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Synthesis and Structure Activity Relationship Studies of Small Molecule Disruptors of EWS- FLI1 Interactions in Ewing's Sarcoma

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42 **KEYWORDS:** Ewing's Sarcoma, EWS-FLI1, YK-4-279, SAR, Biotinylated Conjugate
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ABSTRACT:

EWS-FLI1 is an oncogenic fusion protein implicated in the development of Ewing's sarcoma family tumors (ESFT). Using our previously reported lead compound **2** (YK-4-279), we designed and synthesized a focused library of analogues. The functional inhibition of the analogues were measured by an EWS-FLI1/ NR0B1 reporter luciferase assay and a paired cell screening approach measuring effects on growth inhibition for human cells containing EWS-FLI1 (TC32 and TC71) and control PANC1 cell lines devoid of the oncoprotein. Our data revealed that substitution of electron donating groups at the *para*-position on the phenyl ring was the most favorable for inhibition of EWS-FLI1 by analogs of **2**. Compound **9u** (with a dimethylamino substitution) was the most active inhibitor with a $GI_{50} = 0.26 \pm 0.1 \mu\text{M}$. Further, a correlation of growth inhibition (EWS-FLI1 expressing TC32 cells) and the luciferase reporter activity was established ($R^2 = 0.84$). Finally, we designed and synthesized a biotinylated analogue and determined the binding affinity for recombinant EWS-FLI1 ($K_d = 4.8 \mu\text{M} \pm 2.6 \mu\text{M}$).

INTRODUCTION

Ewing's sarcoma (ES) is a malignant tumor in adolescents and young adults¹ and presents with a high propensity for metastasis, particularly to lung, bone and bone marrow. ES is an orphan disease with more than 200 new cases diagnosed in the United States each year. ES arises from the mesenchymal tissues occurring mostly in the bones, however approximately 10% of the tumors arise in the soft tissues of the chest wall, pelvis and leg²⁻⁴. Currently, adjuvant therapy is required to treat both the primary tumor and the presumed microscopic metastasis. The intensive combination of chemotherapy, surgery and radiation has increased the disease-free survival rate to 70% in patients with localized ES. Patients with metastatic ES have a poor prognosis⁵ and thus the development of specific therapeutic agents targeting ES is urgently needed.

ES are characterized by a nonrandom balanced chromosomal translocation involving the EWS gene and one of the several ETS family of transcription factor genes mainly FLI1⁶, resulting in the oncoprotein EWS-FLI1. The most common translocation in ES is the t(11;22)(q24;q12) translocation and occurs in 90-95% of ES cases. The high frequency of this translocation suggest that the product plays a significant role in the origin of these malignancies⁷.

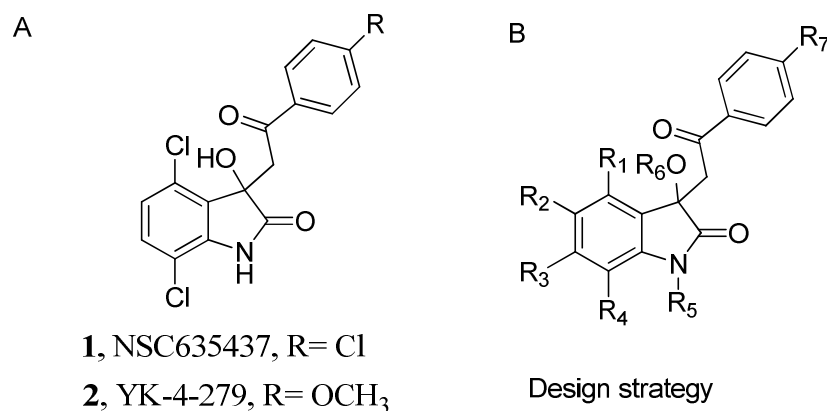
EWS-FLI1 consists of the N-terminal RNA-binding domain of EWS and the C-terminal DNA-binding domain of transcription factors such as ETS family^{8,9}. During this fusion, the transactivation domain of FLI1 is replaced by the N-terminal region of EWS converting FLI1 into an oncoprotein. Elimination of EWS-FLI1 through small interfering and antisense RNA results in the prolonged survival of ES xenograft-bearing mice¹⁰. Consequently, this oncoprotein may provide a unique target in the development of therapeutics against ES.

EWS-FLI1 is predicted to be an intrinsically disordered protein (IDP), i.e. lacks secondary and

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3 tertiary structure under physiological conditions¹¹. The disordered nature of EWS-FLI1 is
4 necessary for optimal transactivation of transcription¹² and the formation of the protein-protein
5 complexes that lead to oncogenesis¹³. Targeting transcription factors requires either the
6 disruption of specific DNA-protein or protein-protein interactions¹⁴. However, due to their
7 higher induced-fit sampling properties, IDPs have potential for binding to small molecules and
8 may possess potentially multiple binding sites for small molecules¹⁵.

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10 We previously reported the discovery of NSC635437 (**1**), identified through screening of small
11 molecules against recombinant EWS-FLI1 using surface plasmon resonance, and we developed
12 YK-4-279 (**2**) with limited SAR (Figure 1A). Both compounds bind EWS-FLI1 and disrupt
13 EWS-FLI1/RHA interaction¹⁷. We have shown that **2** specifically and stereoselectively¹⁸ inhibits
14 the function EWS-FLI1 and induces apoptosis both *in vitro* (GI₅₀ = 0.5-2.0 μM in ES cells) and
15 *in vivo*¹⁷.

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17 In this study, we report the full SAR studies of **2** against two Ewing's Sarcoma cell lines (TC32
18 and TC71) and the transcriptional inhibition of EWS-FLI1. Finally, we designed and synthesized
19 a novel biotinylated analogue of an active inhibitor to measure binding affinity to EWS-FLI1.
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18 **Figure 1:** A. Structures of EWS-FLI1 inhibitors: **1** = NSC635437, GI₅₀ = 20 μM in TC32 cells;
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20 **2** = YK-4-279, K_d = 9.48 μM, GI₅₀ of 0.5-2.0 μM in several ES cells. B. Sites for modifications.
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25 RESULTS

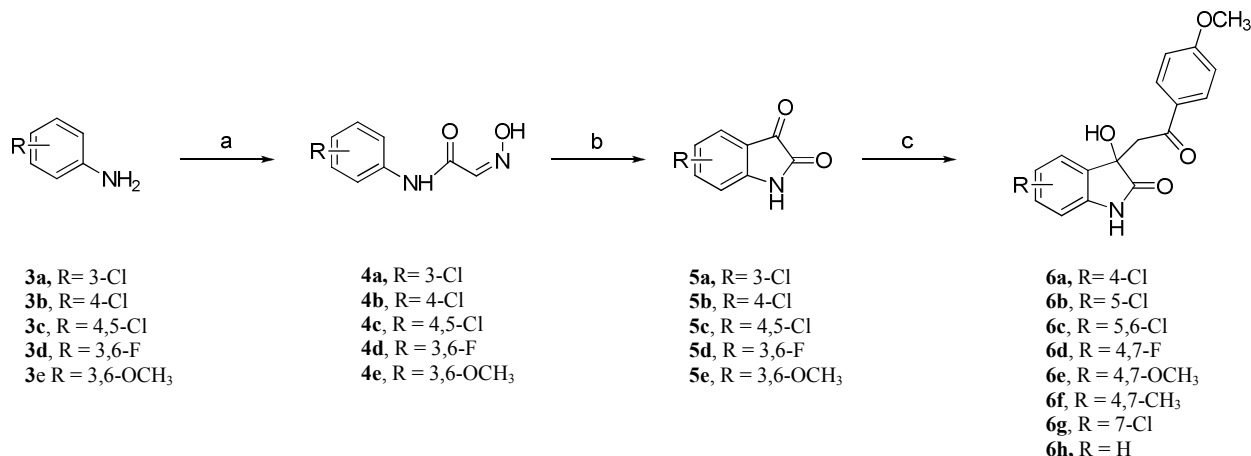
26 Chemistry

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30 Our previous study led to the discovery of compound **1** (Figure 1), 4,7-dichloro-3-(2-(4-
31 chlorophenyl)-2-oxoethyl)-3-hydroxyindolin-2-one, which displayed anti-proliferative activity
32 against various ES cell lines with GI₅₀ of 20 μM in TC32 cells¹⁷. The aromatic *para*-methoxy
33 substitution on the phenyl ring afforded compound **2** which exhibited an increased activity with
34 GI₅₀ of 0.9 μM in TC32 cells¹⁷.
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37 Since EWS-FLI1 is more than 75% disordered¹⁹, a lack of crystal structure or homology model
38 of the protein²⁰ requires a ligand based approach. Thus, we designed a patterned structural study
39 of **2**, to obtain structure activity information and to investigate the electrostatic, steric and
40 hydrogen bonding requirements for inhibition.
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51 To explore the importance of the 4,7-dichloro substituent group on the indoline ring of **2**, we
52 synthesized indolin-2-one substituted compounds **6a-h** (Scheme 1). A corresponding aniline (**3a-**
53 **e**) was reacted with chloral hydrate and hydroxylamine hydrochloride in aqueous sodium sulfate
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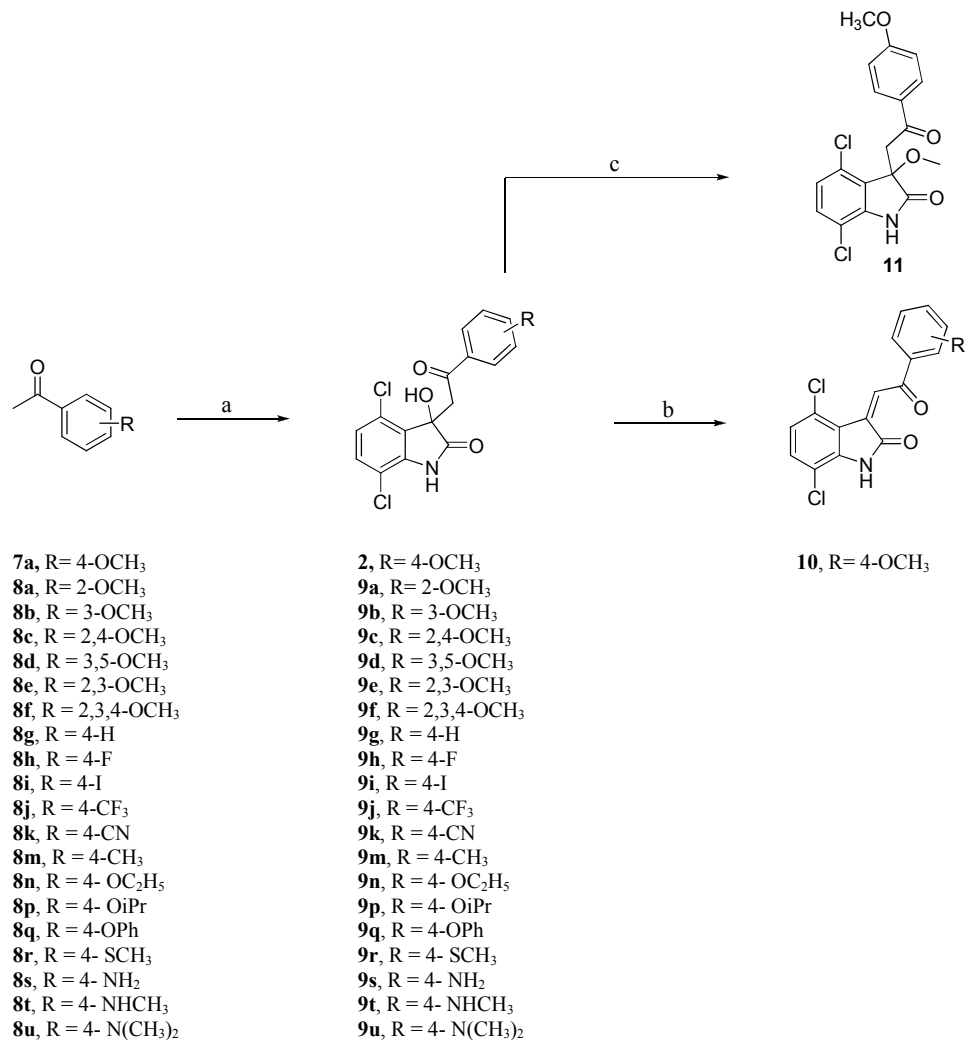
to form isonitrosoacetanilide (**4a-e**) followed by a Sandmeyer reaction with concentrated sulfuric acid to afford the desired isatin (**5a-e**) in good yield (55-90 %) ^{21,22}. We observed that maintaining the temperature at 55 °C is necessary for the synthesis of the 4,7-difluoro and 4,7-dimethoxy substituted intermediates. Isatins **5f-h** were commercially available and the aldol reaction was performed in methanol (with Et₂NH as a catalyst) to give **6a-h** in excellent yield (90-100 %).



Scheme 1. Reagents and conditions: (a) Cl₃CCH(OH)₂, NH₂OH.HCl, Na₂SO₄, H₂O, HCl, 55 °C, overnight, (b) H₂SO₄, 55 °C to 80 °C, (c) 4-methoxy-acetophenone, Et₂NH, MeOH, rt, 90-100 %.

The synthetic strategy for substituting the phenyl ring of **2** is shown in Scheme 2. In general, commercially available 4,7-dichloroisatin was condensed with a substituted acetophenone (**8a-u**) in the presence of a catalytic amount of Et₂NH to afford **9a-u** in excellent yield (90-100 %) ²³.

To understand the importance of the hydroxyl group in the aldol product and to assess the potential for an *in vivo* dehydration reaction, we synthesized the dehydrated compound **10**. This was accomplished by treating **2** with concentrated sulfuric acid as shown in Scheme 2 (58 % yield) ^{23,24}. Of interest was the report that this methodology affords the *Z* isomer ²⁴. We confirmed and assigned the *Z* configuration of **10** by X-ray crystallography (Figure 2).



Scheme 2. Reagents and conditions: (a) 4,7-dichloroisatin, Et₂NH, MeOH, rt, 90-100 %; (b) H₂SO₄, 0 °C, 5 mins, 58 %; (c) Ag₂O, THF, MeI, rt, 58 %.

