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Perturbation of Estrogen Receptor α Localization with Synthetic Nona-Arginine LXXLL-Peptide Coactivator Binding Inhibitors

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SUMMARY

The interaction of estrogen receptor α (ERα) with the consensus LXXLL motifs of transcriptional coactivators provides an entry for functional ERα inhibition. Here, synthetic cell-permeable LXXLL peptide probes are brought forward that allow evaluation of the interaction of specific recognition motifs with ERα in the context of the cell. The probes feature a nona-arginine tag that facilitates cellular entry and induces probe localization in nucleoli. The nucleoli localization provides an explicit tool for evaluating the LXXLL motif interaction with ERα. The probes compete with coactivators, bind ERα, and recruit it into the nucleoli. The physical inhibition of the ERα-coactivator interaction by the probes is shown to be correlated with the inhibition of ERα-mediated gene transcription. This chemical biology approach allows evaluating the ERα-coactivator interaction and inhibitor binding directly in cells.

INTRODUCTION

Estrogen receptor α (ERα) is a member of the nuclear receptor superfamily and is an estrogen-inducible transcription factor. In the normal physiology of the female reproductive system, ERα regulates the growth and function of such tissues as breast, uterus, and ovaries. In addition, ERα has been identified as playing a role in many pathological processes, such as breast cancer (Holst et al., 2007). Because 70% of breast tumors depend on the stimulatory effect of estrogens, anti-estrogen therapies have been developed that significantly reduce breast cancer recurrence (Fisher et al., 2005). However, the long-term effects of tamoxifen, one of the most successful anti-estrogen drugs, in metastatic disease are limited to an average of 15 months (Howell et al., 1995), after which tamoxifen resistance develops in more than 80% of treated women (Clarke et al., 2001). In the induction of gene transcription, ERα acts in complex association with other cellular factors, including coactivators, which are required for the transcriptional activation process to occur (McKenna et al., 1999). The most widely studied group of ERα coactivators includes the p160 protein family, consisting of three members: SRC-1, Tif-2/GRIP1, and SRC-3/AIB1. The importance of these coactivators in ERα signaling has been highlighted by knockout studies and by the discovery of p160 gene amplification in ERα-positive breast cancer (Xu et al., 1998, 2000; Mahajan and Samuels, 2005). These p160 coactivators have the ability to interact with ERα and other nuclear receptors in a hormone-dependent manner via small hydrophobic and amphiphatic α-helical peptide sequences with the common signature motif, LXXLL (L = leucine, and X = any amino acid). Crystallographic studies showed that this motif, also called nuclear receptor box (NR box), binds to a hydrophobic cleft on the ERα ligand binding domain (LBD) surface, under control of activating ligand (Shiau et al., 1998). p160 coactivators contain three NR box copies within their central nuclear receptor interaction domain. Although the three leucines in the LXXLL motif are conserved, the amino acid residues flanking this sequence are not and specify NR box recognition for a particular nuclear receptor in cooperation with specificities of the ligand. These characteristic features have raised the idea that the ERα-coactivator interaction is a specific amendable interface for LXXLL motif–like inhibitors, preventing transactivation by ERα (Pike et al., 2000; Hall et al., 2000; Vaz et al., 2008). The formation of the coactivator binding surface on ERα requires a hormone-dependent conformational change. Inhibitors of coactivator binding (ICBs) (Rodriguez et al., 2004) may only bind to hormone-occupied ERα, thus featuring a different mechanism than the classic anti-estrogen drugs. These ICBs might thus be useful in anti-estrogen therapy for patients with tamoxifen-resistant breast cancers or could provide a possibility to target orphan nuclear receptors for which antagonists are difficult to identify (Kurebayashi et al., 2004).

