further potency underlining the positive influence of at least one bulky substituent.
- Comparing the methyl *vs.* propyl (3 – 5 *vs*. 8 – 10) shows how the increased steric bulk is well tolerated by GSK-3. Extension to hexanenitrile in 6 maintains GSK-3 potency and selectivity over CDKs.
- Conversion of 6 to its corresponding carboxylic acid derivative 7 improves potency towards CDK-9 but not GSK-3, together with the manifestation of DYRK inhibition.
- In addition, removal of the methyl substituent and going from 7 to 13 reverses this effect.
- Bishexanenitrile substitution as per 12 imbues selectivity of action over CDKs and inhibits GSK-3 at an IC_{50} of 750 nM. Derivative 14 is more potent but suffers from polypharmacology. Derivative 12 therefore serves as a lead for future endeavors.

Full Anticancer Evaluation

- Selected compounds were submitted for testing in collaboration with the NCI through the 60-cell screen.
- Compounds 1, 3 – 4, 6 – 9 and 11 – 14 were chosen in order to scope the breadth of structural diversity and kinase inhibition. These compounds were initially screened at a 10 µM dose against 60 cancer cell lines.
- The majority of these derivatives gave mean growth values between 70% and 100% at a single 10 µM dose.
- It is clear the indole *N*-substitution affects the activity.



Compound	R^1	R^2
3	Me	H
4	Me	Me
5	Me	Et
6	Me	$(CH_2)_5CN$
7	Me	$(CH_2)_5COOH$
8	*i*Pr	H
9	*i*Pr	Me
10	*i*Pr	Et
11	$-CH_2(CH_2)_4CH_2-$	
12	$(CH_2)_5CN$	$(CH_2)_5CN$
13	$(CH_2)_5COOH$	H
14	$(CH_2)_5CN$	$(CH_2)_5CN$

Full Anticancer Evaluation

Compound No. (NSC)	Mean Growth at 10 µM (%)	Growth of Selected Cell Lines					
		A498	SK-MEL-2	MDA-MB-435	LOX IMVI	UO-31	SR
1 (762129)	29	-20	-13	13	41	23	12
3 (775309)	78	79	76	17	51	41	31
4 (774887)	98	86	102	95	73	74	75
6 (776694)	91	77	94	68	60	51	67
7 (776691)	96	92	-	102	80	63	90
8 (775308)	94	94	87	102	59	68	86
9 (775307)	91	99	87	104	62	56	-
11 (781334)	103	88	120	111	79	80	92
12 (776693)	68	69	68	16	30	44	18
13 (776692)	98	89	99	102	80	61	100
14 (781333)	87	54	96	105	70	73	-

Compounds shaded purple were taken forward for five-dose screening by the NCI.

Full Anticancer Evaluation

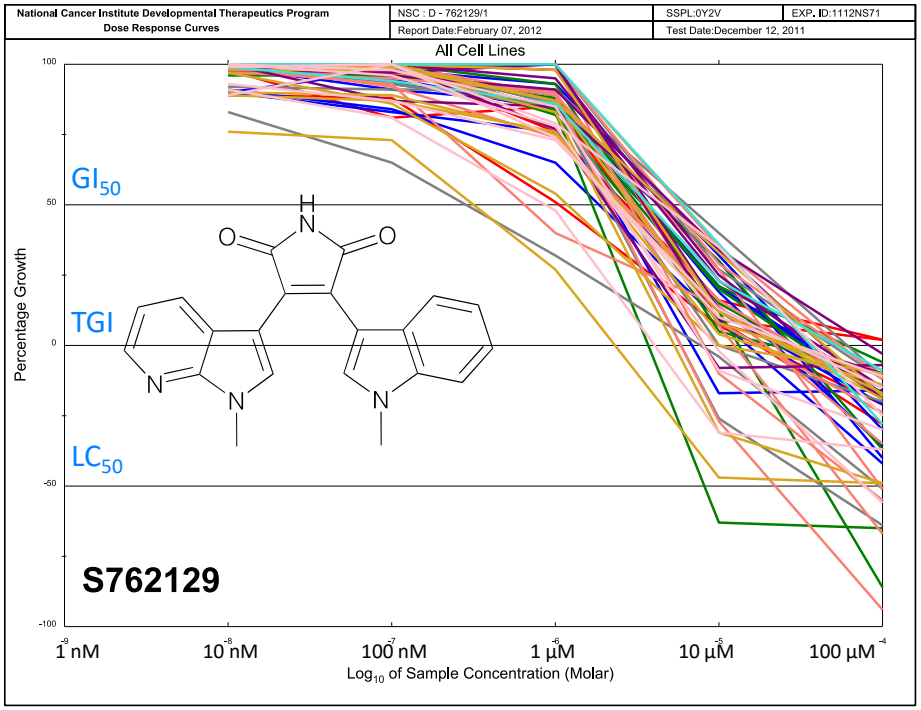
- Inhibition was observed for 3 towards SR (leukemia), MDA-MB-435 (melanoma), UO-31 (renal cancer) and MCF-7 (breast cancer).
- Assimilation of a second methyl substitutent 4 was detrimental to anticancer activity, with complete loss of activity. Some residual activity was noted towards SR, LOX IMVI (non-small cell lung cancer) and UO-31.
- Isopropyl containing derivatives 8 and 9 were also inactive but also showed a similar activity pattern towards LOX IMVI, UO-31 and most of the leukemia cell lines.
- Derivatives containing one extended alkyl chain with a nitrile or carboxylic acid functional group displayed little activity with nitrile 6 being slightly more active than acid derivatives 7 and 13. These compounds showed moderate selectivity for LOX IMVI and UO-31.
- Incorporation of a second nitrile chain as per 12 significantly increased activity. This particular compound progressed onto five-dose screening.
- Considerable activity was noted for 12 towards HL-60 and SR (leukemia), LOX-IMVI and MDA-MB-435 (melanoma), UO-31 (renal cancer) and MCF-7 (breast cancer) cell lines. Replacement of the hydroxymaleimide with a maleimide 14 surprisingly reduced activity

