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Synthesis and Antitumor Effect in Vitro and in Vivo of Substituted 1,3-Dihydroindole-2-ones

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Optimization of the anticancer activity for a class of compounds built on a 1,3-dihydroindole-2-one scaffold was performed. In comparison with recently published derivatives of oxyphenisatin the new analogues exhibited an equally potent antiproliferative activity in vitro and improved tolerability and activity in vivo. The best compounds from this series showed low nanomolar antiproliferative activity toward a series of cancer cell lines (compound (*S*)-**38**: IC_{50} of 0.48 and 2 nM in MCF-7 (breast) and PC3 (prostate), respectively) and potent antitumor effects in well tolerated doses in xenograft models. The racemic compound (*RS*)-**38** showed complete tumor regression at a dose of 20 mg/kg administered iv on days 1 and 7 in a PC3 rat xenograft.

Introduction

Inhibition of cancer cell proliferation is one of the most effective principles in the treatment of cancer using chemotherapy. Many compounds presently used in cancer treatment affect proliferation and/or lead to cancer cell death via apoptosis, necrosis, or alike. A challenge in this approach is to obtain compounds with sufficient selectivity toward cancer cells versus normal cells in order to avoid toxicological problems and side effects commonly seen when using cytotoxics, such as inhibition of rapidly dividing cells (e.g., bone marrow and intestinal stem cells). Thus, during any development of new drugs, an assessment of the toxicological profile is essential in order to determine a sufficient therapeutic window, enabling efficient use.

In the present paper further optimization of a recently described group of compounds based on a 1,3-dihydroindole-2-one scaffold is described.[1,2] These compounds exhibit potent antiproliferative effects in vitro and high efficacy in mouse xenograft models. However, the first generation of this compound class showed only a narrow therapeutic window when tested in rat xenografts and further optimization was thus warranted.

The starting point for this investigation was the recently described 3,3-substituted oxindole compounds such as 6,7-difluoro-3,3-bis(4-hydroxyphenyl)-2-oxindole (**2**, TOP216) (Figure 1, Table 1) which was shown to be a potent inhibitor of cell proliferation. This class of compounds was discovered in a screen for compounds with antiproliferative activity and was later optimized. Compound **2** is a substituted derivative of oxyphenisatin (**1**, 3,3-bis(4-hydroxyphenyl)-2-oxindole) (Figure 1), which has been extensively used in man as a laxative. This nonprescription drug **1** was used for more than 40 years before it was taken off the market because of transient hepatotoxicity.[3−5] However, the hepatic reaction caused by **1** is presumably due to a hypersensitivity response rather than a nonspecific toxic effect.[3−5]

In vitro, compound **2** is found to potently inhibit proliferation in the breast cancer cell line MDA-MB-468 (IC_{50} = 3 nM).[1] Interestingly, this class of compounds has a broad but distinct selectivity for inhibition of cancer cells, some being very sensitive, others almost resistant as further discussed below. Also, compound **2** is found to be well tolerated and shows efficient effects in vivo when tested in breast (MCF-7) and prostate cancer (PC3) xenograft models in mice.[2] To further explore the therapeutic potential of this compound, the maximum tolerated dose (MTD[a]) of **2** was determined in rat, where the compound was found to be much more toxic than in mice. Thus, potent effects in rat xenografts with compounds closely related to **2** (e.g., compound **4**) were only observed at doses close to or above the MTD. Therefore, it was decided to optimize these compounds further with respect to the in vivo effects and the therapeutic window using the rat in the toxicological assessment.

Since the precise mode of action and the biological target of the compounds are not known at present, the optimization was performed using standard cancer cell proliferation assays, followed by pharmacokinetic and toxicological assessment in

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[a] Abbreviations: AUC, area under the curve; DCM, dichloromethane; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; HP-β-CD, (2-hydroxypropyl)-β-cyclodextrin; HRMS, high resolution mass spectrometry; IC_{50}, concentration of a test compound that produces half maximal inhibition; LC−MS, liquid chromatography−mass spectrometry; MTD, maximum tolerated dose; NMR, nuclear magnetic resonance; p-TSA, *p*-toluenesulfonic acid; SAR, structure−activity relationship; SD, standard deviation; SDS−PAGE sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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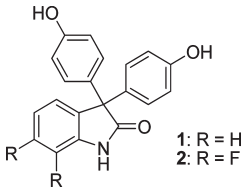
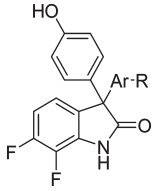


Figure 1. Oxyphenisatin (**1**) and the analogue compound TOP216 (**2**).