Several types of compounds blocking ERα-cofactor interactions have already been developed. The first proof that the concept of inhibiting ERα-cofactor interactions results in the inhibition of ERα-mediated transcription has been obtained with LXXLL peptides overexpressed in cells from transfected DNA constructs (Chang et al., 1999; McInerney et al., 1998; Shao et al., 2000; Connor et al., 2001; Norris et al., 1999). This finding has been enforced by the identification and the use of synthetic ICBs, either peptide based or small molecule (Leduc et al., 2003; Shao et al., 2004; Rodriguez et al., 2004; Geistlinger et al., 2004; Galande et al., 2004, 2005; Gunther et al., 2008; Parent et al.,

2008; LaFrate et al., 2008). Most of these compounds inhibit ERα-coactivator binding in biochemical assays, and a selected set of compounds was also able to inhibit ERα gene transactivation in cellular assays (Gunther et al., 2008; Parent et al., 2008; LaFrate et al., 2008). The translation from biochemical studies on the ERα LBD to cellular activity on the full-length protein is, however, not trivial. For example, it has been reported that the conformation of the coactivator binding groove in the full-length receptor tends to be different from the isolated LBD (Chang et al., 2005; Bapat and Frail, 2003; Chang and McDonnell, 2002). In addition, direct binding of inhibitors to ERα in cells and their capacity to displace coactivators are two processes that are difficult to observe directly. The process of inhibiting the ERα-coactivator interaction thus requires more molecular insight. Therefore, methods need to be developed that directly visualize the interaction of inhibitors with ERα and provide information on the resulting ERα inhibition. Here, we show that synthetic cell-permeable LXXLL-peptide probes with specific localization and visualization tags are ideal molecular probes to inhibit and evaluate ERα-coactivator interactions in living cells and to address the above-mentioned issues. By use of confocal laser scanning microscopy (CLSM), these probes can be used to monitor the association of specific peptide sequences with the full-length ERα and to evaluate their effects on the assembly of ERα signaling protein complex within the organized microenvironment of the living cell. The peptide probes feature a nona-arginine tag that ensures efficient cellular entry of the peptides and induces a specific localization of the peptides into the nucleoli. This specific property of the peptides is used to evaluate the affinity of different LXXLL-motif peptides for the ERα. The peptides bind and translocate ERα into the nucleoli, resulting in the displacement of ERα from the DNA and coactivators. Overall, the peptides function as functional inhibitors as they effectively lower ERα-mediated gene transcription.

RESULTS

Selection and Synthesis of LXXLL-Motif Molecular Probes

For the generation of molecular probes inhibiting the ERα-coactivator interaction, we selected a set of natural LXXLL-motif sequences reported to have a high affinity for ERα (Bramlett et al., 2001) and capable of blocking ERα transcriptional activity when overexpressed in cells—boxes 1 and 2 of the SRC-1 coactivator protein and boxes 1, 2, and 3 of the Tif-2 coactivator protein (Table 1). Phage display peptides D2 and D11, which were previously reported to possess a high affinity for full-length ERα and to bind at the same coactivator binding surface (Chang et al., 1999), were also included in the selection (Table 1). The plasma membrane of mammalian cells is generally impermeable to the vast majority of peptides and proteins. We therefore decided to take advantage of the cell-permeable properties of the nona-arginine (R_9) peptide sequence (Futaki et al., 2001; Tünnemann et al., 2008) and use it as a carrier for the selected LXXLL peptides. The arginine tags were attached to the peptides at their C terminus by means of peptide synthesis. To monitor the localization of the LXXLL peptides within the cells via immunolabeling, they were provided with a biotin epitope connected via an ethylene glycol linker to the C-terminal part of the complete peptide. In addition to the LXXLL molecular probes, two AXXAA-molecular probes, based on SRC-1 Box2 and Tif-2 Box2 and mutated for the recognition motif by alanines, were also generated as negative control peptides.