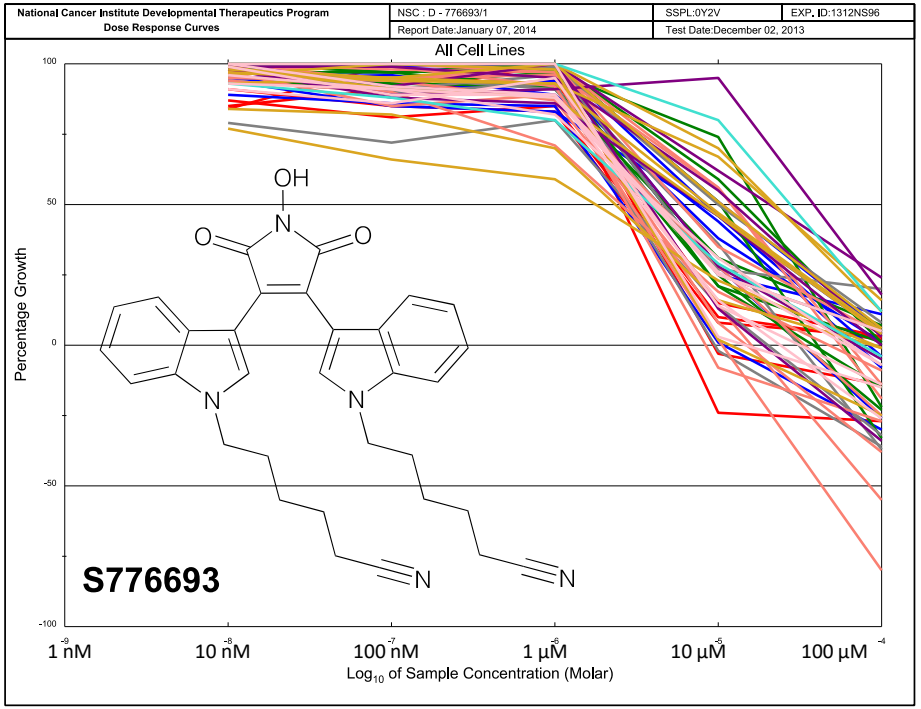
Full Anticancer Evaluation

- The most active compound from this set was 1, which was also submitted for five-dose screening along with 12.
- Derivative 1 has a mean GI_{50} value of 2.8 µM with no clear specificity for any particular cancer sub-type or cell phenotype.
- Cytostatic effect at 10 µM but this does not translate to cytotoxic at higher concentrations – which is typical of most compounds evaluated at five-dose.
- Cell lines with sub-micromolar GI_{50} values include SNB-75 (CNS), MDA-MB-435 (melanoma), A498 (renal) and HS-578 T (breast) cancer cell lines (shaded orange, slide 25) showing the spread of activity. Performance of 1 against SNB-75 and A498 is of interest for future studies however.
- Bishexanenitrile 12 has a relatively higher mean growth at single-dose in comparison to 1. However it posesses low micromolar GI_{50} values for a number of cell lines particularly those in the leukemia and melanoma group (shaded light green, slide 25).
- A number of values are lower for 12 than for 1 on transfer to cellular assay and this is of note. Most cell lines required conc. >100 µM for the death of 50% of the cell population (LC_{50}), with only SK-MEL-5 and UACC-62 showing any appreciable cytotoxicity (shaded purple, slide 25).