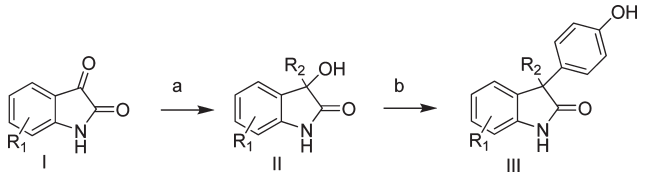
Table 1. SAR: Determination of Antiproliferative Activity of Compounds **1**−**14**



			IC_{50} (nM)[b]	
compd	Ar	R	WST-1[a] MCF-7	PC3
1			73% at 0.5 μM	> 1000
2	Ph	4-OH	20 ± 4.4	699
3	Ph	H	7.1 ± 0.24	8.9 ± 0.35
4	Ph	4-F	2.6 ± 0.09	8.6 ± 0.14
5	Ph	4-Me	1.7 ± 0.01	0.8 ± 0.7
6	Ph	4-OMe	6.3 ± 0.42	2.2 ± 3.4
7	Ph	4-OEt	11 ± 1.5	24 ± 13
8	Ph	4-O-*n*-Pr	356	> 1000
9	Ph	4-O-*n*-Pent	> 1000	> 1000
10	(3-OH-Ph),[c] Ph	4-OMe	196 ± 18	145 ± 25
11	Ph	3,4-di-F	7.8 ± 3.1	19 ± 1.5
12	2-thiophene	H	82 ± 2.0	0.3 ± 0.1
13	4-pyridyl	H	37 ± 3.7	110 ± 184
14	3-pyridyl	H	63 ± 0.63	> 1000

[a] Cancer cells were incubated for the designated time and the number of viable cells assessed using cell proliferation reagent WST-1. [b] IC_{50} values are the mean ± SD of at least two experiments performed in triplicate. [c] 3-OH-Ph in place of 4-OH-Ph.

Scheme 1. Synthesis of 3,3-Diaryl- or 3-Alkyl-3-aryloxindoles **III**[a]



[a] Reagents and conditions: (a) R_2MgX (3 equiv), THF, −78 °C; (b) phenol (5 equiv), p-TSA (7.5 equiv), dichloroethane.

vivo and efficacy determination in xenograft models. Ongoing studies of the mode of action are also presented.

Chemistry

The general synthetic approach to the 3,3-diaryl- or 3-alkyl-3-aryloxindoles **III** is outlined in Scheme 1. The first step involved a Grignard addition to the isatin derivative **I**, using an aryl- or alkylmagnesium halide, and the second step involved an elimination−addition reaction.[6,7] This was accomplished by an acid-catalyzed Friedel−Crafts type condensation of compound **II** with phenol to generate 3,3-diaryl- or 3-alkyl-3-aryloxindoles **III**. The isatin derivatives **I** were either commercially available or synthesized according to literature procedures.[8−14]

Results and Discussion

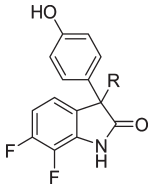
As earlier reported, the SAR studies for substituted analogues of compound **1** have only been explored for the symmetrically substituted 3,3-diphenyl-substituted scaffold. In summary, the most potent compounds are 3,3-bis(4-hydroxyphenyl) moieties with small substituents in the 6 and 7 positions of the oxindole ring.[1] Alternative or further substitution of the phenyl rings and larger substituents in the oxindole do not improve in vitro activity.

Lead Optimization. In the initial optimization approach, aromatic substituents different from 4-hydroxyphenyl were introduced in place of one of the 4-hydroxyphenyl substituents in the 3-position of the lead compound **2**. The effects of these substitutions were determined by the antiproliferative activities of the compounds in MCF-7 (breast) and PC3 (prostate) cancer cell lines (Table 1). A simple removal of one of the hydroxyl groups from the 4-hydroxyphenyl moieties gave a compound **3**, which retained high antiproliferative potency (Table 1). Removal of both hydroxyl groups gave inactive compounds (data not shown), and this illustrates that only one phenolic group is needed for high activity. Substitution of the 3-phenyl group showed that small substituents in the 4-position of the phenyl moiety were well tolerated (compounds **4**−**7**). Compounds **4** and **5** with a 4-fluorophenyl and a 4-Me-phenyl group were the most potent with IC_{50} values of 2.6 and 1.7 nM, respectively, in the MCF-7 WST-1 screening assay. Larger alkoxy substituents, such as propoxy (**8**) and pentoxy (**9**), in the 4-position gave compounds with low or no activity, suggesting a hydrophobic interaction with some limitation in size. Compound **10** showed that the 4-hydroxyphenyl as one of the substituents in the 3-position of the oxindole is crucial for high activity. Further substitution in the second phenyl ring is allowed, as exemplified by compound **11**. However, this did not improve in vitro activity substantially, and compounds that are more structurally distinct from the starting compound **2** were therefore synthesized to tentatively increase the chances for altered activity and toxicological profile.