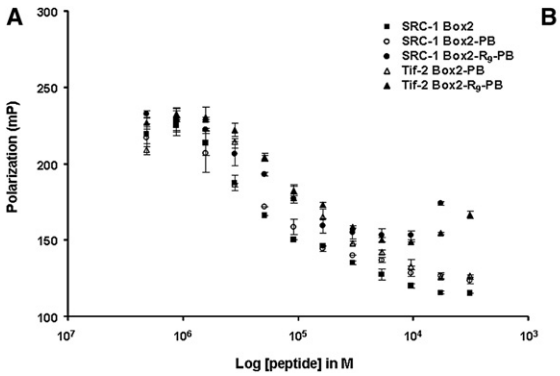
Table 1. Sequences of the Selected LXXLL-Motif Peptides

SRC-1 Box1	Y	S	Q	T	S	H	K	**L**	V	Q	**L**	**L**	T	T	T	A	E	Q	Q
SRC-1 Box2	L	T	E	R	H	K	I	**L**	H	R	**L**	**L**	Q	E	G	S	P	S	D
SRC-1 Box2-Ala	L	T	E	R	H	K	I	A	H	R	A	A	Q	E	G	S	P	S	D
Tif-2 Box1			S	K	G	Q	T	K	**L**	L	Q	**L**	**L**	T	T	K	S	D	
Tif-2 Box2	L	K	E	K	H	K	I	**L**	H	R	**L**	**L**	Q	D	S	S	S	P	V
Tif-2 Box2-Ala	L	K	E	K	H	K	I	A	H	R	A	A	Q	D	S	S	S	P	V
Tif-2 Box3	K	K	K	E	N	A	L	**L**	R	Y	**L**	**L**	D	K	D	D	T	K	
D2	G	S	E	P	K	S	R	**L**	L	E	**L**	**L**	S	A	P	V	T	D	V
D11	V	E	S	G	S	S	R	**F**	M	Q	**L**	**F**	M	A	N	D	L	L	T

The effect of the biotin and nona-arginine tags, incorporated in the peptides, on the binding to the ERα coactivator binding surface was evaluated in a biochemical fluorescent polarization assay with the recombinant LBD of ERα (Figure 1). The assay measures the displacement of a fluorescein-labeled reference peptide (SRC-1 box2) by the nonfluorescent LXXLL peptides under study. The R_9 tag alone did not bind to ERα LBD, as expected. The dose-dependent fluorescence depolarization curves obtained with peptides SRC-1 Box2 and SRC-1 Box2-PEG-biotin were overlapping (Figure 1A), showing that the PEG-biotin spacer does not influence ERα binding. For both the SRC-1 Box2 and the SRC-1 Box2-R_9 peptides and the Tif-2 Box2 and Tif-2 Box2-R_9 peptides, the same binding affinities were found (Figure 1B), showing that the nona-arginine tag also does not disturb the capacity of the peptides to bind to ERα.

Cellular Toxicity and Intracellular Distribution of the Synthetic LXXLL Molecular Probes

To study and evaluate any toxic effects of the synthetic peptide probes, the viability of cells after an overnight incubation with increasing concentrations of peptides was tested using the WST-1 assay (see Table S1 available online) (Berridge et al., 2005). Peptides conjugated to PEG-biotin but lacking the nona-arginine tag were found to be toxic on the human cancer cell line U2OS at concentrations of ~1 mM, whereas all of their nona-arginine counterparts showed toxicity at around 0.1 mM. The nona-arginine tag alone was found to be less toxic to the cells. Interestingly, the two peptides SRC-1 Box2-Ala-R_9 and Tif-2 Box2-Ala-R_9 mutated for the recognition motif by alanines were not lethal to cells at concentrations up to 0.6 mM. This difference in minimal toxic concentration may be explained by a different sequence-dependent cellular uptake capacity of these peptides, but may also be related to their intracellular pharmacological effects. From the dose-dependent toxicity curves obtained, we determined the concentrations of peptides that gave less than 20% of cell death, compared with nontreated cells (Table S1). These peptide concentrations (0.02–0.07 mM), were also tested by fluorescent assisted cell sorting (FACS) for apoptosis induction using the specific fluorescently labeled apoptotic marker Annexin-V (Bedner et al., 1999). Also in this assay (data not shown), the nona-arginine peptides induced less than 20% apoptosis at these concentrations.



