Letters

A Structure-Guided Approach to an Orthogonal Estrogen-Receptor-Based Gene Switch Activated by Ligands Suitable for in Vivo Studies

Olaf Kinzel,* Daniela Fattori, Ester Muraglia, Paola Gallinari, Maria Chiara Nardi, Chantal Paolini, Giuseppe Roscilli, Carlo Toniatti, Odalys Gonzalez Paz, Ralph Laufer, Armin Lahm, Anna Tramontano, Riccardo Cortese, Raffaele De Francesco, Gennaro Ciliberto, and Uwe Koch

IRBM (Merck Research Laboratories Rome), Via Pontina km 30,600, 00040 Pomezia, Rome, Italy

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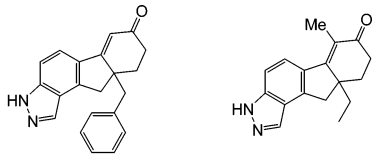
Abstract: A strategy to obtain a fully orthogonal estrogen-receptor-based gene switch responsive to molecules with acceptable pharmacological properties is presented. From a series of tetrahydrofluorenones active on the wild-type estrogen receptor (ER) an inactive analogue is chosen as a new lead compound. Coevolution of receptor mutants and ligands leads to an ER-based gene switch suitable for studies in animal models.

In the field of gene therapy an important goal is to have tight temporal and dose control over the expression of an introduced gene (transgene). This can be achieved by controlling the expression of the transgene with a small molecule (inducer) with well-behaved pharmacokinetic and pharmacodynamic properties. In the presence of the inducer the ligand-dependent transcription system should be activated in a dose-dependent manner, whereas in its absence a low basal expression of the transgene should occur.[1,2] We recently published the development of an estrogen receptor (ER) based gene switch fulfilling all the desired criteria for such a system, namely, the complete orthogonality of the artificial receptor and the exogenous ligand to the endogenous ligand/receptor pair and a low immunogenicity.[3] In this paper we describe the medicinal chemistry effort of this work culminating in the identification of highly selective ligands, active on an estradiol-unresponsive ER mutant and suitable for studies in vivo.

The strategy applied in order to obtain an orthogonal gene switch consisted of six steps: (1) identification of inactive compounds within an active series, focusing on compounds where the activity was abolished because of minor structural changes; the compounds should also have favorable pharmacological properties; (2) determination of the residues of the protein responsible for the loss of activity of the chosen compound through molecular modeling; (3) generation of a mutant library of the ER ligand binding domain (LBD), containing mutations at the previously identified amino acid positions and selection of mutants with increased affinity for the low-affinity ligand;[3] (4) optimization of the chosen lead compound in terms of selectivity profile; (5) introduction of an additional mutation to abolish the remaining affinity for estradiol; (6) second round optimization of the ligands in terms of selectivity for the final mutant.



1a **1b**

Figure 1. Inactive chosen lead compound **1a** and active analogue **1b**.

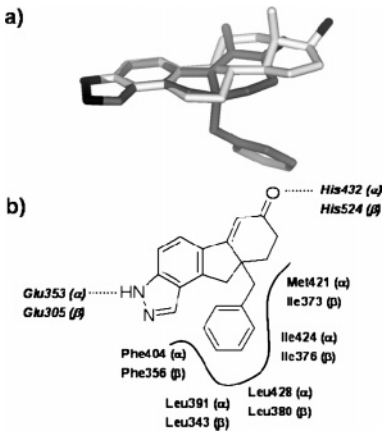


Figure 2. (a) Superposition of estradiol and **1a** and (b) residues most likely to interfere with binding of **1a** (bold). Conserved polar interactions used to define the position of the tetrahydrofluorenone are also shown (italic).

Compound **1a** (Figure 1) belongs to a series of tetrahydrofluorenones, which were prepared as part of our β-selective ER modulator (SERM) project.[4,5] **1a** shows weak binding affinity for hERα and hERβ (IC_{50} > 10 μM for both subtypes). Members of this series of benzopyrazole analogues had demonstrated good oral bioavailability and an acceptable clearance in several species (data not shown). In contrast, **1b** (Figure 1) is an active tetrahydrofluorenone with high affinity for hERβ. A comparison of both compounds shows that the replacement of the ethyl group in position 9a of **1b** by the benzyl group is responsible for the loss of affinity for **1a**. This replacement increases the volume of the ligand by 54 Å^3.