Replacement of one of the 4-hydroxyphenyl groups by heterocycles, such as compounds **12**−**14**, did not improve activity in the MCF-7 cell lines (Table 1) and was not further explored, mainly because of sluggish chemistry that generally gave low yields and complicated purifications. Further replacement of one 4-hydroxyphenyl group with alkyl and cycloalkyl groups gave a new series of interesting compounds (Table 2). Compounds with small *n*-alkyl substituents (**15**, **16**) showed only low activity in the MCF-7 cell lines (Table 2). However, compounds with cyclic substituents (**17**−**19**) were found to be active with an optimum for the cycloheptyl derivative **19** (Table 2). Larger alkyl and alkylaryl groups (**20**−**22**) led to compounds with low activity or inactive compounds (Table 2).

The antiproliferative activities were analyzed for substituted analogues of the most interesting compounds from Tables 1 and 2, such as compounds carrying different substituents in position 4, 5, 6, or 7 of the oxindol (Table 3). Compounds with a substituent at the 4- and 5-position of the oxindole did not show any significant activity (data not shown). With the toxicological effects observed for compound **2** in mind we wanted to substitute the 6,7-difluoro with alternative groups in order to structurally discriminate these compounds from the starting compound but with

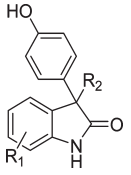
Table 2. SAR: Determination of Antiproliferative Activity of Compounds **15**−**22**



compd	R	IC_{50} (nM)[b] WST-1[a] MCF-7	PC3
15	*n*-propyl	> 1000	> 1000
16	*n*-pentyl	96 ± 7.8	1.1 ± 1.2
17	cyclopentyl	12 ± 1.6	8.2 ± 6.9
18	cyclohexyl	13 ± 2.4	32 ± 17
19	cycloheptyl	1.2 ± 1.9	12 ± 1.2
20	cyclooctyl	> 1000	> 1000
21	methylcyclohexyl	102 ± 70	> 1000
22	methylphenyl	> 1000	> 1000

[a] Cancer cells were incubated for the designated time and the number of viable cells assessed using cell proliferation reagent WST-1. [b] IC_{50} values are the mean ± SD of at least two experiments performed in triplicate.

Table 3. SAR: Determination of Antiproliferative Activity of Compounds **23**−**38**



compd	R_1	R_2	IC_{50} (nM)[b] WST-1[a] MCF-7	PC3
23	H	4-F-Ph	28 ± 6.7	> 1000
24	H	cycloheptyl	247 ± 121	> 1000
25	7-Me	cycloheptyl	> 1000	> 1000
26	7-CF_3	cycloheptyl	9.7	91
27	7-CF_3	imidazolyl	> 1000	> 1000
28	5,7-di-Me	4-F-Ph	40 ± 51	209 ± 94
29	5,7-di-Me	cycloheptyl	> 1000	> 1000
30	5-F,7-Me	cycloheptyl	> 1000	> 1000
31	6,7-di-Me	cycloheptyl	19	> 1000
32	6-F,7-Me	cycloheptyl	20 ± 0.21	30 ± 21
33	6-Cl,7-Me	cycloheptyl	51 ± 27	150
34	6-Me,7-Cl	cycloheptyl	2.8 ± 1	17
35	6-OMe,7-Me	4-F-Ph	6.3	9 ± 2.4
36	6-OMe,7-Me	cyclopentyl	1 ± 0.69	111 ± 179
37	6-OMe,7-Me	cyclohexyl	14 ± 2.5	28 ± 3.9
38	6-OMe,7-Me	cycloheptyl	4.7 ± 0.05	12 ± 15

[a] Cancer cells were incubated for 72 h and the number of viable cells assessed using cell proliferation reagent WST-1. [b] IC_{50} values are the mean ± SD of at least two experiments performed in triplicate.