Synthetic peptides	IC_{50} (μM)
SRC-1 Box2 (control peptide)	4.47 ± 1.3
SRC-1 Box2-**PB**	5.72 ± 0.3
SRC-1 Box2-**R_9-PB**	5.80 ± 2.4
SRC-1 Box1-**R_9-PB**	> 300
Tif-2 Box1-**R_9-PB**	2.53 ± 1.6
Tif-2 Box2-**PB**	8.81 ± 0.2
Tif-2 Box2-**R_9-PB**	9.59 ± 0.08
Tif-2 Box2-Ala-**R_9-PB**	> 300
Tif-2 Box3-**R_9-PB**	> 300
D2-**R_9-PB**	1.85 ± 0.5
D11-**R_9-PB**	> 300
R_9-PB	> 300

R_9 = Nona-arginine Tag
PB = PEG-Biotin

Figure 1. Biochemical Fluorescence Polarization Studies of the ERα-LBD Binding Capacity of the Synthetic Peptides

(A) Representative dose-dependent inhibition curves of selected peptides for the binding of a fluorescein-labeled SRC-1 Box2 peptide to the ERα-LBD.

(B) IC_{50} values of all tested peptides. Error bars represent mean ± standard error.

The cell-entry and intracellular distributions of the synthetic peptides were assessed by immunolabeling of cells treated with the peptides using a fluorescent Cy5-conjugated anti-biotin antibody. CLSM imaging of these cells revealed that the PEG-biotin spacer and the LXXLL peptides lacking a nona-arginine tag did cross the plasma membrane to some extent but clustered into vesicular structures in the cytoplasm (Figure 2). However, the cellular distribution of the nona-arginine-tagged molecular probes was completely different, localizing in both the cytoplasm and the nucleus. In the nucleus, these peptides were mainly found located at the nucleoli, as anticipated (Figure 2) because the nucleolar accumulation of such positively charged peptides has been described elsewhere (Tünnemann et al., 2006). Because this particular localization at the nucleoli was not found for the non-R_9-tagged peptides, we conclude that this feature can be exclusively ascribed to the R_9 tag. Also, in accordance with the results of a recent study (Kosuge et al., 2008), we observed that the concentration of serum used in the culture medium had a significant impact on the distribution of these molecular probes inside cells (data not shown). In addition, we noticed that supplementing the medium with 10% of non-heat-inactivated serum could abrogate the cellular uptake of R_9 peptides, which is most likely due to unspecific binding between the peptides and serum components. However, this did not occur when the medium was beforehand heat inactivated, resulting in an efficient cellular uptake.

Binding Capacity of LXXLL Molecular Probes to ERα in Cells

ERα is typically localized in the nucleus, excluded from the nucleoli, and its uniform distribution in the nucleoplasm rapidly changes into a hyperspeckled foci pattern upon the addition of either agonistic or antagonistic ligands (Stenoien et al., 2000). When bound to estradiol, ERα associates with specific regions in the DNA, called estrogen responsive elements (EREs), via zinc-finger motifs present in its DNA binding domain. Inhibiting peptides expressed from DNA constructs fused to a fluorescent protein are evenly distributed throughout the nucleus, but also in the cytoplasm, and are colocalized with ERα upon hormone addition (Schaufele et al., 2000). We investigated here whether peptide probes of a synthetic nature and featuring a specific localization tag are able to interact with ERα as well, when applied externally to cells, and whether the introduced localization tag could be used to evaluate this interaction in a novel manner. Therefore, U2OS cells transiently transfected with full-length ERα fused to cyan fluorescent protein (CFP) were incubated with the LXXLL-nona-arginine molecular probes in the presence of estradiol. The ERα-binding capacity of these peptides was assessed by colocalization with ERα by CLSM. When cells were not incubated with the molecular probes, ERα localized as expected in the nucleoplasm, in punctuated foci (data not shown). The same ERα distribution was observed in presence of the nona-arginine tag alone, whereas this peptide itself was clearly observed into the nucleoli (Figure 3A), indicating a lack of interaction between the receptor and this peptide. However, incubating the cells with the peptides SRC-1 Box2-R_9, Tif-2 Box1-R_9, Tif-2 Box2-R_9, and D2-R_9 and, to a lesser extent, with the peptide Tif-2 Box3-R_9 resulted in a clear nuclear colocalization of these peptides with the estradiol-liganded ERα into the nucleoli. The affinity of these peptides for ERα is apparently strong enough to bind to ERα and subsequently to translocate it to the alternative location in the nucleoli. This effect occurs in the presence of a concentration of estradiol (10^{-6} M) that normally would result in complete recruitment of ERα to DNA templates. The incorporated nona-arginine tag thus serves both to facilitate cellular uptake of the peptide probes and as a specific localization tag, generating a spacial resolution within the nucleus that enables visualization of peptides/ERα complexes. Using the statistic tools of the CLSM Leica software, we quantified the ERα-nucleoli recruitment by the peptides,