To identify the residues that interfere with binding of the benzyl group, we built models of **1a** and **1b** bound to the hERα and hERβ LBDs using X-ray structures of both LBDs with bound agonists.[6,7] For this purpose the tetrahydrofluorenone scaffold was superimposed with the estradiol scaffold (Figure 2a). Severe steric clashes between the benzyl group of **1a** and five hydrophobic amino acid residues in the binding pocket were identified. These amino acids were selected as sites of replacements by other hydrophobic amino acids in the generation of an ERα-LBD mutant library (together with the mutation L384M to mimic the ERβ subtype) (Figure 2b). Recently a crystal structure of a similar tetrahydrofluorenone has been published that is in good agreement with the superposition shown in Figure 2.[8]

The designed LBD-mutant library was expressed in yeast, and sequence analysis of mutants which conferred sufficient

* To whom correspondence should be addressed. Phone: +39 069 109 3334. Fax: +39 069 109 3654. E-mail: olaf_kinzel@merck.com.

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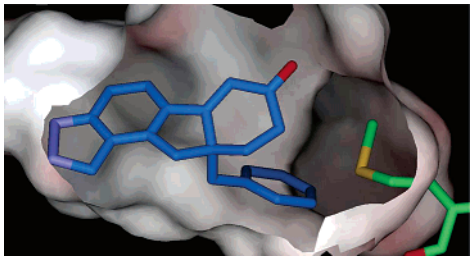
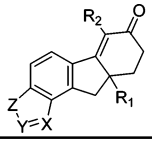


Figure 3. Model of **1a** in the estrogen pocket. The solvent accessible surface area was calculated for Gly421, showing the large pocket that becomes available to accommodate the benzyl moiety of **1a**. Met421 of the wild-type receptor is shown for comparison.

Table 1. SAR of Tetrahydrofluorenones



No.	X	Y	Z	R_1	R_2	MG-ERα IC_{50} (nM)[a]	hERα[b] IC_{50} (nM)	hERβ[b] IC_{50} (nM)
E_2[c]	-	-	-	-	-	46 ±4	1.6 ±0.2	1.4 ±0.2
1a	CH	N	NH	Bn	H	3900 ±400	>10000	>10000
7a	CH	N	NH	Bn	Me	160 ±20	>10000	1200 ±140
7b	CH	N	NH	4-F-Bn	Me	82 ±58	>10000	>1000
7c	CH	N	NH	3-F-Bn	Me	440 ±60	>10000	1600 ±800
7d	CH	N	NH	2-F-Bn	Me	140 ±30	3400 ±700	560 ±30
7e	CH	N	NH	2-naphthyl-methyl	Me	5500[f]	n.d.[d]	n.d.
15a	N	N	NH	Bn	Me	1200 ±250	>10000	5800±1400
15b	N	N	NH	4-F-Bn	Me	340 ±130	>10000	>10000
20a	H	-	OH	Bn	Me	14 ±2	1500±200 2300±200[e]	510 ±60
20b	H	-	OH	4-Cl-Bn	Me	22 ±10	6800[ef]	n.d.

[a] IC_{50} values are the mean of three or more experiments. [b] Full-length receptor. [c] Estradiol. [d] Not determined. [e] IC_{50} on the wtERα-LBD. [f] Only one experiment.

growth in the presence of **1a** revealed the presence of one predominant mutation in the ligand binding pocket. Methionine 421 was replaced by the much smaller glycine (ERα-LBD containing mutations L384M/M421G, called MG-ERα). The IC_{50} of **1a** on the isolated ligand binding domain MG-ERα was found to be 3.9 μM. This increase in affinity can be rationalized by an increase of the volume of the binding pocket by 66 Å^3, matching the 54 Å^3 increase of the volume of **1a** compared to **1b** (Figure 3). As anticiptated, MG-ERα still exhibited high affinity for estradiol, although being reduced by at least 1 order of magnitude (Table 1).

Keeping in mind that MG-ERα would not be our final mutant, we started an effort to improve affinity and selectivity of lead compound **1a** for MG-ERα. Meanwhile additional mutations were evaluated for insertion into MG-ERα to abolish the remaining affinity for estradiol. We reasoned that a compound with high affinity and selectivity for MG-ERα would most likely serve as a lead for a SAR study on the final mutant.