retained activity. The structure−activity relationship (SAR) was carefully investigated for the two very active compounds **4** and **19**. The unsubstituted derivatives (**23**, **24**) lost much of their activity, but some of the activity could be retained by monosubstitution, preferably at the 7-position (e.g., compound **26**, Table 3). On the basis of earlier work, larger or polar substituents reduce the potency.[1] However, as it evident from Table 3, the data indicate that halogens and small lipophilic substituents in the 6 and 7 positions not only are allowed but are important to achieve high antiproliferative activity (compounds **31**−**38**). The preferred substitution pattern was the 6-methoxy and 7-methyl in compounds **35**−**38**, and the cycloheptyl analogue **38** was found to be one of the most potent derivatives in this series in both cell lines, structurally distinct from the starting compound **2** and exhibiting very high antiproliferative activity (IC_{50} of 4.7 and 12 nM in WST-1 MCF-7 and PC3, respectively).

Table 4. SAR: Determination of Antiproliferative Activity of Enantiomers of Compounds **6**, **18**, and **38**

compd	enantiomer	IC_{50} (nM)[b] WST-1[a] MCF-7	PC3
6	1	2.1 ± 0.12	2 ± 0.42
6	2	> 1000	> 1000
18	1	18 ± 11.2	9.2 ± 1.8
18	2	> 1000	> 1000
(*S*)-**38**	1	0.48 ± 0.19	2 ± 1.1
(*R*)-**38**	2	> 1000	> 1000

[a] Cancer cells were incubated for the designated time and the number of viable cells assessed using cell proliferation reagent WST-1. [b] IC_{50} values are mean values ± SD of at least two experiments performed in triplicate.

Stereochemistry. The new compounds discussed above were all racemates with a chiral center at the 3-position of the oxindole. To explore the stereochemical preference of the target, selected potent compounds **6**, **18**, and **38** were resolved by chiral chromatography (Chiracel OD) and the antiproliferative activity was determined for the pure enantiomers. Interestingly, a high eudismic ratio was observed for all three compounds, the first eluting enantiomer being the eutomer (Table 4). The great difference in activity was further confirmed in a clonogenic assay using MCF-7 and A2780 cell lines (Figure 2). The high activity difference for the stereoisomers suggested a specific biological interaction with a receptor or an enzyme. The absolute configuration of the active stereoisomer of compound **38** was determined to be *S* by X-ray crystallography of the corresponding Mosher ester. The diastereoisomeric Mosher esters of **38** were separated by chromatography followed by crystallization and determination of the absolute configuration by X-ray diffraction. The absolute configuration of the Mosher ester could then be correlated back to **38** by hydrolysis of the Mosher ester and analysis using chiral chromatography. Because of the close structural similarity between **6**, **18**, and **38** and because of the similar elution order on Chiralcel OD-H, it is most likely that the active enantiomers possess the same structural configuration, which would be (*R*)-**6** and (*S*)-**18**, respectively.

In Vitro Activity and Mode of Action. As reported, compound **2** has a similar selectivity profile as the mTOR inhibitor CCI-779 with high cytotoxic activity at low nanomolar concentrations toward a subset of human cancer cell lines and with a striking selectivity (>1000-fold) against naturally resistant cell lines, e.g., MDA-MB-468 IC_{50} = 20 nM vs MDA-MB-231 IC_{50} > 3 μM.[1] The molecular basis for the very high selectivity of compound **2** and analogues toward specific cancer cell lines has not yet been elucidated, but it is currently being investigated. In an attempt to learn more about the mode of action of **2** and the new analogues with the same profile, two resistant cell lines, MCF-7/TOP216 and A2780/TOP216, were generated by culturing the parental cell lines with increasing concentrations of **2** (Trojel-Hansen et al., manuscript in preparation). The compound **2** resistant cell lines showed cross-resistance with the new analogues prepared, indicating a similar mode of action. Furthermore, it has been demonstrated that treatment of

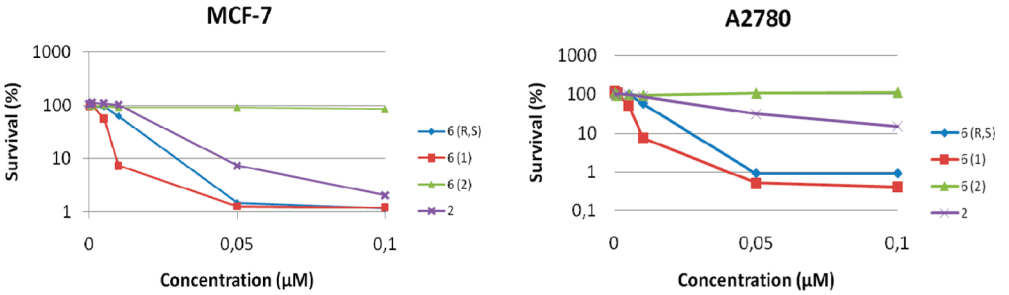


Figure 2. Clonogenic assay for racemate and pure enantiomers (1 or 2) of compound **6** and compound **2**. In vitro colony forming assays were performed in MCF-7 (breast carcinoma) and A2780 (ovarian carcinoma) cancer cells essentially as previous published.[16]