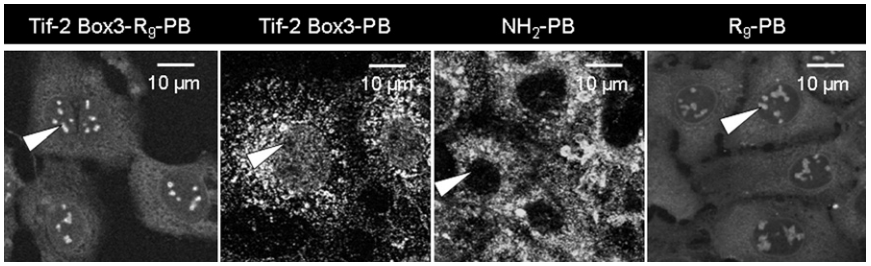


Figure 2. CLSM Imaging of U2OS Cells after Incubation with Biotinylated Peptide Probes

Cells were overnight incubated with peptides featuring different tags and probe elements, followed by immunolabeling with a Cy5-conjugated anti-biotin antibody. PB = PEG-biotin; white arrows indicate nucleoli, and scale bars represent 10 μm.

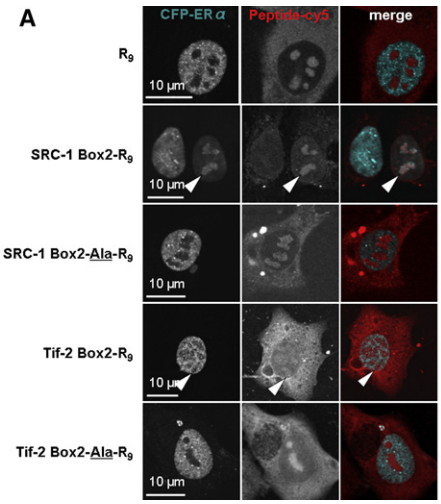


Figure 3. ERα-Binding Capacity of Synthetic Peptides in U2OS Cells

(A) CLSM imaging of cells (positive events) after transient transfection with ERα-CFP and incubation with nona-arginine peptides and estradiol (10^{-6} M). White arrows show ERα sequestration into the nucleoli, and scale bars represent 10 μm.

(B) Quantification of ERα-nucleoli recruitment expressed as the ratio of the ERα-CFP fluorescence intensity per pixel into nucleoli versus the nucleoplasm. Circles represent ratio values per cell ($6 \leq n \leq 19$); medians are indicated by the black line in the middle of the box plots and whose sizes represent ± 25% of the medians (program Profit); asterisks (*) indicate results statistically different from the control ($p < 0.01$).

expressed as the ratio of the CFP-ERα fluorescence intensity per pixel at the nucleoli versus the nucleoplasm (Figure 3B). Interestingly, significant ERα-nucleoli recruitment was found when cells were incubated with all the synthetic R_9 peptides, except for the peptides mutated in their recognition motif by alanines (i.e., SRC-1 box2-Ala-R_9 and Tif-2 Box2-Ala-R_9 and the R_9 alone; Figure 3B and Table S2), showing that the nucleoli recruitment is specifically due to the selective interaction of the peptides with the ERα coactivator binding surface via their LXXLL motifs. The ERα-nucleoli recruitment is concentration dependent (Figure S1A), in line with the expected molecular mechanism of the interaction. Additionally, a Tif-2 Box2 peptide not featuring the R_9-motif does not show ERα-nucleoli recruitment (Figure S1B), as expected on the basis of the nonnucleoli localization of this peptide.