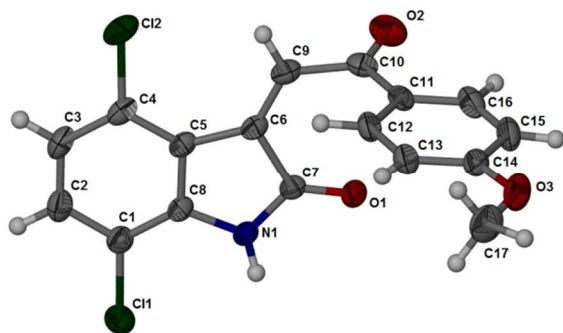
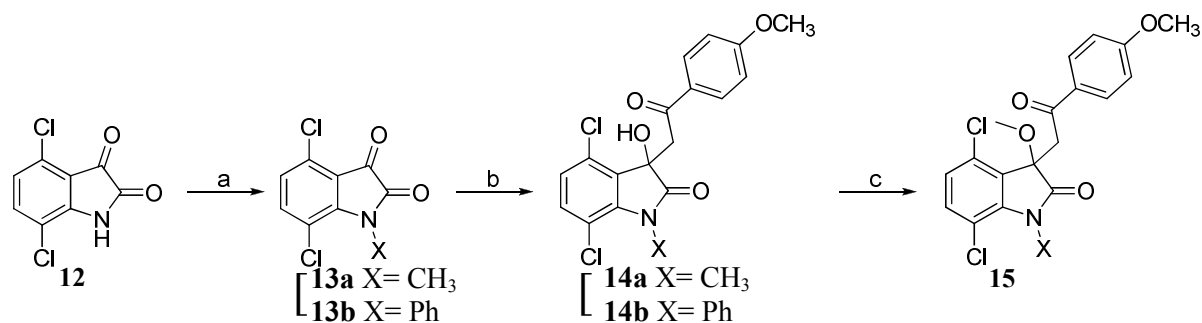


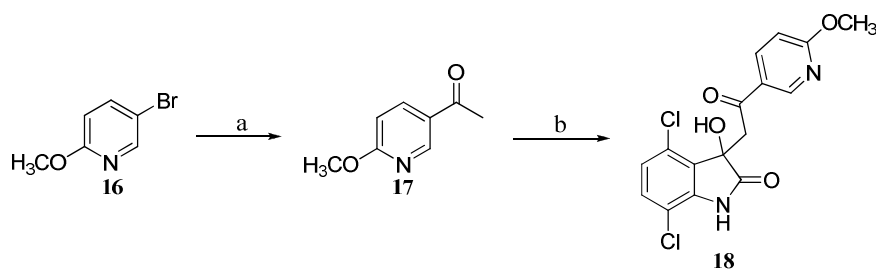
Figure 2: X-ray single crystal structure of **10**, the dehydration product of **2**; the structure determination establishes the *Z* stereochemistry of **10**.

Hydrogen bonding has an important role in ligand receptor interactions²⁵. To check the involvement of the indoline hydroxyl and amine group in potential hydrogen bonding, we synthesized the methylated compounds **11**, **14a-b** and **15**. Compound **11** was synthesized through the O-methylation of **2** by treating the aldol with methyl iodine in silver oxide to afford **11** with a yield of 58 % (Scheme 2)²⁶. Compound **14a** was synthesized by N-methylating the isatin (Scheme 3). The double methylated compound **15** was obtained by treating **14a** with methyl iodine in silver oxide.



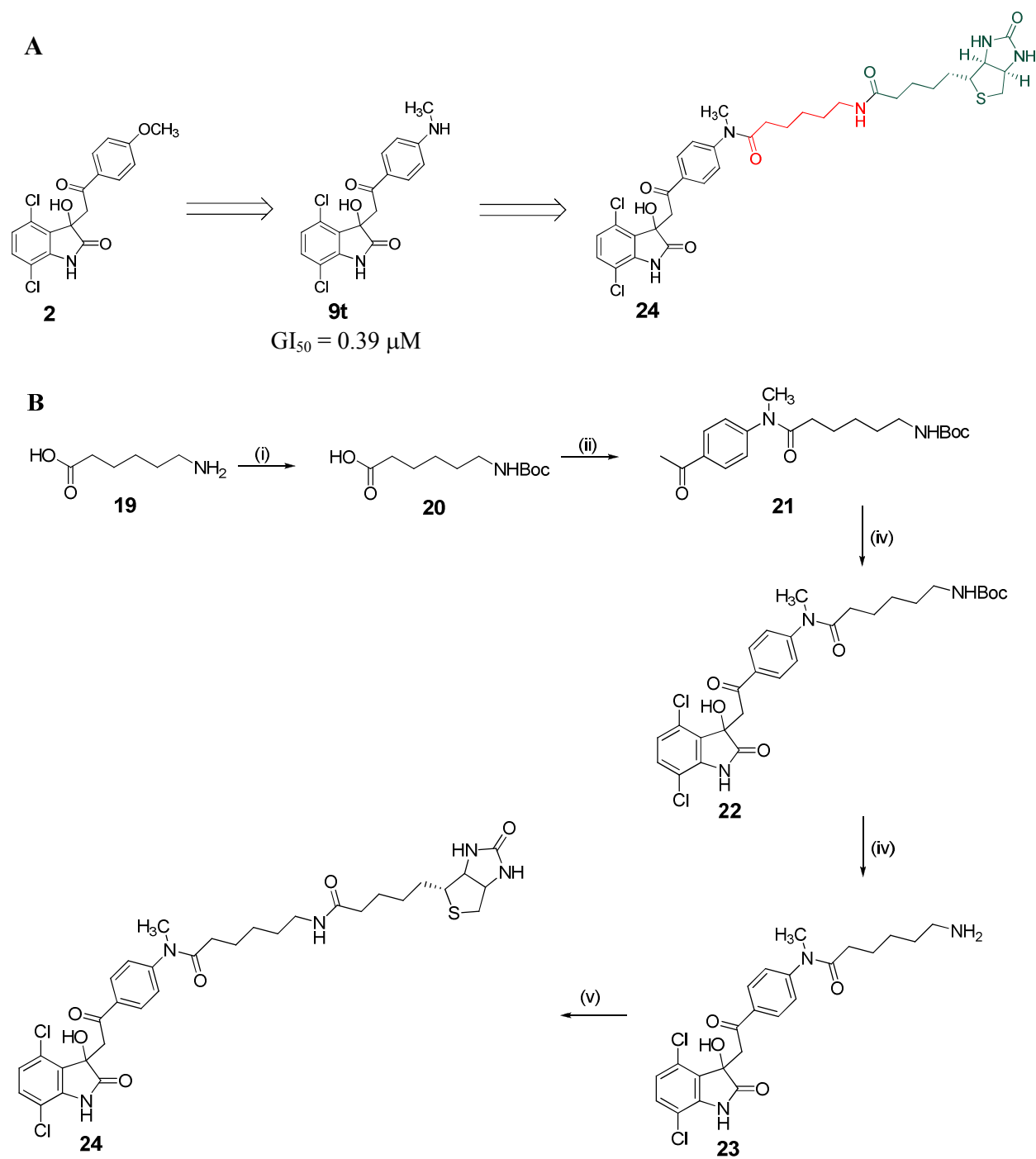
Scheme 3. Reagents and conditions: (a) NaH, MeI, DMF, 0 °C to rt, 54.5 %; (b) 4-methoxyacetophenone, Et₂NH, MeOH, rt, 51-100 %; (c) Ag₂O, THF, MeI, rt, 56 %.

We synthesized analog **18** with a pyridine ring instead of the phenyl ring in an attempt to increase the solubility of the lead compound. The synthesis of **18** began with a bromine lithium exchange in the reaction of **16** with acetaldehyde in the presence of n-BuLi. The resulting alcohol was oxidized in PDC and 4Å molecular sieves to afford **17**. Reaction of **17** with 4,7-dichloroisatin afforded the desired compound **18** in 90 % yield (Scheme 4).



Scheme 4. Reagents and conditions: (a) 1. Acetaldehyde, n-BuLi, THF, -78 °C; 2. PDC, 4Å Mol. Sieves, CH₂Cl₂, 0 °C, (b) 4,7-dichloroisatin, Et₂NH, MeOH, rt, 90 %.

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3 To further assess binding affinity of these derivatives with EWS-FLI1, we synthesized a biotin
4 conjugate of **9t** (Scheme 5). The synthesis started with the Boc (tert-butoxycarbonyl) protection
5 of the 5-aminopentanoic acid (**19**) followed by coupling with 4-acetyl-N-methyl aniline with
6 DCC to give **21**. An aldol addition of **21** with 4,7-dichloroisatin yielded **22** followed by its
7 deprotection to afford **23**. EDCI coupling of biotin to **23** provided the final biotin conjugate
8 analogue **24** in 49% yield.
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Scheme 5. (A) The design strategy of the biotinylated compound **24**. (B) Reagents and conditions (i) $(\text{Boc})_2\text{O}$, Et_3N , MeOH , 60°C , overnight, 90 %; (ii) 4-acetyl-N-methylaniline, DCC , DMAP , CH_2Cl_2 , 0°C to rt , 58 %; (iii) 4,7-dichloroisatin, Et_2NH , MeOH , rt ; 95% (iv) 3:1 CH_2Cl_2 : TFA , rt , 1.5 h; (v) biotin, $\text{EDCI}\cdot\text{HCl}$, DMAP , HOBt , CH_2Cl_2 , 0°C to rt , 49%.

RESULTS AND DISCUSSION

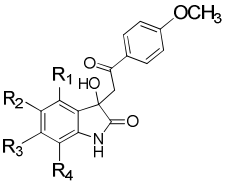
The anti-proliferative properties of the derivatives were assayed by monitoring their ability to inhibit cell growth using a water soluble tetrazolium (WST) assay (see experimental section). Anti-proliferative activities of the small molecules were determined as described in the experimental section in the two human ES cell lines (TC32 and TC71) and their GI_{50} 's were evaluated. The results are summarized in Tables 1-5. Compound **2** was used as comparison in the parallel experiments.

Compounds **6a-h** were synthesized to investigate the importance of different substituent groups in the indoline ring as compared to **2** (Table 1). Of all the compounds in this series, only the dimethoxy substituted compound (**6e**) retained the same potency as **2** (GI_{50} 1.0 μ M) in TC32 cells. Since both electron withdrawing (-Cl) and donating group substitutions (-OCH₃) in the R₁ and R₄ positions were active, differences in steric contributions were considered. Increased size of the aromatic substitution in the R₁ and R₄ positions (compound **6d** versus **2**) appears to contribute to improved inhibitory activity. Because **6e** does not significantly improve the pharmacological properties of **2**, we kept the 4,7-dichloro substitutions on the indoline ring for the rest of our study.

Compound **2** contains a chiral center. In a previous study, we found that the S-(-) was 4-fold (GI_{50} = 0.28 μ M) more potent *in vitro* than the racemic **2**. The R-(+) was almost inactive *in vivo*¹⁸. To further understand the influence of the hydroxyl group and the potential dehydration *in vivo*, we synthesized the dehydrated analog of **2**. The dehydrated compound **10** was as potent as the parent compound **2** (GI_{50} 1.0 μ M in TC32), suggesting that the hydroxyl group is not required for binding. We prepared derivative **11** in which the hydroxyl group was methylated to eliminate potential hydrogen bond donation. This chemical transformation resulted in a complete

loss of inhibitory activity. Further methylations performed are summarized in Table 2. The methylation of the amide group, represented by compound **14a**, resulted in a two-fold reduction in TC32 cell GI₅₀. Furthermore, the replacement of the proton on the amide with a phenyl group (**14b**) resulted in the complete loss of activity apparently due to steric effects. The O-methylated and N-methylated compound **15** was inactive.

Table 1. Chemical Structure and Biological Activities of Compounds **6a-6i**

	Substitutions				GI ₅₀ (μM)		
	R ₁	R ₂	R ₃	R ₄	PANC 1	TC32	TC71
6a	-Cl	-H	-H	-H	NI ^a	NI	NI
6b	-Cl	-Cl	H	-H	NI	NI	NI
6c	-H	-Cl	-Cl	-H	NI	NI	NI
6d	-F	-H	-H	-F	NI	NI	NI
6e	-OCH ₃	-H	-H	-OCH ₃	NI	1.0 ^b	0.94 ^b
6f	-CH ₃	-H	-H	-CH ₃	NI	NI	NI
6g	-H	-H	-H	-Cl	NI	NI	NI
6h	-H	-H	-H	-H	NI	NI	NI

^aNI: No significant inhibition (GI₅₀ > 10 μM). ^bExperimental error is within 20%.

Table 2. Chemical Structure and Biological Activities of O-Methylation and N-Methylation

	Substitutions		GI ₅₀ (μM)		
	R ₁	R ₂	PANC 1	TC32 ^b	TC71 ^b
2	H	H	NI ^a	0.94	0.92
11	-CH ₃	H	NI	NI	NI
14a	H	-CH ₃	NI	2.2	0.71
14b	H	-Ph	NI	NI	NI
15	-CH ₃	-CH ₃	NI	NI	NI

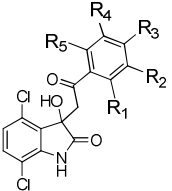
^aNI: No significant inhibition (GI₅₀ > 10 μM). ^bExperimental error is within 20%.

As previously described, we synthesized **18** by replacing the electron rich phenyl ring by an electron deficient pyridine ring. This resulted in a complete loss of growth inhibition for the EWS-FLI1 containing cells lines (data not shown).

We further evaluated the positional effect of the methoxy moiety on the phenyl ring, and the results are summarized in Table 3. The data showed that substitution with 2-methoxy, 3-methoxy and the 2,3-methoxy (**9a**, **9b** and **9e**) reduced inhibition of cell growth. The 3,5-dimethoxy derivative **9d** also resulted in a nearly 10-fold loss of growth activity in TC32 cells. Interestingly, the 2,4-dimethoxy substitution (**9c**) resulted in a GI₅₀ of 2.6 μM but the 2,3,4-trimethoxy derivative (**9f**) resulted in a 3-fold loss of the growth activity as compared to **2**. Taken together, these data suggested that the *para* position contributes more to the potency whereas the *meta* and

ortho substitutions decrease the inhibitory activity. We concluded that the mono substitution at the *para* position on the aromatic ring plays a significant role in the inhibition of EWS-FLI1 in ES cells.

Table 3. Chemical Structure and Biological Activities of Compounds **9a-9f**

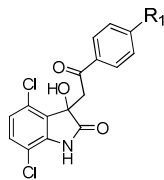
	Substitutions				GI ₅₀ (μM)		
	R ₁	R ₂	R ₃	R ₄	PANC 1	TC32 ^b	TC71 ^b
2	H	H	OCH ₃	H	NI*	0.94	0.92
9a	OCH ₃	H	H	H	NI	NI	NI
9b	H	OCH ₃	H	H	NI	NI	NI
9c	OCH ₃	H	OCH ₃	H	NI	2.6	2.8
9d	H	OCH ₃	H	OCH ₃	NI	9.0	NI
9e	OCH ₃	OCH ₃	H	H	NI	NI	NI
9f	OCH ₃	OCH ₃	OCH ₃	H	NI	2.70	4.4

^aNI: No significant inhibition (GI₅₀ > 10 μM). ^bExperimental error is within 20%.

Further SAR studies of the *para* position on the phenyl ring were performed (**9g-u**) and the results are summarized in Table 4. We found that a substitution of 4-methoxy to an electron withdrawing groups substituents **9h-k**, resulted in the loss of activity. The 4-fluoromethyl derivative (**9j**) displayed a 3-fold loss of inhibitory activity in TC32. However, it should be noted that **9j** also inhibited PANC1 cells growth suggesting that its inhibitory action was not selective. Thus, **9j** could have generalized off-target toxicity.