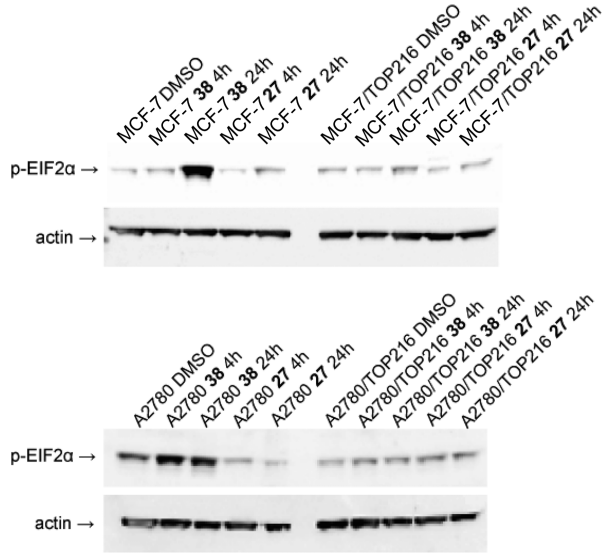


Figure 3. Treatment with the active compound **38** but not the inactive analogue **27** led to an increased p-eIF2α phosphorylation. MCF-7 and A2780 including their **2**-resistant sublines were treated with 100 mM **38** and **27** for 4 and 24 h, respectively. The resistant sublines showed cross-resistance toward **38** as indicated by lack of p-eIF2α induction.

cells with 3,3-diaryloxindoles, compounds with a structural similarity to the compounds in this paper, leads to phosphorylation of the translational initiation factor eIF2α at the regulatory site, serine 51.[15] To investigate whether this mechanism is conserved among the present compounds, human breast cancer MCF-7 cells and human ovary cancer A2780 cells were incubated in the presence of the active compound **38** or the inactive analogue **27**. Treatment with **38** for 24 h leads to a robust induction of the phosphorylation of eIF2α, while **27** had no effect on eIF2α phosphorylation (Figure 3). In the MCF-7 and A2780 subcell lines with induced resistance to **2** (MCF-7/TOP216 and A2780/TOP216, respectively) **38** had no effect on eIF2α phosphorylation, suggesting that the induction of eIF2α phosphorylation is an integral part of the mechanism of compound **38** mediating inhibition of cell proliferation.

In Vivo Antitumor Activity and Toxicology. Selected compounds with potent (nanomolar) activity in the in vitro screen (Tables 1−4) were tested in vivo in a PC3 (human prostate cancer) mouse xenograft model. The PC3 cells were grown on nude NMRI mice, and treatment was initiated at large tumor size (800−1000 mm^3) to observe for tumor regression. The compounds were formulated in 2% DMSO/20% HP-β-CD and isotonic sterile saline and administered iv at 5−20 mL/kg three times weekly. Several of the compounds

Table 5. In Vivo PC3 Xenograft Activities in Mouse and Rat[a]

compd	mouse tumor regression[b] dose (mg/kg)	rat tumor regression[b,c] dose (mg/kg)	rat MTD (mg/kg)	rat therapeutic index[d]
2	5	ND	1	0.4
4	2.5	< 2.5	2.5	2
18	10	< 10	10	2
19	10	ND	> 40	8
26	20	> 20	40	4
35	10	< 10	15	3
38	20	10	70	7

[a] The antitumor effect in vivo was tested in a subcutaneous (sc) xenograft model in nude mice or rat. ND: not determined. [b] Schedule: ×3/week iv, PC3 mouse or rat xenograft model. [c] The effective doses in rats were estimated from mouse xenograft models (mouse to rat conversion factor: 0.5). [d] Therapeutic index: (toxic dose in rats)/(effective dose in rats).