Among the peptides tested, the highest ERα relocation score was found for the SRC-1 Box2-R_9, Tif-2 Box1-R_9, and D2-R_9 peptides, with median ratios of ERα signal into the nucleoli versus the nucleoplasm of 1.2, 0.9, and 0.8, respectively (Figure 3B). These cellular observations are in line with our biochemical binding results (Figure 1B) and with previous reports indicating that ERα interacts with all three LXXLL motifs of the SRC-1 coactivator but has a distinct preference for the second motif (Voegel et al., 1998). Although no binding was observed in our biochemical assay between the ERα LBD and the peptides SRC-1 Box1-R_9, Tif-2 Box3-R_9, and D11-R_9 (IC_{50} >300 μM), these peptides did induce a weak but significant ERα-nucleoli recruitment (Figure 3B). This finding underlines the value of assessing ERα-peptide binding directly in cells, where ERα is expressed as full length and reveals ERα-LXXLL-peptide interaction surfaces beyond the isolated and not posttranslational modified LBD alone.

Displacement of SRC1 and Tif-2 Coactivators by LXXLL Molecular Probes in ERα Binding

To investigate whether the LXXLL-peptide-ERα binding can displace overexpressed full-length coactivators in living cells, we tested the ERα binding of the synthetic molecular LXXLL probes again, but now in the presence of the transfected SRC-1 and Tif-2 coactivators, tagged with yellow fluorescent protein (YFP). ERα indeed still colocalized with the nona-arginine peptides into the nucleoli (Figure 4A) except for the Tif-2 Box3-R_9, R_9, and the alanine mutant peptides (Table S2). We also observed that the full-length SRC-1 coactivator could at least

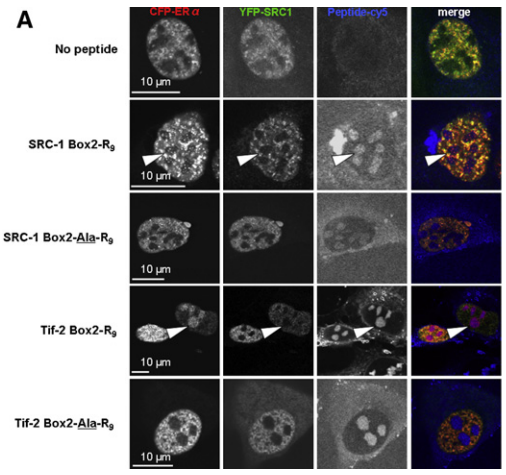


Figure 4. ERα-Binding Capacity of Synthetic Peptides in U2OS Cells in the Presence of Overexpressed Coactivators

(A) CLSM imaging of cells (positive events) transfected with ERα-CFP and SRC-1-YFP, in the presence of estradiol (10^{-6} M). Scale bars represent 10 μm.

(B) Quantification of ERα-nucleoli recruitment in cell cotransfected with SRC-1-YFP expressed as the ratio of the ERα-CFP fluorescence intensity per pixel into nucleoli versus the nucleoplasm. Circles represent ratio values per cell ($5 \leq n \leq 43$). Medians are indicated by the black line in the middle of the box plots, whose sizes represent ± 25% of the medians (program Profit). Asterisks (*) indicate results statistically different from the control (*$p < 0.01$).