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3 The 4-isopropyl substitution **9p** and the bulky phenyl substitution represented by **9q** resulted in
4 the complete loss of activity. Also, the 4-methyl derivative (**9n**) resulted in a complete loss of
5 activity. The 4-ethoxy derivative **9m** displayed a 3-fold decrease of potency in the ES cells
6 suggesting that substitution with the larger group may be unfavorable to the inhibitory activity of
7 the derivatives. Interestingly, the thiomethyl derivative **9r** retained activity comparable to **2** (GI_{50}
8 of 0.98 μM). The 4-amine substitution **9s** on the aromatic ring resulted in the complete loss of
9 activity. However, the methylamine derivative **9t** resulted in more than two-fold increase in
10 potency (GI_{50} 0.34 μM) compared to **2** (GI_{50} 0.92 μM). The activity was also improved with the
11 dimethylamine derivative **9u** with GI_{50} of 0.26 μM in TC32 cells. This activity was equal to the
12 potency of the **S-(-)** enantiomer of **2** (GI_{50} 0.28 μM) in TC32 cells¹⁸. Anti-proliferative results
13 in TC32 cells were comparable to those observed in TC71 cells.
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Table 4. Chemical Structure and Biological Activities of Compounds **9g-9u**

	Substitution		GI ₅₀ (μM) ^{a,b}		
	R ₁	PANC 1	TC32	TC71	
2	-OCH ₃	NI	0.94 (0.18)	0.92 (0.12)	
9g	-H	NI	NI	NI	
9h	-F	NI	NI	NI	
9i	-I	NI	NI	NI	
9j	-CF ₃	11.0 (0.23)	2.9 (0.23)	6.9 (0.19)	
9k	-CN	NI	NI	NI	
9n	-CH ₃	NI	NI	NI	
9m	-OC ₂ H ₅	NI	2.70 (0.21)	3.57 (0.19)	
9p	-OiPr	NI	NI	NI	
9q	-OPh	NI	NI	NI	
9r	-SCH ₃	NI	0.98 (0.17)	0.91 (0.14)	
9s	-NH ₂	NI	NI	NI	
9t	-NHCH ₃	NI	0.34 (0.09)	0.39 (0.10)	
9u	-N(CH ₃) ₂	NI	0.26 (0.1)	0.40 (0.07)	

^aNI: No significant inhibition (GI₅₀ > 10 μM) ^bExperimental error represents S.E.M.

Functional Inhibition of EWS-FLI1

Previously, we have demonstrated that compound **2** acted as a functional inhibitor of EWS-FLI1¹⁷. It has been reported that EWS-FLI1 binds directly to the microsatellite repeats of the promoter NR0B1 to drive oncogenesis in ES²⁷. In this study, COS7 cells were transfected with EWS-FLI1 and NR0B1 reporter-luciferase plasmids as previously reported (Figure 3A)^{17,28}. EWS-FLI1-transfected cells showed a dose-dependent decrease in the promoter activity when treated for 20 h with compound **2** (IC₅₀= 0.35 μM) as compared to no transfection (Figure 3B-C). Control experiments at 48 hours show that the compounds were not cytotoxic to the COS7 cells. COS7 are fibroblast-like cells derived from monkey kidney that are devoid of the EWS-FLI1.

To further assess their inhibitory effects on the transcriptional activities of EWS-FLI1, we selected **6e**, **9c**, **9m**, **9r**, **9t**, **9u**, **10** and **14a** which displayed a GI₅₀ of 2.7 μM or better and **9a**, **11** and **15** which displayed no anti-proliferative activity (Figure 4A-B). As expected, **9a** which displayed no inhibition on cell growth did not inhibit the transcriptional activities of EWS-FLI1. **9r** inhibition was comparable to **2** with IC₅₀ of 0.33 μM whereas **6e** has an IC₅₀ of 0.77 μM. Compounds **9c** and **9m** inhibitory effects were lower than **2**. Unlike **11** that did not display any inhibition, **14a** and **15** showed modest inhibitory effects on the transcriptional activities of EWS-FLI1. Consistent with the ES cell growth inhibition data, **9t** and **9u** exhibited better inhibition of EWS-FLI1 with an IC₅₀ of 0.18 μM and 0.15 μM respectively. The results strongly support that these derivatives inhibit the transcriptional activity of EWS-FLI1.

A plot of the IC₅₀ of the luciferase activity versus the GI₅₀ (Figure 5) was performed to show that the cellular growth inhibition effects of the ES cells observed are dependent on the inhibition of the transcriptional activity of EWS-FLI1. A correlation coefficient of 0.84 provides further

evidence that the effects on the inhibition of the transcriptional activity of EWS-FLI1 and the cell growth inhibition of ES cells are related. This data suggested that the EWS-FLI1 mediated growth of the ES cells can be effectively decreased by inhibiting the transcriptional activity of EWS-FLI1.

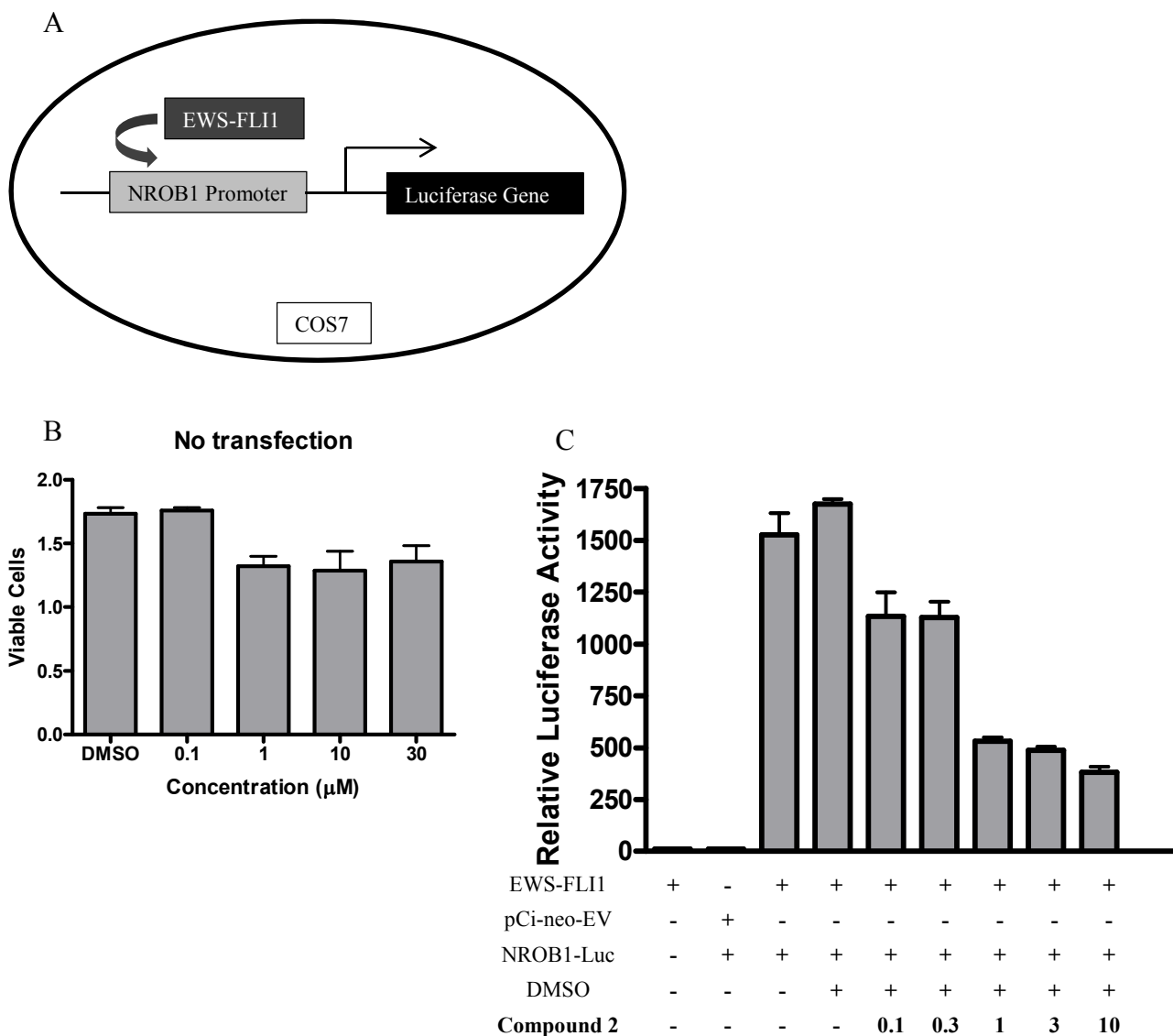


Figure 3. (A) Schematic construct of the luciferase reporter assay for the transcription inhibition of the EWS-FLI1 by small molecule. (B) Cell proliferation assay (WST) indicating negligible effect of compound 2 on COS7 cells. (C) COS7 cells were transfected with NR0B1 luciferase

reporter and empty vector (pCi-neo-EV) or full length EWS-FLI1. Diagram shows a mean \pm SD of a single representative experiment performed in quadruplicate

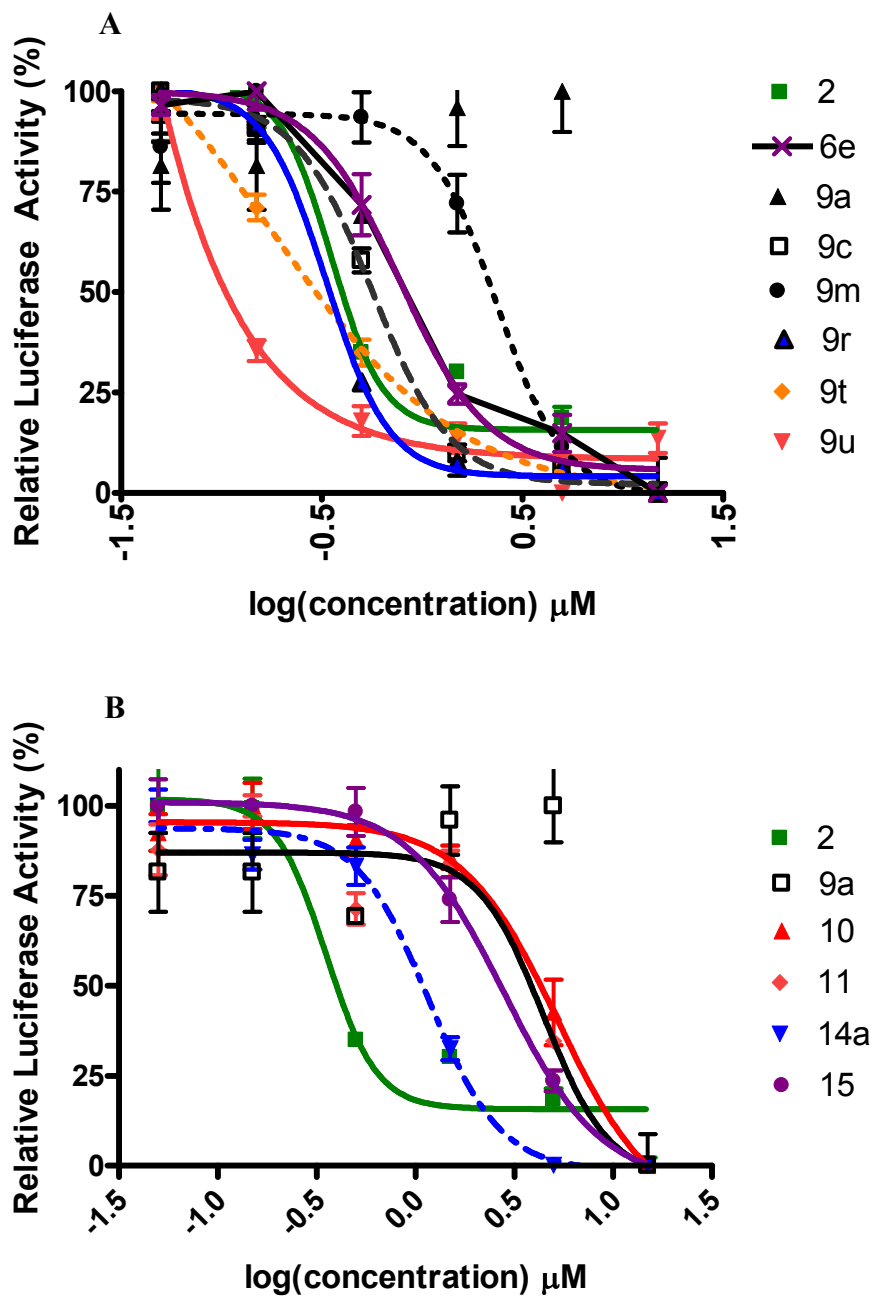
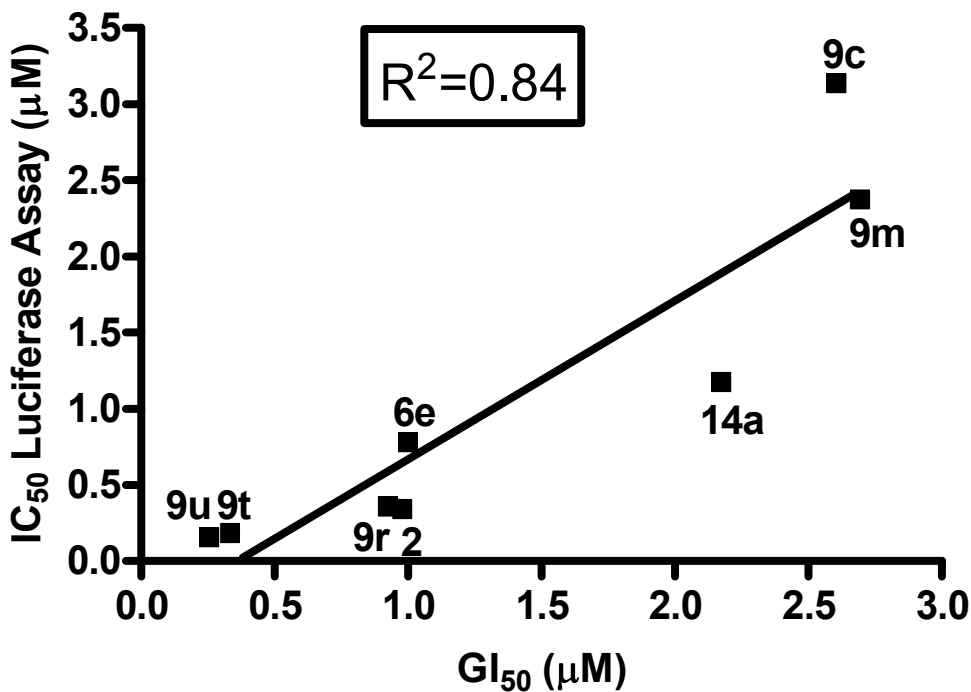


Figure 4. Luciferase assay showing inhibition of EWS-FLI1 transcriptional activity in COS7 cells. (A) Dose-response profiles of compounds 2, 6e, 9a, 9c, 9m, 9r, 9t, 9u for EWS-FLI1 inhibition. (B) Dose-response profiles of compounds 2, 9a, 10, 11, 14a, 15 for EWS-FLI1 inhibition.