tested induced tumor stasis and regression (including cures) at low concentrations (5−20 mg/kg) comparable to or better than those of compound **2** (Table 5). Interestingly, in mice no signs of toxicity were observed at concentrations, where the compound was highly active. To further select the best compounds, the MTD in rat was determined, followed by measurements of efficacy in a prostate cancer (PC3) rat xenograft model. The MTD of **2** was determined to be 1 mg/kg at a single dose after iv administration, leading to a therapeutic index below 1 (Table 5). At doses above the MTD, the main toxicological findings were respiratory distress immediately after treatment followed by a mucous-containing diarrhea. In some cases the rats died with vascular shocklike symptoms shortly after dosing. Compounds with only one phenol group and an aromatic or cycloalkyl substituent in the 3-position (such as **4**, **18**, and **19**) showed a weak but significant improvement in the MTD. Further substitution in the oxindole group also gave compounds with improved toxicological profile, e.g., compounds **26** and **35**. In this series, compound **38** was observed with the highest MTD (70 mg/kg) and a therapeutic index of 7. This compound showed a very high antitumor activity in the rat PC3 xenograft model with no signs of toxicity when administered iv on days 0 and 7 at a dose of 20 mg/kg (Figure 4). In this experiment the PC3 tumors disappeared and no regrowth was observed until the end of study at day 26. The observed increase in body weight was normal. The difference in toxicology between the selected compounds does not seem to be explained by simple pharmacokinetic data, since the $T_{1/2}$ and AUC for the compounds were similar in rat (Table 6). The more dramatic difference in toxicological sensitivity found between species (mouse vs rat) could not be easily explained. However, some distribution differences in the two species were observed with higher concentrations of the drug in the rat lung compared to concentrations in the mouse lung. Also, a more

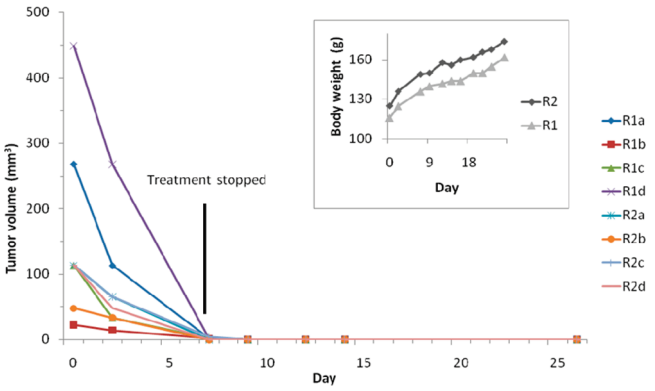


Figure 4. Treatment with compound **38** in rat xenograft PC3 tumor model. Compound **38**, 20 mg/kg iv on days 0 and 7, resulted in tumor regression of PC3 human prostate cancer cell growth on nude rats. Each of two rats (R1 and R2) had four tumor nodes (a−d). The tumor volume at start was from 50 to 450 mm^3. Regression was observed after the first treatment. Rat received an extra treatment on day 7, and regrowth was not observed at day 26 when the experiment was terminated. Treatment with **38** had no effect on body weights or clinical condition of the rats.

Table 6. Pharmacokinetic Parameters for Selected Compounds in Mouse and Rat[a]

	mouse			rat		
compd	dose iv (mg/kg)	$T_{1/2}$ (h)	AUC (h·ng/mL)	dose iv (mg/kg)	$T_{1/2}$ (h)	AUC (h·ng/mL)
2	5	1.7	6769	ND	ND	ND
4	5	2.1	1913	2.5	0.8	2534
12	5	0.4	395	2.5	0.7	2634
17	5	3.7	1849	2.5	1.1	2396
18	5	1.6	3554	5	1.1	1353
19	5	1.3	694	5	2.1	2288
35	ND	ND	ND	10	1.5	3949
38	ND	ND	ND	10	1.5	5760

[a] ND: not determined.

rapid uptake was found for compound **2** in the lung compared to the less toxic compound **38**. This may be one reason for the acute toxicological reactions in the rat. Further pharmacokinetic studies may clarify this possibility and suggest ways to further improve the toxicological profile of these interesting drug leads.

Conclusion

The present study describes the successful optimization of the therapeutic and toxicological effects of 1,3-dihydroindole-2-ones as anticancer agents in vivo. Modification of the substitution pattern on this scaffold gave compound **38**, which showed very potent anticancer effects in a rat PC3 xenograft model at well tolerated doses, suggesting this compound to be an interesting candidate for further preclinical evaluation. Present activities aim for improved pharmacokinetics and possibly distribution to diminish toxicological side effects through the preparation of prodrugs of the most active compounds. Future work is also pursued to elucidate the mode of action of this unique active group of compounds.