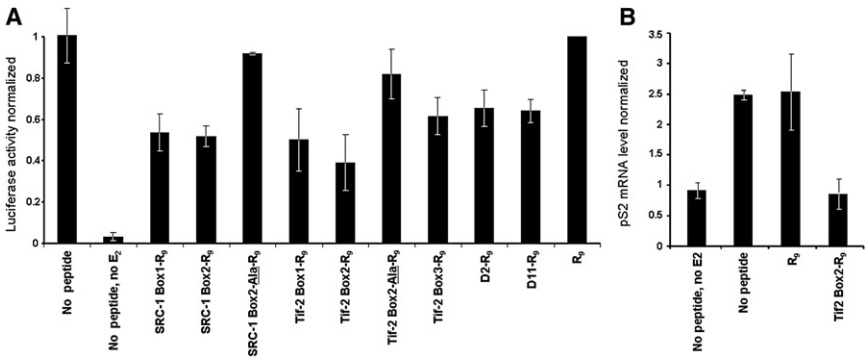


Figure 5. Inhibition of ERα-Gene-Mediated Transactivation by Synthetic Nona-Arginine LXXLL Peptides

(A) U2OS cells were transfected with ERα and the ERα-DNA binding sequence ERE-tk-Luc. Luciferase activities are expressed as the ratio of *Firefly* luciferase signal to *Renilla* luciferase signal and are normalized to the ratio obtained when cells were solely treated with the R_9-tag peptide.

(B) Inhibition by R_9 peptides of an endogenous ER-mediated *pS2* gene in MCF-7 cells. MCF-7 cells were cultured with 70 μM of the indicated peptides in the presence of 10 nM estradiol (E2) (unless indicated). Cells were lysed after 3 hr of estradiol treatment, after which RNA was isolated and prepared for QPCR analysis of endogenous ER-mediated *pS2* mRNA levels (normalized). Error bars represent mean ± standard error.

partially be recruited to the nucleoli together with ERα in cells treated with the most potent ERα-binding peptides (data not shown). This could be due to the recruitment of ERα as a dimer to the nucleoli in which one monomer is bound to an arginine-tagged peptide and the other monomer is still bound to SRC-1, or to the ligand-independent interaction between SRC-1 and the AF-1 domain of ERα (Webb et al., 1998; Zwart et al., 2007a). Among the set of peptides tested, the strongest nucleoli recruitment effect for ERα was observed with the Tif-2 Box2-R_9 peptide (median ratios, 2) and the SRC-1 Box2-R_9 peptide (median ratios, 0.7) (Figure 4B), with 95% and 88% of cells, respectively, exhibiting a ratio higher than that found for cells treated with the control peptide R_9 (Figure S2).

Similar experiments performed on cells cotransfected with CFP-ERα and full-length YFP-Tif-2 coactivator followed by treatment with the synthetic SRC-1 Box2-R_9 or Tif-2 Box2-R_9 peptides showed that these peptides could also efficiently recruit ERα into the nucleoli in the presence of Tif-2 coactivator (Figure S3). It is interesting to note that ERα nucleoli-recruitment upon the Tif-2 Box2-R_9 peptide treatment was found to be less efficient when cells expressed the corresponding Tif-2 coactivator (median ratios, 0.6) than the SRC-1 coactivator (median ratios, 2), possibly indicating a higher affinity of the Tif-2 coactivator or a different binding mechanism.

Inhibition of ERα Gene Transactivation by Synthetic LXXLL Motif Molecular Probes

The synthetic nona-arginine peptides induced sequestration of ERα into the nucleoli. We therefore presumed a concomitant decrease of ERα transcriptional activity when perturbed with these peptides. Moreover, because these peptides are in fact mimicking the interactions between ERα and endogenous cofactors, we expected that they might function in a dominant negative manner when added to cells, disrupting these interactions and blocking the ERα transcriptional activity. To study such an activity, a firefly luciferase reporter gene controlled by EREs was cotransfected with an expression vector for ERα. Cells were incubated in the presence or absence of estradiol, and the reporter gene product was quantified in the resulting cell lysates by measuring the luminescence generated from the oxyluciferin formed (Rishi et al., 1995). All the peptides, except for the control R_9 and alanine mutant peptides, decreased ERα gene-mediated transcription (Figure 5A). The largest inhibition, compared with untreated cells, was obtained with the Tif-2 Box2-R_9 peptide (61.0% of inhibition), the Tif-2 Box1-R_9 peptide (49.8% of inhibition), and the SRC-1 Box2-R_9 peptide (48.2% of inhibition). For comparison, when overexpressed in U2OS cells from a transfected DNA construct, an SRC-1 Box2 peptide exhibited a degree of inhibition (51.5%; Figure S4) similar to that of the synthetic SRC-1 Box2-R_9 peptide on ERα-mediated gene transcription. The concentration dependence of the inhibition of the ERα transcriptional activity by the peptides was verified with the Tif-2 peptide constructs (Figure S5A), and no inhibition was observed with external applied Tif-2 Box2 peptide without an arginine tag (Figure S5B).