A summary of the SAR on MG-ERα with **1a** as the lead compound is given in Table 1. The introduction of a methyl group at the 6-position resulted in **7a** with a 17-fold increase in affinity for MG-ERα, being inactive on hERα and displaying an 8-fold selectivity over hERβ. The exploration of the SAR around the benzyl substituent resulted in the identification of **7b** with an IC_{50} of 82 nM on MG-ERα and a slightly improved selectivity over hERβ (>12-fold). More bulky substituents such

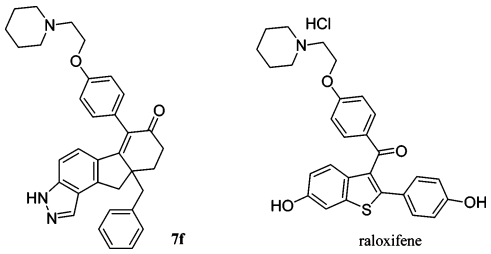


Figure 4. Compound **7f** and raloxifene.

as 2-naphthylmethyl were not tolerated (**7e**). Replacements of the pyrazole ring were investigated as well. Compound **15a**, the triazole analogue of **7a**, showed an 8-fold lower affinity, but the introduction of a 4-fluoro substituent on the benzyl ring largely restored affinity and resulted in **15b** with an IC_{50} of 300 nM on MG-ERα and no activity on hERα and hERβ up to 10 μM. Finally, replacement of the pyrazole by a 7-hydroxy group resulted in compounds with the highest affinity and selectivity. Introduction of a chloro substituent in the 4-position on the benzyl ring yielded **20b** as the most potent and selective compound (>300-fold selective for MG-ERα over wtERα-LBD).

To abolish the remaining affinity of MG-ERα for estradiol, the additional mutation G521R was introduced, leading to a triple-mutated ERα-LBD (called MGR-ERα). It is known in the literature that estrogen receptors that contain the mutation G521R in the LBD exhibit a strongly reduced affinity for estradiol but remain responsive to certain ER antagonists such as 4-OH-tamoxifen and raloxifene.[9,10]

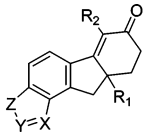
To test our compounds on MGR-ERα, a functional assay in mammalian cells was set up. (Affinity determination of our ligands on MGR-ERα by a competitive radiometric binding assay was hampered by the loss of affinity for radiolabeled estradiol.) For this purpose MGR-ERα was grafted into our previously described gene switch HEA-1[11] (replacement of the HEA-1-LBD with MGR-ERα), which was transfected in HeLa cells.[3] In the presence of the ligands the expression of a reporter protein (SEAP, human-secreted alkaline phosphatase) was activated, protein concentrations were measured at different ligand concentrations, and the EC_{50} values were determined. The MGRα-containing gene switch was unresponsive to estradiol up to 10 μM, but to our disappointment, when **7a** was tested in this assay, the gene switch also remained unresponsive up to 2 μM, suggesting a substantial loss of affinity of **7a** for MGR-ERα.

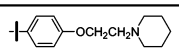
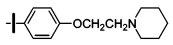
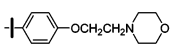
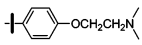
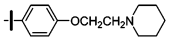
Undeterred by this result, we synthesized a hybrid molecule of **7a** and raloxifene, yielding **7f** (Figure 4).

When **7f** was tested on MGR-ERα in the cell-based assay, an EC_{50} of 300 nM was obtained. But at the same time **7f** exhibited an increased affinity for hERα and hERβ compared to **7a** (Table 2). Encouraged by this result, we concentrated on compounds with bulky R_2 side chains, and a SAR study was performed to restore selectivity versus hERα/hERβ.

For practical reasons we continued to measure affinity for MG-ERα, while interesting compounds were further tested in the cell-based assay on MGR-ERα. A summary of the results is given in Table 2. The hydroxy compound **20c** showed a much improved selectivity (defined as IC_{50} ratios of MG-ERα/hERα or MG-ERα/hERβ) compared to **7f** (17-fold/8-fold against 6-fold/1.2-fold), and a small SAR study around the side chain R_2 in the hydroxy series identified **20g** with still further improved selectivity (30-fold/14-fold). As expected, installation of a chloro substituent at the 4-position of the benzyl ring (**20h**) gave the most selective compound (38-fold/76-fold), confirming