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3 inhibition. Diagrams show a mean \pm SD of single representative experiment performed in
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6 quadruplicate, with compound **2** graphed in comparison and **9a** as negative control.
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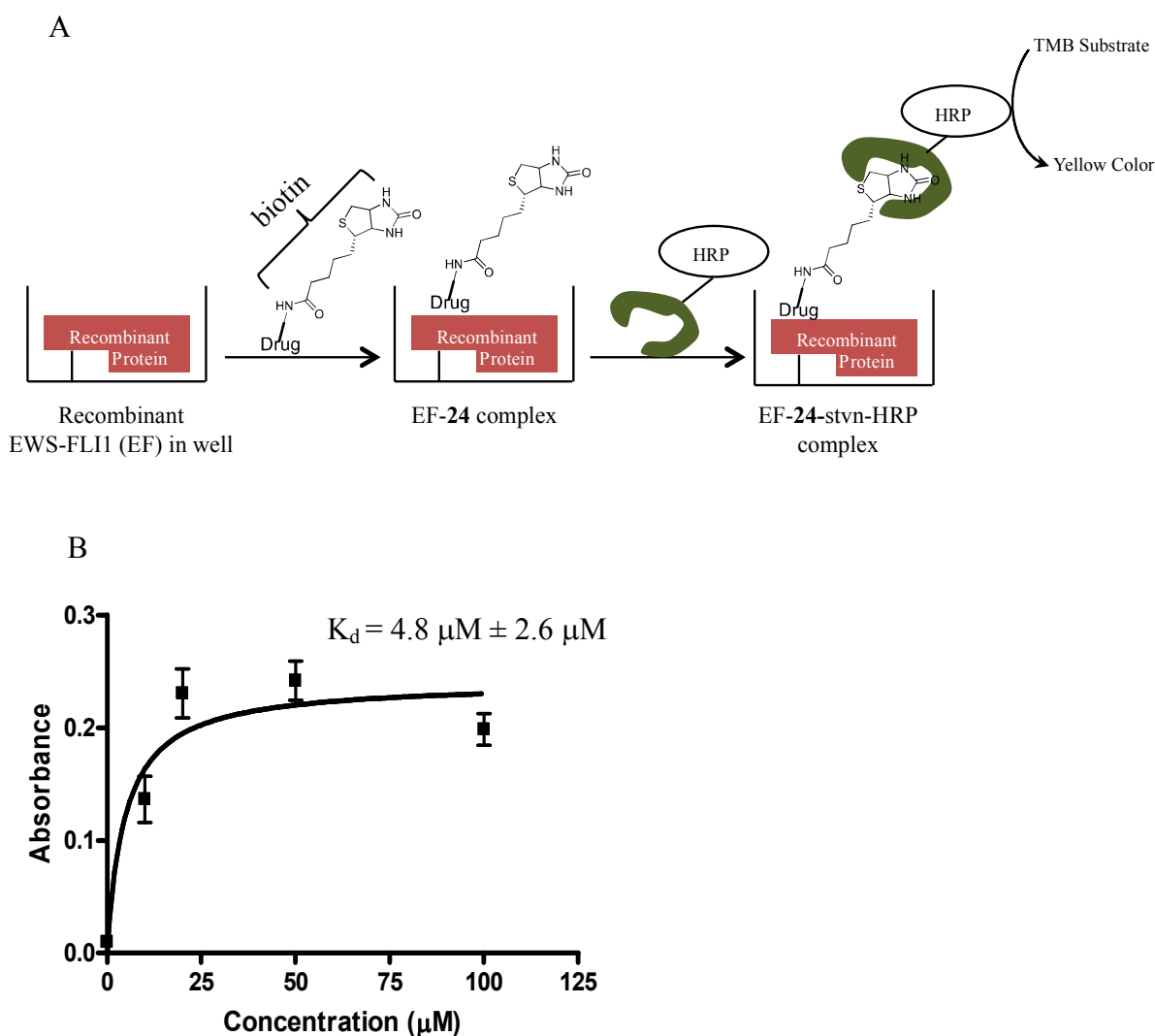
33 **Figure 5.** Correlation of the analogs on growth inhibition in TC32 cells and Luciferase reporter
34 activity in COS7 cells.
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40 **Design and evaluation of biotinylated probe of compound 24**

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44 We envisioned the synthesis of a biotin conjugate that could be used to detect binding of the
45 derivative and the recombinant EWS-FLI1 (Scheme 5A). We developed a synthetic route to
46 append a five carbon linker terminating with an amine to compound **9t**. The amine was exploited
47 to link biotin to the **9t**-linker molecule to afford **24** (Scheme 5A). We evaluated the binding of **24**
48 to recombinant EWS-FLI1 as shown in Figure 6A. A 96-well plate was coated with the
49 recombinant protein that was allowed to bind with the biotinylated conjugate. The binding of **24**
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3 to EWS-FLI1 was detected with HRP streptavidin conjugate. We detected a dose dependent
4 binding of **24** to immobilized EWS-FLI1 (Figure 6B). No binding was detected when BSA was
5 immobilized. The saturation was reached at 20 μ M. Concentration-dependent binding of **24** to
6 immobilized EWS-FLI1 (Figure 6B) was exhibited by biotinylated **24** over a range of 1-100 μ M
7 resulting in a K_d of $4.8 \pm 2.6 \mu$ M.
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Figure 6. (A) Schematic overview of ELISA-like assay to probe the binding of the biotin

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3 conjugate to recombinant EWS-FLI1; (B) Saturation curve of biotin conjugate (**24**) to
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5 recombinant EWS-FLI1
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10 CONCLUSION

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12 This research provides a solid ground for the discovery of new and effective agents to treat
13 Ewing's sarcoma. The hit compound **1** was discovered through a surface plasmon resonance
14 initial screening against recombinant EWS-FLI1. Compound **2** with increased activity reduces
15 the growth of ES cells and ES orthotopic xenografts¹⁷. Compound **2** was used as our lead
16 compound for further detailed SAR studies.
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20 We modified **2** with the aim to clarify the relevant structural determinants for small molecule
21 inhibition of EWS-FLI1. On the indoline of **2**, our investigation highlights the importance of the
22 4,7-position and the significance of the free amine for optimal inhibition. We found that removal
23 of the alcohol by dehydration shows that the hydroxyl group is not required for binding. The loss
24 of activity resulting from the removal of hydrogen bond donation supports the finding that the
25 alcohol group does not act as a hydrogen bond donor.
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29 Additionally, we have observed that the *para*-position on the phenyl ring was the most favorable
30 for inhibition of EWS-FLI1 by **2**. Electron donating groups at the *para*-position significantly
31 contribute to a higher inhibitory activity. Compounds **9t** and **9u** had over 3-fold higher potency
32 than the lead **2**. It appears that the electron density of the substitutions may play a more
33 significant role in the inhibitory action of the derivatives. The electron density of 4-
34 dimethylamine > 4-methylamine > 4-methoxy correlates with increasing inhibitory activity
35 (compound **9u** > compound **9t** > compound **2**).
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39 We have successfully correlated the cell growth inhibition of the ES cells by the active
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3 derivatives with the luciferase reporter activity, statistically linking observed activity to the
4 inhibition of the transcriptional activity of EWS-FLI1. Finally, we showed that a derivative binds
5 to the target using a novel biotin conjugate. The conjugate can be utilized as a screening tool to
6 better understand the binding of small molecules to the EWS-FLI1.
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14 **Experimental Section**

15 **Chemistry**

16 **General Chemistry Methods**

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20 All solvents and reagents were used as obtained from commercial sources unless otherwise
21 indicated. Commercial reagents and anhydrous solvents were used without further purification.
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23 Analytical thin layer chromatography (TLC) was performed on silica gel 60 F254 aluminum
24 sheets. Removal of solvents was conducted by using a rotary evaporator, and residual of solvents
25 was removed from nonvolatile compounds using a vacuum manifold. All reported yields are
26 isolated yields. ^1H and ^{13}C NMR spectra were recorded on a Varian 400MR spectrometer
27 operating at 400 MHz for ^1H and 100 MHz for ^{13}C . Deuterated dimethyl sulfoxide or deuterated
28 methanol or deuterated chloroform was used as the solvent for NMR experiments. ^1H Chemical
29 shifts values (δ) are referenced to the residual nondeuterated components of the NMR solvents (δ
30 = 2.54 ppm for DMSO, δ = 7.26 ppm for CDCl_3 and δ = 3.31 ppm for CD_3OD). The ^{13}C
31 chemical shifts (δ) are referenced to CDCl_3 (central peak, δ = 77.16 ppm) or CD_3OD (δ = 49.00
32 ppm) as the internal standard. The HRMS data were obtained on a Waters Q-TOF Premier mass
33 spectrometer. Column chromatography was performed using silica gel (35–75 mesh). The purity
34 of final compounds was evaluated by C, H, N, analysis (Atlantic microlabs). The purity of all the
35 final compounds was confirmed to be ≥ 95 by combustion.
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6 **General Procedure A.** To a solution of chloral hydrate in water were added sodium sulfate (6
7 equiv.), and appropriate aniline (1 equiv.) followed by a solution of hydroxylamine
8 hydrochloride solution (3 equiv.) in water. The reaction mixture was heated at 55 °C with stirring
9 overnight and then cooled to room temperature. The resulting precipitate was filtered and dried
10 to obtain the isonitrosoacetanilide intermediates. The isonitrosoacetanilide was added to a
11 stirring solution of concentrated sulfuric acid slowly to keep the temperature in the range of 60-
12 70 °C. Upon completion of the addition, the mixture was heated to 80 °C for 10 minutes and then
13 poured onto crushed ice and stirred for 30 minutes. The precipitated was filtered and washed
14 three times with water and dried under vacuum to afford the solids **5a-5e**.
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29 **General Procedure B.** To a solution of 4,7-dichloroisatin (1.0 equiv.) was added appropriate
30 acetophenone (4.0 equiv.) and catalytic amount diethylamine (10 drops) in methanol. The
31 mixture was stirred at room temperature until the starting material (4,7-dichloroisatin)
32 disappeared completely. The resulted solution was concentrated and purified in flash
33 chromatography eluting with Hexane/Ethyl acetate to afford product in quantitative yield. The
34 product was recrystallized with Hexanes / Ethyl acetate to give a pure product.
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46 *4-chloroindoline-2,3-dione (5a).* Compound **5a** was prepared via general procedure A from **3a**
47 (500 mg, 3.88 mmoles), chloral hydrate (965 mg, 5.83 mmoles), hydroxylamine hydrochloride
48 (808 mg, 11.6 mmoles), sodium sulfate (3.42 g, 23.3 mmoles) and 1N HCl (8mL) following the
49 general procedure A to yield a dark brown solid (580 mg, 82.3 %). ¹H NMR (DMSO-*d*₆, 400Hz)
50 δ 11.17 (s, 1H), 7.53 (t, 1H, J=16.4), 7.06 (d, 1H, J=8), 6.87 (d, 1H, J=8).
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6 *5-chloroindoline-2,3-dione (5b)*, Compound **5b** was prepared via general procedure A from **3b**
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8 (500 mg, 3.88 mmoles), chloral hydrate (965 mg, 5.83 mmoles), hydroxylamine hydrochloride
9
10 (808 mg, 11.64 mmoles), sodium sulfate (3.42 g, 23.3 mmoles) and 1N HCl (8mL) following the
11
12 general procedure A to yield a brown solid (600 mg, 85 %). ¹H NMR (DMSO-*d*₆, 400Hz)
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14 δ 11.24 (s, 1H), 7.62-7.59 (m, 1H), 7.535(s, 1H), 6.92 (d, 1H, J = 8.4).
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20 *5,6-dichloroindoline-2,3-dione (5c)*; Compound **5c** was prepared via general procedure A from
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22 **3c** (500 mg, 2.31 mmoles), chloral hydrate (574 mg, 3.47 mmoles), hydroxylamine
23
24 hydrochloride (482 mg, 6.94 mmoles), sodium sulfate (2.04 g, 13.9 mmoles) and 1N HCl (4 mL)
25
26 following the general procedure A to yield a brown solid (370 mg, 74%). ¹H NMR (DMSO-*d*₆,
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28 400Hz) δ 11.24 (s, 1H), 7.7 (d, 1H, J = 9.2), 6.86 (d, 1H, J = 8.4).
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34 *4,7-difluoroindoline-2,3-dione (5d)*; Compound **5d** was prepared via general procedure A from
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36 **3d** (400 μL, 3.96 mmoles), chloral hydrate (722 mg, 4.36 mmoles), hydroxylamine
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38 hydrochloride (827 mg, 11.9 mmoles), sodium sulfate (3.49 g, 23.8 mmoles) and 1N HCl (8 mL)
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40 following the general procedure A to yield an orange solid (507 mg, 70 %). ¹H NMR (DMSO-*d*₆,
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42 400Hz) δ 11.704 (s,1H), 7.60-7.55 (m, 1H), 6.88-6.23 (m, 1H). ¹⁹F NMR (DMSO-*d*₆, 367Hz): -
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51 *4,7-dimethoxyindoline-2,3-dione (5e)*; Compound **5e** was prepared via general procedure A from
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53 **3e** (400 mg, 2.61 mmoles), chloral hydrate (433.2 mg, 2.61 mmoles), hydroxylamine
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55 hydrochloride (548 mg, 7.83 mmoles), sodium sulfate (2.3 g, 15.7 mmoles) and 1N HCl (5.5
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mL) following the general procedure A to yield a brown solid (184 mg, 34 %). ^1H NMR (DMSO- d_6 , 400Hz) δ 11.70 (s, 1H), 7.32 (d, 1H, J=9.2), 6.62 (d, 1H, J=9.2), 3.80 (s, 3H), 3.78 (s, 3H).

4-chloro-3-hydroxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)indolin-2-one (6a) ; Compound **6a** was prepared via general procedure B from **5a** (50 mg, 0.27 mmoles) and 4-methoxyacetophenone (165.4 mg, 1.10 mmoles) to yield a white solid (84 mg, 92 %); mp 224-226 °C. ^1H NMR (DMSO- d_6 , 400Hz) δ 10.51 (s, 1H), 7.90 (d, 2H, J=8.8), 7.22 (t, 1H, J=16), 7.04 (d, 2H, J=8.8), 6.86 (q, 2H), 6.25 (s, 1H), 4.37 (d, 1H, J=18), 3.85 (s, 3H), 3.63 (d, 1H, J=18). ^{13}C NMR (DMSO- d_6 , 100Hz): 195.17, 177.98, 163.88, 145.65, 131.06, 130.66 (2C), 130.09, 129.18, 128.27, 122.41, 114.47 (2C), 108.97, 74.38, 56.03, 44.35. HRMS (ES) m/z calcd for $\text{C}_{17}\text{H}_{14}\text{ClNO}_4$ (M - H) $^+$ 330.0533; found, 330.0524. Anal. calcd for $\text{C}_{17}\text{H}_{14}\text{ClNO}_4$ C, 61.55; H, 4.25; N, 4.22. Found: C, 61.6; H, 4.34; N, 4.26.