Experimental Section

Reaction conditions and yields were not optimized. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 spectrometer (300 MHz). Chemical shifts are reported in parts per million (δ) and referenced according to deuterated solvent for ^1H spectra ($CDCl_3$, 7.26; CD_3OD, 3.31; $(CD_3)_2SO$, 2.50) and ^{13}C spectra ($CDCl_3$, 77.23; CD_3OD, 49.00; $(CD_3)_2SO$, 39.52). The value of a multiplet, defined doublet (d), triplet (t), double doublet (dd), double triplet (dt), quartet (q), or not (m) at the approximate midpoint is given unless a range is quoted. bs indicates a broad singlet. The purity of the compounds was determined using an LC−MS (Bruker Esquire 3000+ ESI ion trap with an Agilent 1200 HPLC system) and was confirmed to be ≥95% for all compounds. HRMS was carried out on a Micromass Q-Tof micro mass spectrometer. The HPLC system for the semipreparative resolution of the Mosher ester of compound **38** consisted of a Jasco 880 pump, a Rheodyne 7125 injector equipped with a 5 mL loop, and a Shimadzu SPD-6A UV detector connected to a Hitachi-D2000 Chromato integrator.

General Procedure A: Grignard Reaction to Form Tertiary Alcohols of General Formula II, Followed by Friedel−Crafts Reaction with Phenol. To a stirred solution of isatin derivative **I** in dry THF under nitrogen at −78 °C was added 3 equiv of Grignard reagent. After 30 min, the dry ice bath was removed and the mixture was left to reach room temperature over 4−14 h.[7] Excess Grignard reagent was quenched with water, and the reaction mixture was acidified with 1 N HCl or saturated NH_4Cl solution, extracted with EtOAc (×2), dried over $MgSO_4$, filtered, and concentrated. The residue was purified by chromatography (1% MeOH in DCM or mixtures of petroleum ether and EtOAc) to afford racemic tertiary alcohol **II**.

To a solution of tertiary alcohol of general formula **II** in dichloroethane was added phenol (5 equiv) and p-TSA (7.5 equiv). The reaction mixture was heated to 90 °C for 2−4 h and then cooled to room temperature. The solid (mainly p-TSA) was filtered off and washed with dichloroethane or DCM. The solution was concentrated and the residue was purified by chromatography (1% MeOH in DCM or mixtures of petroleum ether and EtOAc) to afford racemic compounds of general formula **III**.

(*RS*)-3-Cycloheptyl-3-(4-hydroxyphenyl)-6-methoxy-7-methylindolin-2-one (38). Preparation of **38** was performed according to general procedure A using 6-methoxy-7-methylindoline-2,3-dione and cycloheptylmagnesium bromide.

(*RS*)-3-Cycloheptyl-3-hydroxy-6-methoxy-7-methylindolin-2-one: ^1H NMR ($(CD_3)_2SO$) δ 10.21 (bs, 1H), 7.00 (d, J = 8.3 Hz, 1H), 6.49 (d, J = 8.3 Hz, 1H), 5.58 (s, 1H), 3.75 (s, 3H), 2.08 (m, 1H), 2.01 (s, 3H), 1.88 (m, 1H), 1.73 (m, 1H), 1.55 (m, 1H), 1.50−1.20 (m, 8H), 0.75 (m, 1 H). Yield 35%.

Product 38: ^1H NMR ($(CD_3)_2SO$) δ 10.38 (bs, 1H), 9.28 (bs, 1H), 7.13 (m, 2H), 7.07 (d, J = 8.2 Hz, 1 H), 6.67 (m, 2H), 6.61 (d, J = 8.2 Hz, 1H), 3.79 (s, 3H), 2.27 (m, 1H), 2.05 (s, 3H), 1.58 (m, 3H), 1.41 (m, 2H), 1.13 (m, 3H), 0.93 (m, 1H), 0.68 (m, 1H). HRMS m/z calcd for $C_{23}H_{27}NO_3$ [M + Na], 388.1889; found, 388.1936. Yield 90%.

Cell Culture. Human breast carcinoma MCF-7, ovarian carcinoma A2780, and prostate PC3 cell lines were grown according to American Type Culture Collection guidelines. Cell culture media were from Invitrogen unless otherwise stated. MCF-7 was maintained in DMEM and A2780 in RPMI 1640 with GlutaMax. Media were supplemented with 10% (v/v) FCS (Perbio, Thermo Fischer Scientific), penicillin (100 U/mL), streptomycin (0.1 mg/mL) and cells incubated at 37 °C in an atmosphere containing 5% CO_2.