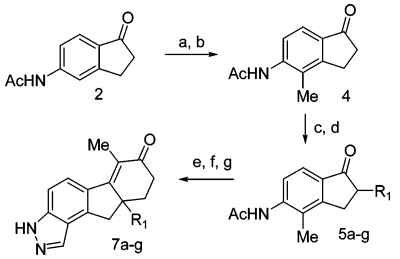
Table 2. SAR of Tetrahydrofluorenones with Bulky Side-chains R_2



No.	X	Y	Z	R_1	R_2	MG-ERα IC_{50} (nM) [a]	MGR-ERα EC_{50} (nM)	hERα [b] IC_{50} (nM)	hERβ [b] IC_{50} (nM)
7f	C H	N	NH	Bn		110 ±10	300 ±40	620 ±90	130 ±10
20c	H	-	OH	Bn		22 ±3	14 ±2	380 ±40	180 ±20
20d	H	-	OH	Bn	n-butyl	25 ±4	n.d. [c]	260 ±40	310 ±40
20e	H	-	OH	Bn	4-OH-phenyl	29 ±4	11 ±3	220 ±20	100 ±20
20f	H	-	OH	Bn		25 ±4	n.d.	710 ±80	230 ±35
20g	H	-	OH	Bn		40 ±5	n.d.	1200 ±110	560 ±60
20h	H	-	OH	4-Cl-Bn		29 ±3	41 ±2	1100 ±90	2200 ±140

[a] IC_{50} values are the mean of three or more experiments. [b] Full-length receptor. [c] Not determined.

Scheme 1[a]

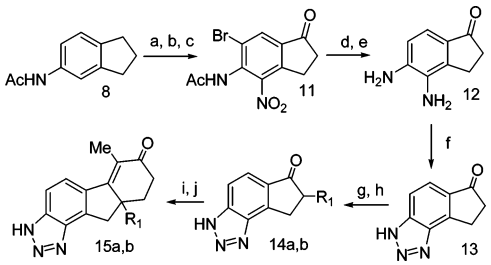


[a] Reagents and conditions: (a) NBS, CH_3CN, 60°C; (b) $PdCl_2(PPh_3)_2$, PPh_3, LiCl, $SnMe_4$, DMF, 100°C; (c) ArCHO, NaOMe, MeOH; (d) Pd/C, H_2; (e) ethyl vinyl ketone, NaOMe, MeOH, 60°C; (f) 6 N aqueous HCl/HOAc, 1:1, 80°C; (g) (i) $NOBF_4$, CH_2Cl_2, −35 to 4 °C; (ii) KOAc, db-18-crown-6, CH_2Cl_2, −35°C to room temp.

the results obtained in the series lacking the bulky side chain. **20c**, **20e**, and **20h** were tested in the cell-based assay on MGR-ERα (Table 2). Their EC_{50} values correlated well with their IC_{50} values measured on MG-ERα, suggesting that the additional mutation present in MGR-ERα has no detrimental effect on the affinity of ligands with bulky side chains R_2. The data obtained in mammalian cells also suggest a good cell penetration of the molecules.

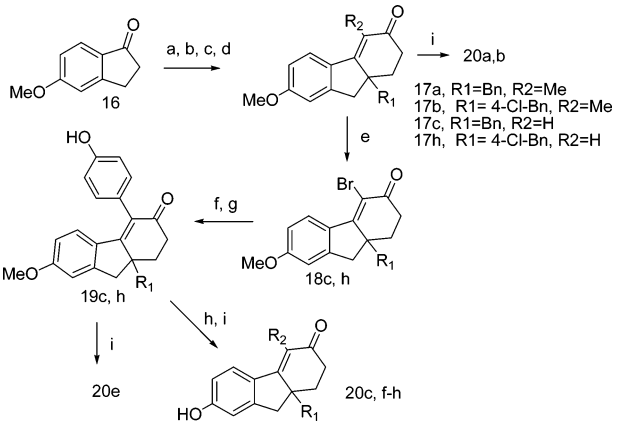
Compounds **20c**, **20e**, and **20h** were evaluated for their suitability for in vivo studies: **20c** and **20e** were dosed iv in rats (dose 3 mg/kg). **20c** displayed a moderate clearance of 22 mL min^{-1} kg^{-1} and a terminal half-life of 2 h. Meanwhile, **20e** displayed a clearance of 39 mL min^{-1} kg^{-1} and a terminal half-life of 1.5 h. The plasma concentration of **20c** after 6 h was more than 10-fold its EC_{50} in mammalian cells, and that of **20e** was more than 4-fold. Compound **20h** was dosed in male Balb-c mice (4 mg/kg, ip). The plasma concentration after 4 h was 1.5-fold its EC_{50} in mammalian cells with a C_{max} of 0.5 μM after 30 min. These data demonstrate that the obtained class of compounds is suitable for in vivo studies in animal models, ideally using ip dosing.