5-chloro-3-hydroxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)indolin-2-one (6b); Compound **6b** was prepared via general procedure B from **5b** (50 mg, 0.27 mmoles) and 4-methoxyacetophenone (165 mg, 1.10 mmoles) to yield a white solid (85 mg, 93.4 %); mp 191 °C. ^1H NMR (DMSO- d_6 , 400Hz) δ 10.37 (s, 1H), 7.88 (d, 2H, J=8.8), 7.38 (s, 1H), 7.23 (q, 1H), 7.04 (d, 2H, J=8.8), 6.83 (d, 1H, J=8.4), 6.16 (s, 1H), 4.11 (d, 1H, J=17.6), 3.84 (s, 3H), 3.59 (d, 1H, J=17.6). ^{13}C NMR (DMSO- d_6 , 100Hz): 195.28, 178.57, 163.79, 142.38, 134.57, 130.75 (2C), 129.49, 129.02, 125.55, 124.35, 114.35 (2C), 111.21, 73.57, 56.04, 45.85. HRMS (ES) m/z calcd for $\text{C}_{17}\text{H}_{14}\text{ClNO}_4$ (M - H) $^+$ 330.0533; found, 330.0529. Anal. calcd for $\text{C}_{17}\text{H}_{14}\text{ClNO}_4$ C, 61.55; H, 4.25; N, 4.22. Found: C, 61.46; H, 4.55; N, 4.09.

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5,6-dichloro-3-hydroxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)indolin-2-one (6c); Compound **6c** was prepared via general procedure B from **5c** (50 mg, 0.23 mmoles) and 4-methoxyacetophenone (139 mg, 0.92 mmoles) to yield a white solid (79 mg, 95 %); mp 214-215 °C. ¹H NMR (DMSO-*d*₆, 400Hz) δ 10.64 (s, 1H), 7.91 (d, 2H, J=8.4), 7.47 (d, 1H, J=8.4), 7.04 (d, 2H, J=9.2), 6.85 (d, 1H, J=8.4), 6.38 (s, 1H), 4.37 (d, 1H, J=18), 3.85 (s, 3H), 3.68 (d, 1H, J=18). ¹³C NMR (DMSO-*d*₆, 100Hz): 195.18, 177.79, 163.97, 144.21, 131.17, 130.77, 130.73 (2C), 130.50, 129.01, 114.50 (2C), 110.15, 75.03, 73.24, 56.05, 44.34. Anal. calcd for C₁₇H₁₃Cl₂NO₄ C, 55.76; H, 3.58; N, 3.82. Found: C, 55.82; H, 3.60; N, 3.88.

4,7-difluoro-3-hydroxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)indolin-2-one (6d); Compound **6d** was prepared via general procedure B from **5d** (50 mg, 0.27 mmoles) and 4-methoxyacetophenone (164 mg, 1.09 mmoles) to yield a light pink solid (83.6 mg, 93 %); mp 170 °C ¹H NMR (DMSO-*d*₆, 400Hz) δ 10.96 (s, 1H), 7.87 (d, 2H, J=8.8), 7.17-7.11 (m, 1H), 7.01 (d, 2H, J=9.2), 6.8-6.62 (m, 1H), 6.45 (s, 1H), 4.03 (d, 1H, J=17.6), 3.82 (s, 3H), 3.66 (d, 1H, J=18). ¹³C NMR (DMSO-*d*₆, 100Hz): 195.18, 177.82, 163.99, 149.24, 130.76, 129.09 (2C), 119.78, 118.11, 114.49, 109.47 (2C), 109.23, 104.99, 73.48, 56.06, 45.17. ¹⁹F NMR (DMSO-*d*₆, 367Hz): -138.62. HRMS (ES) *m/z* calcd for C₁₇H₁₃F₂NO₄ (M - H)⁺ 332.0734; found, 332.0735. Anal. calcd for C₁₇H₁₃F₂NO₄ C, 61.26; H, 3.93; N, 4.20. Found: 61.45; H, 3.93; N, 4.30

3-hydroxy-4,7-dimethoxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)indolin-2-one (6e); Compound **6e** was prepared via general procedure B from **5e** (50 mg, 0.24 mmoles) and 4-methoxyacetophenone (144 mg, 0.96 mmoles) to yield a brown solid (31.4 mg, 35 %);

¹H NMR (DMSO-*d*₆, 400Hz) δ 10.16 (s, 1H), 7.82 (d, 2H, J=9.2), 6.99 (d, 2H, J=9.2), 6.81 (d,

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3 1H, J=9.2), 6.39 (d, 2H, J=8.8), 5.85 (s, 1H), 4.17 (d, 1H, J=16.8), 3.80 (s, 3H), 3.73 (s, 3H),
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5 3.59 (s, 3H), 3.41 (d, 1H, J=16.8). Anal. calcd for C₁₉H₁₉NO₆: C, 63.86; H, 5.36; N, 3.92. Found:
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7 C, 63.78; H, 5.69; N, 3.90.
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12 *3-hydroxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)-4,7-dimethylindolin-2-one (6f)*; Compound **6f**
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14 was prepared via general procedure B from **5f** (50 mg, 0.28 mmoles) and 4-
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16 methoxyacetophenone (172 mg, 1.14 mmoles) to yield a brown solid (87.4 mg, 96 %); mp 135-
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18 136 °C. ¹H NMR (DMSO-*d*₆, 400Hz) δ 10.18 (s, 1H), 7.86 (d, 2H, J=9.2), 6.99 (d, 2H, J=8.8),
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20 6.84 (d, 1H, J=7.6), 6.52 (d, 1H, J=7.6), 5.91 (s, 1H), 4.04 (d, 1H, J=17.2), 3.80 (s, 3H), 3.54 (d,
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22 1H, J=17.2), 2.20 (s, 3H), 2.13 (s, 3H). ¹³C NMR (DMSO-*d*₆, 100Hz): 195.085, 179.15, 163.72,
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24 141.92, 132.01 (2C), 130.68, 130.43, 129.55, 128.73, 123.84, 116.36, 114.37 (2C), 74.70, 56.00,
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26 45.03, 17.42, 16.57. Anal. calcd for C₁₉H₁₉NO₄.1/4 EtOAc: C, 69.15; H, 6.09; N, 4.03. Found: C,
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28 69.10; H, 6.04; N, 4.15.
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37 *7-chloro-3-hydroxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)indolin-2-one (6g)*. Compound **6g** was
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39 prepared via procedure B from commercially available 7-chloroisatin (50 mg, 0.27 mmoles) and
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41 4-methoxyacetophenone (165.4 mg, 1.10 mmoles) to yield a white solid (84 mg, 92 %). ¹H NMR
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43 (DMSO-*d*₆, 400Hz) δ 10.66 (s,1H), 7.87 (d, 2H, J=8.8), 7.25 (dd, 2H, J=12.4), 7.02 (d, 2H,
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45 J=8.8), 6.90 (t, 1H, J=15.6), 6.18 (s, 1H), 4.06 (d, 1H, J=18), 3.83 (s, 3H), 3.60 (d, 1H, J=18). ¹³C
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47 NMR (DMSO-*d*₆, 100Hz): 195.25, 178.75, 163.82, 141.17, 134.44, 130.85 (2C), 129.43, 129.29,
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49 122.94, 122.53, 114.28, 114.05, 74.03, 56.04, 46.04. HRMS (ES) *m/z* calcd for C₁₇H₁₄ClNO₄ (M
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51 - H)⁺ 330.0533; found, 330.0330. Anal. calcd for C₁₇H₁₄ClNO₄.0.1EtOAc C, 61.37; H, 4.38; N,
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53 4.11. Found: C, 61.27; H, 4.48; N, 4.19.
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6 *3-hydroxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)indolin-2-one (6h)*. Compound **6h** was prepared
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8 via general procedure B from commercially available isatin (50mg, 0.34 mmol) and 4-
9 methoxyacetophenone (204.1 mg, 1.56 mmol) to yield a white solid (75 mg, 79.01 %); 187 °C.
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11 ¹H NMR (DMSO-*d*₆, 400Hz) δ 10.20 (s, 1H), 7.85 (d, 2H, J=9.2), 7.24 (d, 1H, J=7.2), 7.15 (d,
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13 1H, J=15.6), 6.99 (d, 2H, J=8.8), 6.84-6.77 (m, 2H, J=7.6, 7.6), 5.99 (s, 1H), 3.99 (d, 1H,
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15 J=17.2), 3.80 (s, 3H), 3.51 (d, 1H, J=17.2). ¹³C NMR (DMSO-*d*₆, 100Hz): 195.17, 178.83,
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17 163.67, 143.4, 132.31, 130.68 (2C), 129.66, 129.28, 123.97, 121.49, 114.3 (2C), 109.19, 73.50,
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19 55.98, 45.84. HRMS (ES) *m/z* calcd for C₁₇H₁₅NO₄ (M + H)⁺ 298.1079; found, 298.1076. Anal.
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21 calcd for C₁₇H₁₅NO₄: C, 68.68; H, 5.09; N, 4.71. Found: C, 68.42; H, 5.25; N, 4.71.
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30 *4,7-dichloro-3-hydroxy-3-(2-(2-methoxyphenyl)-2-oxoethyl)indolin-2-one (9a)*. Compound **9a**
31 was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmol) and **8a**
32 (256 μL, 1.84 mmol) to yield a white solid (150 mg, 89.05 %); mp 183-184 °C. ¹H NMR
33 (DMSO-*d*₆, 400Hz) δ 10.91 (s, 1H), 7.53 (t, 1H, J=8.8), 7.35 (d, 1H, J=8.8), 7.27 (d, 1H, J=8.8),
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35 7.16 (d, 1H, J=8.4), 6.94 (t, 1H, J=7.6), 6.86 (d, 1H, J=8.8), 6.34 (s, 1H), 4.33 (d, 1H, J=18.4),
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37 3.90 (s, 3H), 3.66 (d, 1H, J=18.4). ¹³C NMR (DMSO-*d*₆, 100Hz): 197.7, 177.9, 159.2, 143.03,
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39 135.00, 130.76, 129.84, 129.81, 128.64, 126.47, 123.58, 120.92, 113.19, 113.06, 74.92, 56.36,
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41 50.04. HRMS (ES) *m/z* calcd for C₁₇H₁₃Cl₂NO₄ (M - H)⁺ 364.0143; found, 364.0142. Anal.
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43 calcd for C₁₇H₁₃Cl₂NO₄: C, 55.76; H, 3.58; N, 3.82. Found: C, 55.57; H, 3.52; N, 3.83;
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54 *4,7-dichloro-3-hydroxy-3-(2-(3-methoxyphenyl)-2-oxoethyl)indolin-2-one, (9b)*. Compound **9b**
55 was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmol) and **8b**
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3 (253 μL , 1.84 mmoles) to yield a light pink solid (163 mg, 96.7 %); mp 154-155 $^{\circ}\text{C}$. ^1H NMR
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5 (DMSO- d_6 , 400Hz) δ 10.97 (s, 1H), 7.53 (d, 1H, J=7.6), 7.42 (t, 1H, J=28), 7.31 (s, 1H), 7.28 (d,
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7 1H, J=8.4), 7.19 (d, 1H, J=10), 6.88 (d, 1H, J=8.8), 6.43 (s, 1H), 4.34 (d, 1H, J=18.4), 3.75 (s,
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9 3H), 3.71 (d, 1H, J=18.4). ^{13}C NMR (DMSO- d_6 , 100Hz): 196.75, 177.8, 159.89, 143.06, 137.25,
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11 130.88, 130.52, 129.7, 128.66, 123.64, 120.99, 120.58, 113.29, 112.3, 74.99, 55.75, 44.9.
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13 HRMS (ES) m/z calcd for $\text{C}_{17}\text{H}_{13}\text{Cl}_2\text{NO}_4$ (M - H) $^+$ 364.0143; found, 364.0142. Anal. calcd for
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15 $\text{C}_{17}\text{H}_{13}\text{Cl}_2\text{NO}_4 \cdot 1/4 \text{EtOAc}$: C, 55.69; H, 3.89; N, 3.61. Found: C, 55.45; H, 3.56, N, 3.82.
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23 *4,7-dichloro-3-(2-(2,4-dimethoxyphenyl)-2-oxoethyl)-3-hydroxyindolin-2-one* (**9c**). Compound
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25 **9c** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmoles) and **8c**
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27 (332 mg, 1.84 mmoles) to yield a white solid (142 mg, 78.3 %); mp 195-196 $^{\circ}\text{C}$. ^1H NMR
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29 (DMSO- d_6 , 400Hz) δ 10.86 (s, 1H), 7.46 (d, 1H, J= 8.8), 7.26 (d, 1H, J=8.8), 6.85 (d, 1H, J=8.8),
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31 6.628 (s, 1H), (6.52 (dd, 1H, J=10.8), 6.28 (s, 1H), 4.29 (d, 1H, J=18.8), 3.92 (s, 3H), 3.79 (s,
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33 3H), 3.62 (d, 1H, J=18.8). ^{13}C NMR (DMSO- d_6 , 100Hz): 196.01, 178.88, 166.01, 162.39,
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35 143.91, 132.98, 131.42, 130.98, 129.28, 124.31, 119.95, 113.94, 107.50, 99.69, 75.80, 57.27,
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37 56.92, 50.78. HRMS (ESI) m/z calcd for $\text{C}_{18}\text{H}_{15}\text{Cl}_2\text{NO}_5$ (M + H) $^+$ 396.0406; found, 396.0389.
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40 Anal. calcd for $\text{C}_{18}\text{H}_{15}\text{Cl}_2\text{NO}_5 \cdot \text{EtOAc}$: C, 54.56; H, 4.79; N, 2.89. Found: C, 53.51; H 4.02; N
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43 3.42.
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52 *4,7-dichloro-3-(2-(3,5-dimethoxyphenyl)-2-oxoethyl)-3-hydroxyindolin-2-one* (**9d**). Compound
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54 **9d** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmoles) and **8d**
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56 (332 mg, 1.84 mmoles) to yield a white solid (112 mg, 67.2 %); mp 204-205 $^{\circ}\text{C}$. ^1H NMR
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(DMSO-*d*₆, 400Hz) δ 10.96 (s, 1H), 7.28 (d, 1H, J= 8.4), 6.99 (s, 2H), 6.88 (d, 1H, J=8.8), 6.73 (s, 1H), 6.41 (s, 1H), 4.34 (d, 1H, J=18.4), 3.75 (s, 6H), 3.71 (s, 1H, J= 18.4). ¹³C NMR (DMSO-*d*₆, 100Hz): 196.59, 177.78, 161.06 (2C), 143.06, 137.86, 130.87, 129.72, 128.67, 123.65, 113.29, 106.32, 105.93 (2C), 75.02, 55.96 (2C), 44.96. HRMS (ES) *m/z* calcd for C₁₈H₁₅Cl₂NO₅ (M - H)⁺ 394.0249; found, 394.0251. Anal. calcd for C₁₈H₁₅Cl₂NO₅: C, 54.56; H, 3.82; N, 3.54. Found: C, 54.76; H, 3.91; N, 3.56.