Full Anticancer Evaluation

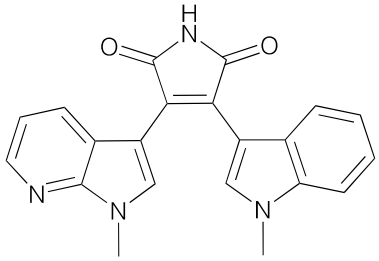
Dose Response Curves for derivatives 1 and 12



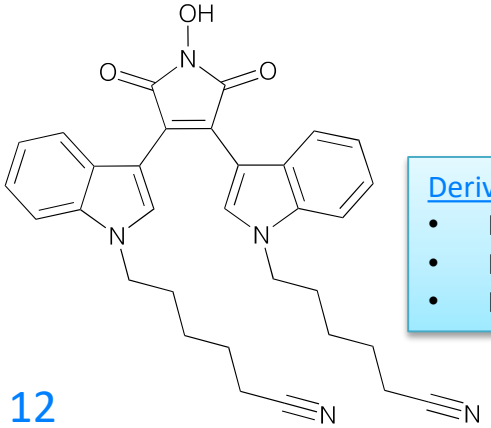


- Each compound was tested against the 60-cell line panel at concentrations ranging from 100 µM to 10 nM.
- Dose response curves were then generated for each cell line.
- Three *in vitro* parameters were then calculated: GI_{50}, TGI and LC_{50}.

Full Anticancer Evaluation



Derivative 1:
- Mean GI_{50} = 2.8 µM
- Mean TGI = 20.9 µM
- Mean LC_{50} = 89.1 µM



Derivative 12:
- Mean GI_{50} = 5.6 µM
- Mean TGI = 50.1 µM
- Mean LC_{50} = 97.7 µM

Selected GI_{50}, TGI and LC_{50} data for 1 and 12

Cancer subtype	Cell Line	GI_{50} (µM)		TGI (µM)		LC_{50} (µM)	
		1	12	1	12	1	12
Leukemia	HL-60 (TB)	4.05	2.54	17.9	6.44	>100	>100
	SR	1.04	3.55	18.4	9.48	>100	>100
Melanoma	MDA-MB-435	0.648	2.74	19.3	8.31	>100	>100
	SK-MEL-5	2.15	2.92	5.80	10.6	21.8	42.8
	UACC-62	4.22	3.23	22.0	16.0	96.7	84.4
CNS	SNB-75	0.291	2.84	7.97	20.0	94.6	>100
Breast	HS-578T	0.847	3.97	4.06	>100	>100	>100
Renal	A498	0.320	2.36	2.34	86.0	>100	>100

- Light orange: submicromolar GI_{50} values of 1.
- Light green: highlighted activity of 12 *vs.* leukemia and melanoma cancer cell lines
- Purple: highlighted LC_{50} values of SK-MEL-5 and UACC-62 cancer cell lines.

Conclusions

- Molecular space around the BIM pharmacophore has been explored through the synthesis and evaluation of 28 novel derivatives, with the subsequent development of anticancer leads.
- Elimination of the polypharmacology associated with the maleimide pharmacophore has been achieved through oxygen insertion into the N-H bond.
- Changes to the indole nucleus suggest that 7-azaindole incorporation is most beneficial to GSK-3 and CDK activity as the maleimide 1. Oxygen insertion into the maleimide N-H bond of aza derivative 2 removes the polypharmacology associated with the maleimide headgroup and retains activity towards GSK-3 and PIM1.
- Conformational restriction through the incorporation of a connecting macrocycle disappointingly has no benefit with a complete loss of kinase activity noted.
- Bishexanenitrile substituted BIM 12 now serves as a valuable lead towards future synthetic endeavors. Excellent GSK-3 kinase activity and progression to five dose screening suggest GSK-3 as a potential mode of its anticancer activity.
- Oxygen insertion into the maleimide N-H bond is well tolerated and capable of low nanomolar kinase inhibition (in particular GSK-3 kinase).

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