Scheme 2[a]



[a] Reagents and conditions: (a) Br_2, HOAc, 10 °C; (b) CrO_3, HOAc, 15−17 °C; (c) HNO_3 (100%), −40°C; (d) 6 N HCl, MeOH, reflux; (e) Pd/C, H_2, NaOAc, EtOAc; (f) $NaNO_2$, HCl, EtOH, 0−5°C; (g) ArCHO, NaOMe, MeOH; (h) Pd/C, H_2; (i) ethyl vinyl ketone, NaOMe, MeOH, 60°C; (j) 6 N aqueous HCl/HOAc, 1:1, 80 °C.

Scheme 3[a]



[a] Reagents and conditions: (a) ArCHO, NaOMe, MeOH; (b) Pd/C, H_2, EtOAc for **17a**,**c**; Se, $NaBH_4$, EtOH/THF, 0−50 °C for **17b**,**h**; (c) ethyl vinyl ketone, NaOMe, MeOH, 60 °C for **17a**,**b**; methyl vinyl ketone, DBU, THF, 55 °C for **17c**,**h**; (d) 6 N aqueous HCl/HOAc, 1:1, 80 °C, for **17a**,**b**; pyrrolidine, HOAc, THF, 55 °C, for **17c**,**h**; (e) Br, $NaHCO_3$, CCl_4, 0 °C; (f) aryl-$SnBu_3$, $Pd(PPh_3)_4$, toluene, 100 °C; (g) MeOH/2 N aqueous HCl, 5:1, 60°C; (h) amino alcohol, DIAD, PPh_3, THF, 0−25°C; (i) $AlCl_3$, 2-propanethiol, CH_2Cl_2.

The synthesis of the pyrazole-containing ligands followed the strategy developed for the synthesis of the hER-β-active compounds [4,5] and is summarized in Scheme 1. Selective bromination of N-protected 5-amino-1-indanone (**2**) at the 4-position with NBS and subsequent replacement of the bromo substituent by a methyl group via Stille cross-coupling led to intermediate **4**. Introduction of the alkyl substituents R_1 was achieved by an aldol condensation with the corresponding arylaldehydes and a subsequent catalytic reduction of the double bond. A two-step Robinson annulation with ethyl vinyl ketone and a concomitant N-deacetylation led to intermediates **5a**−**g**. The final pyrazole ring formation was achieved under mild conditions by formation of the diazonium tetrafluoroborate intermediates, which were then cyclized under phase-transfer conditions.[12]

The triazole-containing ligands were synthesized as shown in Scheme 2. To obtain the key intermediate **12**, a transient protection of the 6-position of **8** with a bromo substituent was necessary because the alternative direct nitration of **8** or **2** gave mixtures of regioisomers that were difficult to separate. After formation of the triazole ring the introduction of the alkyl substituents R_1 was achieved in the same manner as in the pyrazole series by aldol condensation with the corresponding arylaldehydes and subsequent reduction of the double bond. A two-step Robinson annulation led to the final triazole com-

pounds, although in low yields because of a partial conjugate addition of ethyl vinyl ketone to the unprotected triazole ring.

The series of hydroxytetrahydrofluorenones was prepared mostly according to the synthesis of the hER-β-active compounds (Scheme 3).[4,5]

In summary we have successfully demonstrated an approach for the construction of an orthogonal gene switch, responsive to molecules with acceptable pharmacological properties. The essence of this approach lies in the early consideration of the properties of the ligand and the subsequent construction of a suitable receptor for the chosen ligand. Similar to our work, directed evolution approaches have been successfully applied in the creation of highly orthogonal gene switches.[13−15] In these studies though the pharmacological properties of the ligands were not taken into consideration. By contrast, our concept consists of the coevolution of the receptor and of the ligand. To our knowledge the work presented here is the first example where the ligands of a completely orthogonal estrogen-receptor-based gene switch are small molecules with modest clearance and acceptable half-life in vivo, suitable for studies in animal models.