4,7-dichloro-3-(2-(2,3-dimethoxyphenyl)-2-oxoethyl)-3-hydroxyindolin-2-one (**9e**). Compound **9e** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmoles) and **8e** (230 mg, 1.27 mmoles) to yield a white solid (132.2 mg, 72.6 %); mp 203-204 °C. ¹H NMR (DMSO-*d*₆, 400Hz) δ 10.96 (s, 1H), 7.28 (d, 1H, J= 8.8), 7.24 (d, 1H, J=8), 7.07 (t, 1H, J=16), 6.89 (t, 2H, J=8.4), 6.40 (s, 1H), 4.33 (d, 1H, J=18.8), 3.8 (s, 6H), 3.67 (s, 1H, J= 18.8); ¹³C NMR (DMSO-*d*₆, 100Hz): 198.32, 177.87, 135.24, 148.57, 143.06, 131.87, 130.86, 129.64, 128.7, 124.57, 123.63, 120.31, 117.48, 113.26, 74.83, 61.42, 59.47, 49.40. HRMS (ES) *m/z* calcd for C₁₈H₁₅Cl₂NO₅ (M - H)⁺ 394.0249; found, 394.0254. Anal. calcd for C₁₈H₁₅Cl₂NO₅: C, 54.56; H, 3.82; N, 3.54. Found: C, 54.29; H, 3.75; N, 3.53.

4,7-dichloro-3-hydroxy-3-(2-oxo-2-(2,3,4-trimethoxyphenyl)ethyl)indolin-2-one (**9f**). Compound **9f** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmoles) and **8f** (336 μ L, 1.84 mmoles) to yield a white solid (163.5 mg, 83.4 %); mp 162-163 °C. ¹H NMR (DMSO-*d*₆, 400Hz) δ 10.91 (s, 1H), 7.27 (q, 2H), 6.87 (q, 2H), 6.36 (s, 1H), 4.33 (d, 1H, J=18.4), 3.88 (s, 3H), 3.81 (s, 3H), 3.73 (s, 3H), 3.67 (s, 1H, J= 18.4). ¹³C NMR (DMSO-*d*₆, 100Hz): 195.89, 177.97, 158.10, 154.17, 143.09, 142.0, 130.73, 129.93, 128.58, 125.37, 123.99,

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3 123.56, 113.2, 108.34, 74.92, 61.82, 60.87, 56.57, 49.06. HRMS (ESI) m/z calcd for
4 $C_{19}H_{17}Cl_2NO_6$ ($M + H$)⁺ 426.0511; found, 426.0504. Anal. calcd for $C_{19}H_{17}Cl_2NO_6$: C, 53.54; H,
5 4.02; N, 3.29. Found: C, 53.50, H 4.00, N 3.21.
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12 *4,7-Dichloro-3-hydroxyl-3-(2-oxo2-phenylethyl)-1,3-dihydroindol-2-one* (**9g**). Compound **9g**
13 was prepared via general procedure B from 4,7-dichloroisatin (100mg, 0.46 mmoles) and **8g**
14 (253 μ L, 1.84 mmoles) to yield a white solid (163 mg, 96.7 %); mp 198-200 °C. ¹H NMR
15 (DMSO-*d*₆, 400 MHz) δ 10.97 (s, 1H), 7.88 (dd, 2H, $J = 0.8, 1.6$ Hz), 7.62 (m, 1H), 7.48 (t, 2H,
16 $J = 8.4, 7.2$ Hz), 7.27 (d, 1H, $J = 8.8$ Hz), 6.87 (d, 1H, $J = 8.8$ Hz), 6.43 (s, 1H), 4.36 (d, 1H, $J =$
17 18.4 Hz), 3.68 (d, 1H, $J = 18.4$ Hz). Anal. calcd for $C_{16}H_{11}Cl_2NO_3$: C, 57.17; H, 3.30; N, 4.17.
18 Found: C, 56.99, H, 3.81, N, 3.75.
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32 *4,7-Dichloro-3-[2-(4-fluorophenyl-2-oxoethyl)]-3-hydroxyl-1,3-dihydroindol-2-one* (**9h**).
33 Compound **9h** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46
34 mmoles) and **8h** (256 mg, 1.84 mmoles) to yield light a yellow solid (140 mg, 85.9 %); mp 176-
35 178 °C. ¹H NMR (DMSO-*d*₆, 400 MHz) δ 10.98 (s, 1H), 7.98 (m, 2H), 7.29 (m, 3H), 6.87 (d,
36 1H, $J = 8.4$ Hz), 6.43 (s, 1H), 4.34 (d, 1H, $J = 18.4$ Hz), 3.68 (d, 1H, $J = 18.4$ Hz). Anal. calcd
37 for $C_{16}H_{10}Cl_2FNO_3$: C, 54.26; H, 2.85; N, 3.95. Found: C, 54.58, H, 3.50, N, 3.53.
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49 *4,7-Dichloro-3-hydroxyl-3-[2-(4-iodophenyl-2-oxoethyl)]-1,3-dihydroindol-2-one* (**9i**).
50 Compound **9i** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46
51 mmoles) and **8i** (455.2 mg, 1.84 mmoles) to yield light a yellow solid (230 mg, 100 %); mp 190-
52 192 °C. ¹H NMR (DMSO-*d*₆, 400 MHz) δ 10.98(s, 1H), 7.87 (d, 2H, $J = 8.8$ Hz), 7.64 (d, 2H, J
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3 = 8.8 Hz), 7.27 (d, 1H, $J = 8.8$ Hz), 6.87 (d, 1H, $J = 8.8$ Hz), 6.44 (s, 1H), 4.30 (d, 1H, $J = 18.4$
4 Hz), 3.65 (d, 1H, $J = 18.4$ Hz). Anal. calcd for $C_{16}H_{10}Cl_2INO_3 \cdot EtOAc$: C, 43.62; H, 3.27; N,
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6 2.78. Found: C, 43.96, H, 2.88, N, 2.88.
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12 *4,7-dichloro-3-hydroxy-3-(2-oxo-2-(4-(trifluoromethyl)phenyl)ethyl)indolin-2-one, (9j)*
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15 Compound **9j** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46
16 mmoles) and **8j** (346.2 mg, 1.84 mmoles) to yield a white solid (165 mg, 86.8 %); mp 176.5-178
17 °C. 1H NMR (DMSO- d_6 , 400Hz) δ 11.01 (s, 1H), 8.09 (d, 2H, $J=7.6$), 7.86 (d, 2H, $J=8.4$), 7.291
18 (d, 1H, $J=8.8$), 6.88 (d, 1H, $J=8.8$), 6.49 (d, 1H), 4.40 (d, 1H, $J=18$), 3.76 (d, 1H, $J=18$); ^{13}C
19 NMR (DMSO- d_6 , 100Hz): 196.6, 177.68, 143.03, 138.97, 131.04, 129.46, 129.27 (3C), 128.76,
20 126.34, 126.31, 123.4 (2C), 113.4, 74.9, 45.07. HRMS (ES) m/z calcd for $C_{17}H_{10}Cl_2F_3NO_3$ (M -
21 H) $^+$ 401.9912; found, 401.991. Anal. calcd for $C_{17}H_{10}Cl_2F_3NO_3$: C, 50.52; H, 2.49; N, 3.47.
22 Found, C, 49.88; H, 2.6; N, 3.44.
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36 *4-(2-(4,7-dichloro-3-hydroxy-2-oxoindolin-3-yl)acetyl)benzotrile (9k)*. Compound **9k** was
37 prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmoles) and **8k** (455.2
38 mg, 1.84 mmoles) to yield a white solid (93 mg, 64 %); mp 195-196 °C. 1H NMR (DMSO- d_6 ,
39 400Hz): δ 11.01 (s,1H), 8.06 (d, 2H, $J=10$), 7.97 (d, 2H, $J=10.5$), 7.30 (d, 1H, $J=15$), 6.89 (d, 1H,
40 $J=11$), 6.49 (s, 1H), 4.39 (d, 1H, $J=23$), 3.79 (d, 1H, $J=23$). ^{13}C NMR (DMSO- d_6 , 100Hz):
41 196.57, 177.61, 142.94, 138.88, 133.37 (2C), 131.03, 129.39, 129.03 (2C), 128.73, 123.72,
42 118.47, 116.17, 113.36, 74.94, 45.05. HRMS (ES) m/z calcd for $C_{17}H_{10}Cl_2N_2O_3$ (M - H) $^+$
43 358.9990; found, 358.9990. Anal. calcd for $C_{17}H_{10}Cl_2N_2O_3$: C, 56.53; H, 2.79; N, 7.76. Found:
44 C, 56.29; H, 2.77; N, 7.6
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8 *4,7-Dichloro-3-hydroxy-3-(2-oxo-2-p-tolyloethyl)-1,3-dihydroindol-2-one (9m)*. Compound **9m**
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10 was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmol) and **8m**
11 (248.2 mg, 1.84 mmol) to yield light a yellow solid (152 mg, 100 %); mp 189-192 °C; ¹H
12 NMR (DMSO-*d*₆, 400 MHz) δ 10.95 (s, 1H), 7.78 (d, 2H, *J* = 8.4 Hz), 7.27 (m, 3H), 6.86 (d,
13 1H, *J* = 8.8 Hz), 6.41 (s, 1H), 4.33 (d, 1H, *J* = 18.4 Hz), 3.64 (d, 1H, *J* = 18.4 Hz), 2.33 (s, 3H).
14
15 Anal. calcd for C₁₇H₁₃Cl₂NO₃.1/2 EtOAc: C, 57.82; H, 4.31; N, 3.55. Found: C, 57.60, H, 4.23,
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17 N, 3.56.
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27 *4,7-dichloro-3-(2-(4-ethoxyphenyl)-2-oxoethyl)-3-hydroxyindolin-2-one (9n)*. Compound **9n** was
28 prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmol) and **8n** (302.2
29 mg, 1.84 mmol) to yield a white solid (164 mg, 93.8 %); mp 162-163 °C. ¹H NMR (400Hz,
30 DMSO-*d*₆) δ 10.93 (s, 1H), 7.86 (d, 2H, *J*=9.2), 7.28 (d, 1H, *J*=8.8), 6.99 (d, 2H, *J*=9.2), 6.88 (d,
31 1H, *J*=8.8), 6.38 (s, 1H), 4.34 (d, 1H, *J*=18), 4.12 (q, 2H), 3.64 (d, 1H, *J*=18), 1.34 (t, 3H, *J*=14).
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33 ¹³C NMR (100Hz, DMSO-*d*₆): 194.7, 177.5, 162.8, 142.7, 130.4 (2C), 130.3, 129.4, 128.3,
34 128.2, 123.1, 114.4 (2C), 112.7, 74.6, 63.6, 43.9, 14.45. HRMS (ES) *m/z* calcd for
35 C₁₈H₁₅Cl₂NO₄ (M - H)⁺ 378.0300; found, 378.0307. Anal. calcd for C₁₈H₁₅Cl₂NO₄.35 EtOAc:
36 C, 56.69; H, 4.36; N, 3.41. Found: C, 56.38; H, 4.08; N, 3.56.
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51 *4,7-dichloro-3-hydroxy-3-(2-(4-isopropoxyphenyl)-2-oxoethyl)indolin-2-one (9p)*. Compound **9p**
52 was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmol) and **8p**
53 (327.5 mg, 1.84 mmol) to yield a white solid (152.2 mg, 83.9 %); mp 168-169 °C. ¹H NMR
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(DMSO-*d*₆, 400Hz) δ 10.93 (s, 1H), 7.84 (d, 2H, J=8.8), 7.28 (d, 1H, J=8.8), 6.97 (d, 2H, J=8.8), 6.88 (d, 1H, J=8.8), 6.38 (s, 1H), 4.74-4.68 (m, 1H), 4.33 (d, 1H, J=18), 3.64 (d, 1H, J=18), 1.263 (s, 3H), 1.248 (s, 3H). ¹³C NMR (DMSO-*d*₆, 100Hz): 195.13, 177.92, 162.39, 143.13, 130.83 (2C), 130.8, 129.9, 128.65, 128.55, 123.61, 115.66 (2C), 113.26, 104.9, 75.09, 70.24, 44.41, 22.14. HRMS (ESI) *m/z* calcd for C₁₉H₁₇Cl₂NO₄ (M + H)⁺ 394.0613; found, 394.0602. Anal. calcd for C₂₀H₁₉Cl₂NO₄.1/2 EtOAc C, 58.38; H, 5.16; N, 3.07. Found: C, 57.79; H, 4.59; N, 3.48.

4,7-dichloro-3-hydroxy-3-(2-oxo-2-(4-phenoxyphenyl)ethyl)indolin-2-one (9q). Compound **9q** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmoles) and **8q** (390.5 mg, 1.84 mmoles) to yield a white solid (137 mg, 82.9 %); mp 169-170 °C. ¹H NMR (DMSO-*d*₆, 400Hz) δ 10.95 (s, 1H), 7.93 (d, 2H, J=8.4), 7.46 (t, 2H, J=15.2), 7.28 (d, 1H, J=8), 7.26 (d, 1H, J=8), 7.23 (d, 1H, J=8), 7.12 (d, 1H, J=8), 6.99 (d, 2H, J=8.4), 6.89 (d, 1H, J=8.4), 6.41 (s, 1H), 4.34 (d, 1H, J=18), 3.66 (d, 1H, J=18). ¹³C NMR (DMSO-*d*₆, 100Hz): 195.40, 177.84, 162.23, 155.19, 143.09, 140, 130.85, 130.8 (2C), 130.68, 129.78, 128.65, 125.37, 123.61, 120.62 (2C), 117.55 (2C), 113.28, 75.05, 44.58. HRMS (ESI) *m/z* calcd for C₂₂H₁₅Cl₂NO₄ (M - H)⁺ 426.0300; found, 426.0306. Anal. calcd for C₂₂H₁₅Cl₂NO₄: C, 61.70; H, 3.53; N, 3.27. Found: C, 61.96; H, 3.56; N, 3.37.

4,7-dichloro-3-hydroxy-3-(2-(4-(methylthio)phenyl)-2-oxoethyl)indolin-2-one, (9r). Compound **9r** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmoles) and **8r** (307.5 mg, 1.84 mmoles) to yield a light yellow solid (152 mg, 86.4 %); mp 182-182 °C. ¹H NMR (DMSO-*d*₆, 400Hz) δ 10.96 (s, 1H), 7.82 (d, 2H, J=7.6), 7.32 (d, 2H, J=7.6), 7.284 (d, 1H,

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3 J=10), 6.88 (d, 1H, J=10), 6.43 (s, 1H), 4.36(d, 1H, J=18), 3.68 (d, 1H, J=18). ¹³C NMR
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5 (DMSO-*d*₆, 100Hz): 195.83, 177.84, 146.8, 143.08, 132.07, 130.86, 129.75 (2C), 128.81(2C),
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7 128.65, 125.34, 123.62, 113.28, 75.04, 44.53, 14.31. HRMS (ESI) *m/z* calcd for C₁₇H₁₃Cl₂NO₃S
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9 (M + H)⁺ 382.0071; found, 382.0059. Anal. calcd for C₁₇H₁₃Cl₂NO₃S: C, 53.41; H, 3.43; N,
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11 3.66. Found, C, 53.63; H, 3.46; N, 3.74.
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18 *3-[2-(4-Aminophenyl-2-oxoethyl)]-4,7-dichloro-3-hydroxyl 1,3-dihydroindol-2-one (9s)*.