WST-1 Proliferation Assay. Cells were seeded in 96-well plates at 3×10^3 cells/well in 100 μL of culture medium. The following day compounds were serially diluted in culture medium and an amount of 100 μL of each dilution was added per well in triplicate to the cell culture plates. Plates were incubated for 72 h at 37 °C in a 5% CO_2 atmosphere and the number of viable cells assessed using cell proliferation reagent WST-1 (Roche, Mannheim, Germany). An amount of 10 μL of reagent was added to each well, and after a 1 h incubation period, absorbance was measured at 450 nm, subtracting absorbance at 690 nm as a reference. Data were analyzed using GraphPad Prism (GraphPad

Software, CA) and Calcusyn (Biosoft, Cambridge, U.K.) as appropriate.

Western Blotting. Cells were lysed in ELB buffer on ice for 15 min, sonicated for 5−10 s, and centrifuged at 20000*g* for 15 min at 4 °C. Protein extracts (20 μg, as determined by Bio-Rad protein assay (Bio-Rad)) were diluted in sample buffer (4 × Novex Nupage sample buffer), heated at 95 °C for 5 min, and separated by SDS−PAGE followed by blotting onto a nitrocellulose membrane using the NuPAGE Novex BisTris (XCell SureLock) system (Invitrogen). Membranes were blocked with 5% nonfat milk in Tris-buffered saline/0.1% Tween (TBS-T) for 1 h, incubated with primary antibody overnight at 4 °C, washed 3 times in TBS-T, and incubated with horseradish peroxidase labeled secondary antibodies for 1 h at room temperature. The membranes were then washed 3×10 min in TBS-T. Detection was achieved using ECL SuperSignal West Femto maximum sensitivity substrate (Pierce) together with a ChemiDoc XRS/Quantity One documentation system (Bio-Rad).

Clonogenic Assays. In vitro colony forming assays were performed essentially as previous published.[15] Briefly, HCT116 cells were cultured with compounds for the indicated times and seeded onto 35 mm dishes in 3% (w/v) agar containing a sheep erythrocyte feeder layer. Agar plates were cultured for 14−21 days at 37 °C and colonies counted using a digital colony counter and Sorcerer image analysis software (Perceptive Instruments Ltd., SuVolk, U.K.). Data were analyzed using GraphPad Prism (GraphPad Software, CA) and Calcusyn (Biosoft, Cambridge, U.K.) as appropriate.

Xenograft Studies. The antitumor effect in vivo was tested in a PC3 (schedule, ×3/week iv) subcutaneous (sc) xenograft model in nude mice (female, NMRI/nude, Tarconic) or nude rats (NIHRNU-M, female, Taconic). 1e7 PC3 (CRL-1435, ATCC) human prostate cancer cells were grown in RPMI + 10% FBS, washed once with PBS, and suspended in 100 μL of PBS + 100 μL of Matrigel (BD) and injected sc. Treatment started at tumor volumes around 800−1000 mm^3. The compounds were formulated in 2% DMSO and 20% HP-β-CD and were isotonic at 10 mL/kg iv bolus injection ×3/week. Tumor diameters were measured during tumor growth and tumor volumes (Tv) estimated according to the formula $Tv = (width^2 \times length)/2$. Mice were observed for tumor regression after 1 week or else sacrificed. The experiments were conducted at TopoTarget A/S, Copenhagen, Denmark, and approved by the Experimental Animal Inspectorate, Danish Ministry of Justice.

Pharmacokinetic Analysis. Mouse or rat plasma samples were prepared for analysis by protein precipitation on Sirocco plates (Waters, Milford, MA). Waters Acquity UPLC system with Quattro Premier MS−MS system was used for separation and detection. Acetonitrile containing 1 μg/mL internal standard (compound **2**) was used in the ratio 3:1 (v/v) for precipitation. Separation was performed with an acetonitrile−0.05% formic acid gradient on an Acquity UPLC BEH C18, 2.1 mm × 50 mm, 1.7 μm reversed phase column (Waters A/S) operating at 40 °C. Detection was performed using electrospray MRM in the positive mode. Pharmacokinetic parameters were calculated using noncompartmental analysis methods as included in WinNonlin, version 5.02 (Pharsight, CA).

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Supporting Information Available: Experimental procedures, analytical and spectral data for all intermediate and final compounds, and X-ray crystallographic analysis results. This material is available free of charge via the Internet at http://pubs.acs.org. X-ray crystallographic information of fractional atomic coordinates, list of anisotropic displacement parameters, and a complete list of geometrical data have been deposited in Cambridge Crystallographic Data Centre (No. CCDC 779154).

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