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Supporting Information Available: Experimental procedures and characterization of intermediates and final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Toniatti, C.; Bujard, H.; Cortese, R. Gene therapy progress and prospects: transcription regulatory systems. *Gene Ther*. **2004**, *11*, 649−657.
(2) Weber, W.; Fussenegger, M. Artificial mammalian gene regulation networks−novel approaches for gene therapy and bioengineering. *J. Biotechnol*. **2002**, *98*, 161−187.
(3) Gallinari, P.; Lahm, A.; Koch, U.; Paolini, C.; Nardi, M. C.; Roscilli, G.; Kinzel, O.; Fattori, D.; Muraglia, E.; Toniatti, C.; Cortese, R.; De Francesco, R.; Ciliberto, G. A functionally orthogonal estrogen receptor-based transcription switch specifically induced by a non-steroid synthestic ligand. *Chem. Biol*. **2005**, *12*, 883−893.
(4) Parker, D. L., Jr.; Wildonger, K. J.; Meng, D.; Ratcliffe, R. W. Preparation of 1,2,9,9a-tetrahydro-3*H*-fluoren-3-ones and related compounds as estrogen receptor modulators. PCT Int. Appl. WO 2002041835 A2, 2002.
(5) Parker, D. L., Jr.; Ratcliffe, R. W.; Wilkening, R. R.; Wildonger, K. J. Preparation of 1,2,9,9a-tetrahydro-3*H*-fluoren-3-ones and related compounds as estrogen receptor modulators. PCT Int. Appl. WO 2001082923 A1, 2001.
(6) Brzozowski, A. M.; Pike, A. C.; Dauter, Z.; Hubbard, R. E.; Bonn, T.; Engstrom, O.; Ohman, L.; Greene, G. L.; Gustafsson, J. A.; Carlquist, M. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **1997**, *389*, 753−758.
(7) Shiau, A. K.; Barstad, D.; Loria, P. M.; Cheng, L.; Kushner, P. J.; Agard, D. A.; Greene, G. L. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **1998**, *95*, 927−937.
(8) Wilkening, R. R.; Ratcliffe, R. W.; Tynebor, E. C.; Wildonger, K. J.; Fried, A. K.; Hammond, M. L.; Mosley, R. T.; Fitzgerald, P. M. D.; Sharma, N.; McKeever, B. M.; Nilsson, S.; Carlquist, M.; Thorsell, A.; Locco, L.; Katz, R.; Frisch, K.; Birzin, E. T.; Wilkinson, H. A.; Mitra, S.; Cai, S.; Hayes, E. C.; Schaeffer, J. M.; Rohrer, S. P. The discovery of tetrahydrofluorenones as a new class of estrogen receptor β-subtype selective ligands *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3489−3494.
(9) Danielian, P. S.; White, R.; Hoare, S. A.; Fawell, S. E.; Parker, M. G. Identification of residues in the estrogen receptor that confer differential sensitivity to estrogen and hydroxytamoxifen. *Mol. Endocrinol*. **1993**, *7*, 232−240.
(10) Littlewood, T. D.; Hancock, D. C.; Danielian, P. S.; Parker, M. G.; Evan, G. I. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res*. **1995**, *23*, 1686−1690.
(11) Roscilli, A. G.; Rinaudo, C. D.; Cimino, M.; Sporeno, E.; Lamartina, S.; Ciliberto, G.; Toniatti, C. Long-term and tight control of gene expression in mouse skeletal muscle by a new hybrid human transcription factor. *Mol. Ther*. **2002**, *6*, 653−663.
(12) Bartsch, R. A.; Yang, I.-W. Phase transfer catalyzed synthesis of indazoles from *o*-alkylbenzenediazonium tetrafluoroborates. *J. Heterocycl. Chem*. **1984**, *21*, 1063−1064.
(13) Schwimmer, L. J.; Rohatgi, P.; Azizi, B.; Selely, K. L.; Doyle, D. F. Creation and discovery of ligand−receptor pairs for transcriptional control with small molecules. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 14707−14712.
(14) Chockalingam, K.; Chen, Z.; Katzenellenbogen, J. A.; Zhao, H. Directed evolution of specific receptor−ligand pairs for use in the creation of gene switches. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5691−5696.
(15) Chockalingam, K.; Zhao, H. Creating new specific ligand−receptor pairs for transgene regulation. *Trends Biotechnol*. **2005**, *23*, 333−335.

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