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20 Compound **9s** was prepared via general procedure B from 4,7-dichloroisatin (90 mg, 0.43
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22 mmoles) and **8s** (116 mg, 0.86 mmoles) to yield a white solid; mp 240-243 °C. ¹H NMR
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24 (DMSO-*d*₆, 400 MHz) δ 10.85 (s, 1H), 7.56 (d, 2H, *J* = 8.8 Hz), 7.24 (d, 1H, *J* = 8.8 Hz), 6.84
25
26 (d, 1H, *J* = 8.8 Hz), 6.49 (d, 2H, *J* = 8.8 Hz), 6.28 (s, 1H), 6.10 (s, 2H), 4.20 (d, 1H, *J* = 18.0 Hz),
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28 3.45 (d, 1H, *J* = 17.6 Hz). Anal. Calcd for C₁₆H₁₂Cl₂N₂O₃: C, 54.72; H, 3.44; N, 7.98. Found: C,
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30 54.71, H, 3.44, N, 7.76.
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37 *4,7-dichloro-3-hydroxy-3-(2-(4-(methylamino)phenyl)-2-oxoethyl)indolin-2-one (9t)* Compound

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39 **9t** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmoles) and **8t**
40
41 (274.5 mg, 1.84 mmoles) to yield a white solid (154 mg, 91.4 %); mp 214-215 °C. ¹H NMR
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43 (DMSO-*d*₆, 400Hz) δ 10.86 (s, 1H), 7.67 (d, 2H, *J*=8.8), 7.27 (d, 1H, *J*=8.8), 6.88 (d, 1H, *J*=8.8),
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45 6.69 (d, 1H, *J*=8.8), 6.53 (d, 2H, *J*=7.6), 6.31 (s, 1H), 4.27 (d, 1H, *J*=17.6), 3.52 (d, 1H, *J*=17.6),
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47 2.72 (s, 3H). ¹³C NMR (DMSO-*d*₆, 100Hz): 193.66, 178.10, 154.59, 143.18, 130.67 (2C),
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49 130.25, 128.61, 123.68, 123.51, 113.16, 75.27, 60.23, 43.95, 29.57, 21.25, 14.58. HRMS (ESI)
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51 *m/z* calcd for C₁₇H₁₄Cl₂N₂O₃ (M + H)⁺ 365.0381; found, 365.0440. Anal. calcd for
52
53 C₁₇H₁₄Cl₂N₂O₃: C, 55.91; H, 3.86; N, 7.67. Found: C, 55.88; H, 3.98; N, 7.51.
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4,7-dichloro-3-(2-(4-(dimethylamino)phenyl)-2-oxoethyl)-3-hydroxyindolin-2-one (**9u**).

Compound **9u** was prepared via general procedure B from 4,7-dichloroisatin (100 mg, 0.46 mmoles) and **8u** (300 mg, 1.84 mmoles) to yield a white solid (142 mg, 81.4%); mp 205-207. ¹H NMR (DMSO-*d*₆, 400Hz) δ 10.95 (s, 1H), 7.79 (d, 2H, J=8), 7.29 (t, 3H, J=6.8), 6.88 (d, 1H, J=8.8), 6.412 (s, 1H), 4.37 (d, 1H, J=18), 3.68 (d, 1H, J=18), 2.34 (s, 6H). ¹³C NMR (DMSO-*d*₆, 100Hz): 196.44, 177.84, 144.72, 143.08, 133.5, 130.85, 129.85 (2C), 129.76, 128.64, 128.43 (2C), 123.61, 113.26, 75.01, 44.64, 21.61 (2C). HRMS (ES) *m/z* calcd for C₁₈H₁₆Cl₂N₂O₃ (M + H)⁺ 379.0381; found, 379.0607. Anal. calcd for C₁₈H₁₆Cl₂N₂O₃: C, 57.01; H, 4.25; N, 7.39. Found: C, 57.21; H, 4.80; N, 6.72.

(Z)-4,7-dichloro-3-(2-(4-methoxyphenyl)-2-oxoethylidene)indolin-2-one (**10**). To a solution of 2 (100 mg, 0.27 mmoles) in 96% sulfuric acid (5 mL) was stirred at r.t. for 5 min, and was then quenched with ice and extracted with CHCl₃ (3x25-50 mL). The combined organic layers were washed with brine, dried over Na₂SO₄ and concentrated under pressure. The dried organic mixture was purified in a flash chromatography eluting with Hexane/Ethyl acetate to afford a yellow solid (54.9 mg, 58 %); mp 253-254 °C. ¹H NMR (DMSO-*d*₆, 400Hz) δ 11.3 (s,1H), 7.97 (s, 1H), 7.88 (d, 2H, J=8.8), 7.41 (d, 1H, J=8.4), 7.11 (d, 1H, J=11), 7.04 (d, 2H, J=8.8), 3.83 (s, 3H). ¹³C NMR (100Hz, DMSO-*d*₆): 193.15, 165.9, 164.1, 142.3, 138.5, 131.5 (2C), 131.4, 130.4, 129.0, 128.2, 124.2, 119.7, 114.5 (2C), 113.7, 56.1. HRMS (ES) *m/z* calcd for C₁₇H₁₁Cl₂NO₃ (M + H)⁺ 349.0194; found, 349.0170. Anal. calcd for C₁₇H₁₁Cl₂NO₃: C, 58.64; H, 3.18; N,4.02. Found: C, 58.35; H, 3.06; N, 4.11.

Crystal data for **10**, C₁₇H₁₁Cl₂NO₃: *M* = 348.17 g/mol, yellow, prism, 0.65 × 0.56 × 0.49 mm³,

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3 monoclinic, $P2_1/c$ (No. 14), $a = 8.9990$ (3) Å, $b = 15.8170$ (5) Å, $c = 11.6532$ (4) Å, $\beta = 111.787$
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5 (1)°, $V = 1540.20$ (9) Å³ (from 9955 reflections, $\theta = 2.3$ – 29.7°), $Z = 4$, $D_c = 1.501$ g/cm³, $F_{000} =$
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8 712, Bruker APEXII CCD, Mo- $K\alpha$ radiation, $\lambda = 0.71073$ Å, $T = 223$ (2) K, $2\theta_{\max} = 56.0^\circ$,
9
10 14380 reflections collected, 3711 unique ($R_{\text{int}} = 0.0186$). Final $GooF = 1.043$, $R_I = 0.0351$, wR_2
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12 = 0.0953, R indices based on 3313 reflections with $I > 2\sigma(I)$ (refinement on F^2), 252 parameters,
13
14 0 restraints, $(\Delta/\sigma)_{\max} < 0.001$, $\Delta\rho_{\max} = 0.41$ e Å⁻³, $\Delta\rho_{\min} = -0.57$ e Å⁻³. L_p and absorption
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16 corrections applied, $\mu = 0.44$ mm⁻¹.
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23 *4,7-dichloro-3-methoxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)indolin-2-one (11)*. To a solution of
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25 **2** (75 mg, 0.20 mmol) in THF (5 mL) was added MeI (50 µL) at rt. The mixture was treated with
26
27 1.6 mmol of Ag₂O. The progress of reaction was monitored by TLC (12 h). When complete, the
28
29 reaction mixture was filtered over celite. The filtrate was washed with saturated solutions of
30
31 sodium sulfite, sodium bicarbonate and brine, dried and evaporated. The residue was purified on
32
33 silica gel column using hexane/EtOAc (9:1) as an eluent to afford an oil **11** (44.8 mg, 58 %) ¹H
34
35 NMR (CDCl₃, 400Hz) δ 7.86 (d, 2H, J=8.8), 7.80 (s, 1H), 7.22 (d, 1H, J=8.8), 6.91 (d, 2H,
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37 J=9.2), 4.52 (s, 1H, 17.6), 3.85 (s, 3H), 3.84 (d, 1H, 17.6), 3.14 (s, 3H). Anal. calcd for
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39 C₁₈H₁₅Cl₂NO₄: C, 56.86; H, 3.98; N, 3.68;. Found: C, 56.75; H, 3.97; N, 3.70.
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46 *4,7-dichloro-1-methylindoline-2,3-dione (13)*. To a solution of 4,7-dichloroisatin (500 mg, 2.31
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48 mmoles) in DMF at 0 °C, was added sodium hydride (110 mg, 4.62 mmoles). The mixture was
49
50 stirred for 30 minutes followed by dropwise addition of methyl Iodine (215 µL, 3.46 mmoles).
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52 The reaction was monitored by TLC; was quenched with ammonium chloride, extracted with
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54 ethyl acetate, washed with brine, dried over sodium sulfate and purified in a flash
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3 chromatography eluting with hexane/ethyl acetate to afford (**13**) yield, (54.51 %) 290 mg. ¹H
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5 NMR (CDCl₃, 400Hz) δ 7.45 (d, 1H, J=8.8), 7.02 (d, 1H, J=8.8), 3.65 (s, 3H).
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11 *Synthesis of 4,7-dichloro-3-methoxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)indolin-2-one (14a)*

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13 Compound **14a** was prepared via general procedure B from **13a** (75 mg, 0.33 mmoles) and **7a**
14
15 (195.8 mg, 1.31 mmoles) to yield a white solid (109.4 mg, 91.4 %); mp 158.5-159 °C. ¹H NMR
16
17 (DMSO-*d*₆, 400Hz) δ 7.88 (d, 2H, J=8.8), 7.32 (d, 1H, J=8.8), 7.01 (d, 2H, J=8.8), 6.96 (d, 1H,
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19 J=8.8), 6.44 (s, 1H), 4.45 (d, 1H, J=18), 3.82 (s, 3H), 3.70 (d, 1H, J=18), 3.48 (s, 3H). ¹³C NMR
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21 (DMSO-*d*₆, 100Hz): 195.2, 177.04, 164.02, 142.4, 132.9, 130.8 (2C), 130.4, 129.02, 128.8,
22
23 124.64, 114.5 (2C), 113.6, 73.6, 56.05, 44.7, 29.87. HRMS (ES) *m/z* calcd for C₁₈H₁₅Cl₂NO₄ (M
24
25 + H)⁺ 380.0456; found, 380.0441. Anal. calcd for C₁₈H₁₅Cl₂NO₄: C, 56.86; H, 3.98; N, 3.68.
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27 Found: C, 56.74; H, 4.01; N, 3.64.
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35 *4,7-dichloro-3-hydroxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)-1-phenylindolin-2-one (14b).* (**14b**).

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37 Compound **14b** was prepared via general procedure B from N-benzyl-4,7-dichloroisatin (100
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39 mg, 0.33 mmoles) and **7a** (202 mg, 1.31 mmoles) to yield a white solid (152 mg, 100 %); mp
40
41 154-156 °C; ¹H NMR (DMSO-*d*₆, 400 MHz) δ 7.94 (d, 2H, *J* = 8.8 Hz), 7.56 (d, 2H, *J* = 8.8
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43 Hz), 7.30 (m, 4H), 7.25 (d, 2H, *J* = 8.8 Hz), 6.98 (d, 1H, *J* = 8.8 Hz), 6.68 (s, 1H), 5.22 (s, 2H),
44
45 4.49 (d, 1H, *J* = 18.4 Hz), 3.84 (d, 1H, *J* = 18.4 Hz). Anal. calcd for C₂₃H₁₆Cl₃NO₃: C, 59.96; H,
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47 3.50; N, 3.04. Found: C, 59.96, H, 3.48, N, 3.01.
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54 Synthesis of 4,7-dichloro-3-methoxy-3-(2-(4-methoxyphenyl)-2-oxoethyl)-1-methylindolin-2-
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56 one (**15**). To a solution of **14a** (75 mg, 0.20 mmol) in THF (5 mL) was added MeI (127 μL) at rt.
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3 The mixture was treated with 1.6 mmol of Ag₂O (86.3 mg, 0.33 mmoles). The progress of
4 reaction was monitored by TLC (12 h). When complete, the reaction mixture was filtered over
5 celite. The filtrate was washed with saturated solutions of sodium sulfite, sodium bicarbonate
6 and brine, dried and evaporated. The residue was purified on silica gel column using
7 hexane/EtOAc (9:1) as an eluent to afford a white solid (46.5 mg, 57 %); mp 152-153 °C. ¹H
8 NMR (DMSO-*d*₆, 400Hz) δ 7.64 (d, 2H, J=8.8), 7.12 (d, 1H, J=8.8), 6.82 (d, 2H, J=7.6), 6.79 (d,
9 1H, J=8.8), 4.45 (d, 1H, J=18), 3.76 (s, 3H), 3.70 (d, 1H, J=20.4), 3.60 (s, 3H), 2.97 (s, 3H). ¹³C
10 NMR (DMSO-*d*₆, 100Hz): 194.68, 174.77, 164.14, 143.11, 134.09, 130.92 (2C), 129.61,
11 128.76, 125.49, 124.92, 114.51 (2C), 114.16, 79.93, 56.08, 51.85, 44.35, 29.98. HRMS (ES) *m/z*
12 calcd for C₁₉H₁₇Cl₂NO₄ (M - H)⁺ 426.0300; found 426.0306. Anal. calcd for C₁₉H₁₇Cl₂NO₄: C,
13 57.88; H, 4.35; N, 3.55. Found, C, 57.62; H, 4.49; N, 3.56.
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32 *1-(6-methoxypyridin-3-yl)ethanol*. To a suspension of 5-bromo-2-methoxypyridine (206 μL, 1.59
33 mmol) in THF (5mL) was added n-BuLi in Hexane (702 μL, 1.75 mmol) dropwise over 15 min
34 at -78 °C. The mixture was stirred for 1h at -78 °C and to the resulting solution was added
35 acetoaldehyde (98 μL, 1.75 mmol). The mixture was stirred for 2h at -78 °C and then warmed to
36 rt over 40 min. The reaction mixture was quenched with cold water and the aqueous layer was
37 extracted with Et₂O (3 x 35 mL). The combined organic layers were washed with brine and were
38 dried over Na₂SO₄ and concentrated under pressure. Purification of the semi-solid residue by
39 silica gel chromatography (hexane/EtOAc) afforded a white solid (180 mg, 73.67 %). ¹H NMR
40 (CDCl₃, 400Hz) δ 8.83 (d, 1H, J=8.4), 8.74 (d, 1H, J=8), 8.07 (dd, 1H), 5.16 (dd, 1H), 4.07 (s,
41 3H), 3.25 (s, 3H).
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1-(6-methoxypyridin-3-yl)ethanone (17). To a suspension of newly synthesized 1-(6-methoxypyridin-3-yl)ethanol (150 mg, 0.97 mmol) in CH₂Cl₂ (5mL) was added pyridium dichloromate (733 mg, 1.95 mmol) and 4 Å molecular sieve (100mg) at 0°C. The mixture was stirred 0 °C and monitor with TLC. Upon the completion of the reaction, the reaction mixture was filtered and washed with DCM. The filtered solution was washed with brine and dried over Na₂SO₄ and concentrated under pressure. Purification of the semi-solid residue by silica gel chromatography (hexane/EtOAc) afforded a white solid (90mg, 61 %). ¹H NMR (CDCl₃, 400Hz) δ 8.75 (s, 1H), 8.12 (d, 1H, J=10.8), 6.77 (d, 1H, J=8.8), 3.98 (s, 3H), 2.54 (s, 3H).

Synthesis of 4,7-dichloro-3-hydroxy-3-(2-(6-methoxypyridin-3-yl)-2-oxoethyl)indolin-2-one (18).

Compound **18** was prepared via general procedure B from 4,7-dichloroisatin (65mg, 0.3 mmoles) and **17** (90mg, 0.595 mmoles) to yield a white solid (79.8 mg, 72.45 %); mp 203-204 °C; ¹H NMR (DMSO-*d*₆, 400 MHz) δ ppm 10.98 (s, 1H), 8.85 (s, 1H), 8.12 (dd, 1H, J=11.2), 7.31 (d, 1H, J=8.8), 6.91 (dd, 2H, J=10.8), 6.45 (s, 1H), 4.37 (d, 1H, J=18), 3.94 (s, 3H), 3.72 (d, 1H, J=18). ¹³C NMR (DMSO-*d*₆, 100Hz): 194.9, 177.8, 166.9, 149.7, 143.03, 138.8, 130.9, 129.7, 128.7, 126.1, 123.7, 113.3, 111.3, 75.0, 54.5, 44.6. HRMS (ES) *m/z* calcd for C₁₆H₁₂Cl₂N₂O₄ (M + H)⁺ 367.0252; found, 367.0235. Anal. calcd for C₁₆H₁₂Cl₂N₂O₄.0.1 EtOAc C, 52.39; H, 3.43; N, 7.45. Found: C, 52.51; H, 3.4; N, 7.58.

5-(tert-butoxycarbonylamino)pentanoic acid (20). To a solution of 6-aminohexanoic acid (10g, 76.23 mmoles) in dry MeOH (90 mL) was added TEA (15.94 mL) and Boc₂O (19.96g, 91.47 mmol). The reaction mixture was reflux at 60 °C overnight. Subsequently the reaction mixture was washed with 2N HCl and the pH was adjusted to 4 and extracted with Ethyl Acetate (3 × 75

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3 mL). The combined organic extracts were washed with 1 N aqueous NaOH (3 × 75 mL)
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5 followed by extraction with ethyl acetate. The aqueous layer was washed with 1 N HCl (3 × 100
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7 mL) followed by extraction with ethyl acetate. The organic phase was washed with brine, dried
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9 with Na₂SO₄ and the volatiles removed by means of a rotary evaporator to afford a colorless oil
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11 (18.67 g, 90% yield). ¹H NMR (CD₃OD, 400 MHz) δ 5.10 (s, 1H), 4.12 (dd, 1H, J=21.2), 3.05 (t,
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13 2H, J=14), 2.31 (t, 2H, J=14), 1.65 (m, 2H), 1.50 (m, 2H), 1.43 (s, 9H), 1.39 (m, 2H).
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20 *Tert-butyl 6-(4-acetylphenylamino)-6-oxohexylcarbamate (21)*. To a solution of 20 (1.10 g, 4.75
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22 mmoles) in CH₂Cl₂ were added DCC (2.10g, 10.19 mmoles) and DMAP (166 mg, 1.36
23
24 mmoles) at 0 °C with N₂ inlet. The reaction mixture was stirred for 30 mins and 4-acetyl-N-
25
26 methylaniline (500 mg, 3.4 mmoles) was subsequently added. The resulting mixture was stirred
27
28 at room temperature for 12 hours. The resulting mixture was filtered and concentrated and
29
30 purified by silica gel chromatography (hexane/EtOAc) afforded a white solid (712 mg, 58 %). ¹H
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32 NMR (CDCl₃, 400Hz) δ 7.85 (d, 2H, J=8.8), 7.12 (d, 2H, J=8.8), 3.29 (s, 3H), 3.28 (s, 3H), 2.88
33
34 (t, 2H, J=14), 1.97 (t, 2H, J=13.2), 1.45 (m, 2H), 1.24 (s, 9H), 1.22 (m, 2H), 1.07 (m, 2H).
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41 *tert-butyl 6-((4-(2-(4,7-dichloro-3-hydroxy-2-oxoindolin-3-yl)acetyl)phenyl)(methylamino)-6-*
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43 *oxohexylcarbamate (22)*. Compound **22** was prepared via general procedure B from 4,7-
44
45 dichloroisatin (143 mg, 0.55 mmoles) and 21 (600 mg, 1.65 mmoles) to yield a yellow solid (305
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47 mg, 95 %). ¹H NMR (400Hz, DMSO-*d*₆) δ 10.97 (s, 1H), 7.95 (d, 2H, J=8.4), 7.43 (d, 2H, J=8.4,
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49 7.29 (d, 1H, J=8), 6.99 (d, 1H, J=8), 6.69 (t, 1H, J=10.8), 6.44 (s, 1H), 4.40 (d, 1H, J=18.4), 3.73
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51 (d, 1H, J=18.4), 3.17 (s, 3H), 2.84(q, 2H) 2.12 (t, 2H, J=13.1), 1.46 (m, 2H), 1.32 (s, 9H), 1.28
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53 (m, 2H), 1.14 (m, 2H).
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7-amino-N-(4-(2-(4,7-dichloro-3-hydroxy-2-oxoindolin-3-yl)acetyl)phenyl)-N-

methylheptanamide (23). To a suspension of **22** (300 mg, 0.518 mmoles) in CH₂Cl₂ (3 mL) was added treated with TFA (1 mL) and the reaction mixture was stirring for 1.5 h at room temperature. The solvent and the TFA were removed in vacuo. The crude product was purified by silica gel chromatography (CH₂Cl₂/MeOH) afforded a yellow solid in a quantitative yield. ¹H NMR (DMSO-*d*₆, 400Hz) δ 7.97 (d, 2H, J=8.4), 7.44 (d, 2H, J=8.4), 7.30 (d, 1H, J=8.8), 6.89 (d, 1H, J=8.8), 6.45 (s, 1H), 4.4 (d, 1H, J=18.4), 3.74 (d, 1H, J=18.4), 3.30 (s, 2H), 3.18 (s, 3H), 2.72 (t, 2H, J=18.4), 2.12 (t, 2H, J=6.8), 1.49 (m, 4H), 1.22 (m, 2H).

N-(4-(2-(4,7-dichloro-3-hydroxy-2-oxoindolin-3-yl)acetyl)phenyl)-N-methyl-6-(5-((4R)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanamide (24).

To a solution of biotin (66.5 mg, 0.27 mmoles) in DCM was added EDCI-HCl (52.2 mg, 0.27 mmoles), and HOBt (41.7 mg, 0.27 mmoles). After stirring at RT for 30 min the **23** (100 mg, 0.20 mmoles) was added followed by DMAP (33.2 mg, 0.27 mmoles). The reaction was stirred at RT for 16 h, followed by flash column purification. The resulting fractions were evaporated to afford a white powder. ¹H NMR (CD₃OD, 400Hz): 11.02 (s, 1H), 8.02 (d, 2H, J= 8.8), 7.87 (t, 1H, J= 10.8), 7.41 (d, 2H, J=8.8), 7.25 (d, 1H, J=8.8), 6.90 (d, 1H, J=8.8), 4.83 (d, 1H, J=18), 4.58 (d, 1H, J=18), 4.95 (q, 1H), 4.30 (dd, 1H, J=12.4), 3.84 (dd, 1H, J=19.6), 3.32-3.30 (m, 2H), 3.27 (s, 3H), 3.20-3.15 (m, 1H), 3.13 (q, 2H), 2.92 (dd,dd, 1H), 2.7 (d, 1H, J=12.8), 2.18 (t, 4H, J=14.4), 1.74-1.63 (m, 2H), 1.59-1.55 (m, 4H), 1.44-1.36 (m, 4H), 1.28-1.77 (m, 2H). ¹³C NMR (CD₃OD, 100Hz): 195.68, 178.39, 174.50, 173.60, 164.6, 148.40, 142.33, 130.41 (2C), 129.34, 128.92, 128.64, 127.23, 123.57 (2C), 113.79, 75.10, 61.98, 60.21, 55.57, 44.18, 39.64, 38.78,

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3 38.66, 36.29, 35.41, 33.56, 28.64, 28.33, 28.07, 26.02, 25.50, 24.72. Anal. calcd for
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5 $C_{33}H_{39}Cl_2N_5O_6S$: C, 56.25; H, 5.58; N, 9.94. Found: C, 56.20; H, 5.61; N, 9.98
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10 **X-ray Crystallography**

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12 Single crystals of **10** were grown by slow evaporation from ethyl acetate. Single crystal
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14 diffraction data were collected at 223(2) K on a Bruker APEX II CCD X-ray diffractometer
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16 equipped with a 700 Series Oxford Cryostream low temperature device and employing Mo-
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18 $K\alpha$ radiation (0.71073 Å). Notably, initial attempts to collect data at 100(2) K were
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20 unsuccessful due to deterioration of the crystals at this temperature, presumably the result of a
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22 temperature-induced phase change. The structure was solved by direct methods using SHELXS
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24 and all structural refinements were conducted using SHELXL-97-2²⁹. All non-hydrogen atoms
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26 were modeled with anisotropic displacement parameters. All hydrogen atoms were located in
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28 the final difference Fourier map and were refined, without restraints, with isotropic displacement
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30 parameters. The program X-Seed was used as a graphical interface for the SHELX software
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32 suite and for the generation of figures³⁰. CCDC-988096 contains the supplementary
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34 crystallographic data. This data can be obtained free of charge via
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36 <http://www.ccdc.cam.ac.uk/conts/retrieving.html>, or from the Cambridge 150
37
38 Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, U.K.; fax: (+44) 1223-
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40 336-033; or e-mail: deposit@ccdc.cam.ac.uk.
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51 **Biological Evaluation**

52 **Cell Culture**

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55 TC32, TC71 cells were grown in RPMI with 10% fetal bovine serum (FBS) and 1% HEPES.
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3 PANC1 and COS7 cells were grown in DMEM with 10% FBS. Cells were grown at 37°C in
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5 humidified atmosphere of 5% CO₂ in a VWR CO₂ incubator.
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10 **Cell Proliferation Assays**

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12 The effect of the new derivatives on TC32, TC71 and Panc1 cells viability were assessed. Cells
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14 were grown in colorless media and plated at a density of 5,000-15,000 cells/well, depending on
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16 cell line, in a 96-well plate. Analogs or vehicle alone (DMSO) were added to cells in appropriate
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18 growth media the day after plating. Viable cells were quantified using MTT or WST-1 per the
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20 manufacturer's protocol after three days of treatment. GI₅₀ values were calculated by nonlinear
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22 regression analysis using Prism Graphpad 4.0.
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29 **Reporter Assay**

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31 EWS-FLI1 activity was assessed using NR0B1 luciferase construct containing EWS-FLI1
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33 binding sites. COS7 cells were transiently transfected with NR0B1 luciferase reporter and full
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35 length EWS-FLI1 or empty vector control (pCI-neo-EV) with X-tremegene 9 (Roche) according
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37 to the manufacturer's protocol. One and half hours after transfection, cells were treated with
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39 0.05, 0.15, 0.5, 1.5, 5 and 15 μM of appropriate compounds or DMSO. Luciferase activity was
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41 measured 20 hours after treatments. All the luciferase assays were performed using a luciferase
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43 assay kit according to the manufacturer's protocol (Promega). IC₅₀ values were calculated by
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45 sigmoidal dose-response curve fit using Prism Graphpad 4.0.
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52 **Protein Purification**

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55 EWS-FLI1 was expressed from in BL21 cells to an OD of 0.6-0.8 and induced with 1mM IPTG.
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3 Following 2 hours of shaking at 27 °C, the protein was pelleted by centrifugation at 9500RPM.
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5 The inclusion bodies were treated with Bugbuster (Novagen) following the manufacturer's
6
7 instructions. The resulting pellet was denatured in binding buffer containing 20 mM Tris-HCl,
8
9 500 mM NaCl, 6 M guanidinium, and 50 mM imidazole, pH 7.4 and filtered through a 0.22
10
11 micron filter. Protein was loaded on a HiTrap Chelating HP column (GE) that is pre-charged
12
13 with 0.1 M NiSO₄ and subject to purification on AKTA explorer or AKTA purifier system.
14
15 Protein was loaded on the column at 0.5 mL/min and washed with buffer contain 20 mM Tris-
16
17 HCL, 500 mM NaCl, 8 M urea, and 50 mM imidazole. Next, the protein on the column was
18
19 refolded using a slow gradient to a buffer containing 20 mM Tris-HCl, 500 mM NaCl, and 50
20
21 mM imidazole at 0.5 mL/minute. Column elution done in a buffer containing 2 M imidazole was
22
23 performed with the protein eluting in fractions equivalent to 1.2 M imidazole.
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32 **Binding Assay**

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34 A 96 well plate (Maxisorb) was coated with 100 µL recombinant EWS-FLI1 overnight at a
35
36 concentration of 20 nM per well. Coated wells were blocked with 150 µL 4% bovine albumin
37
38 serum (BSA) in PBS at for 1h room temperature. The biotin conjugate **24** was diluted from stock
39
40 DMSO in assay buffer (A15). After washing the conjugate was added to each well and incubated
41
42 for 1h. The complex was washed five times in a washing buffer followed by the incubation of
43
44 1:5000 dilution of HRP-streptavidin (Thermo Scientific, N100) for 1h at room temperature. After
45
46 washing five times, the bound biotin conjugate was detected using the colorimetric phosphatase
47
48 substrate. After development, the absorbance was measured at 405nm. The data was analyzed
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50 using prism Graphpad.
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Notes

The authors declare the following financial interest(s): P.T., Y.K., U. A., J.T., and M.B. are co-inventors on the following patent: PCT/US2013/036234.

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ABBREVIATIONS

ESFT, Ewing's sarcoma family tumors; ES, Ewing's sarcoma; ETS, E26 transformation-specific; FLI1, friend leukemia 1; IDP, intrinsically disordered protein; RHA, RNA helicase A; n-BuLi, n-butyllithium; PDC, Pyridinium dichromate; DCC, *N,N'*-dicyclohexylcarbodiimide; EDCI, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; WST, water soluble tetrazolium.

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Table of Contents graphic