We also examined the effect of the most efficacious peptide from the luciferase reporter assay (Tif-2 Box2-R_9) on the expression of an endogenous ERα target gene in the breast cancer cell line MCF-7 through quantitative polymerase chain reaction (QPCR) analysis (Figure 5B). MCF-7 cells treated with estradiol or with estradiol and the R_9 peptide as reference showed the expected up-regulation of ERα-mediated *pS2* mRNA levels. MCF-7 cells treated with estradiol and the Tif-2 Box2-R_9 peptide featured *pS2* mRNA levels analogous to cells not treated with estradiol, showing the effective down-regulation of the *pS2* gene expression by the inhibitor peptide. Together, the results show that inhibition of the ERα-coactivator interactions in cells by exogenous synthetic peptides resulted in an inhibition of ERα-mediated gene transcription, the extent of which depends on the amino acid sequences of the peptides. There is thus a clear relationship between the potential of the LXXLL molecular probes to bind and recruit ERα to the nucleoli and their capacity to antagonize ERα-mediated gene transcription. The results show that a cell-based study of the ERα binding and antagonizing activity of inhibitors directly evaluates their physiological effects.

DISCUSSION

We have designed a set of synthetic peptidic LXXLL molecular probes bearing both a nona-arginine tag, promoting cell-entry and specific nucleoli localization, and a biotin tag, allowing the visualization of their intracellular localization. Neither the arginine tag nor the biotin epitope changed the binding affinity of the

peptides for the ERα LBD, showing that these tags are located at sufficient distance from the binding pharmacophore. Even though all the LXXLL-peptide sequences under study are reported to have an affinity for the full-length ERα (Chang et al., 1999; Bramlett et al., 2001), not all of the sequences were found to bind the ERα LBD strongly. The fact that the full-length receptor can have a different surface topology as the isolated LBD (Chang et al., 2005; Bapat and Frail, 2003; Chang and McDonnell, 2002) is probably reflected in these observations, especially since a cellular activity of all these LXXLL-peptide sequences could be shown in our studies.

In order to perform subsequent cellular studies without the occurrence of any toxicity side effects, the concentrations at which these probes showed cellular toxicity were determined. Interestingly, the peptides that featured both a nona-arginine tag and an LXXLL-motif showed higher toxicity to the cells than either the nona-arginine tag alone or the peptide featuring an arginine tag but with the LXXLL motif mutated to an AXXAA motif. Since no strong differences in cellular uptake are to be expected between the LXXLL and AXXAA peptides, the different pharmacological behavior is more likely related to their intracellular effects. This furthermore indicates that the effects observed for the molecular probes are highly sequence specific.

CLSM imaging of cells incubated with these molecular probes showed that they successfully entered the cells and adopted a particular localization into the nucleoli. This particular nucleoli localization was not found for the non-R_9-tagged peptides and is therefore exclusively due to the R_9 tag. The efficient cellular uptake and specific nucleoli localization provide a strong methodological tool to study any interactions of these molecular probes with proteins, as it can be expected that either the localization of the probes or that of the proteins will be perturbed upon binding. Transient transfection of cells with ERα together with their incubation with the LXXLL probes showed that these peptides indeed resulted in perturbation of the ERα through recruitment to the nucleoli. This specific localization of ERα was not observed for a peptide consisting of the nona-arginine tag alone, nor was it observed for the AXXAA-motif peptides or peptides not featuring the nona-arginine tag. The ERα recruitment to the nucleoli by the nona-arginine LXXLL probes was furthermore found to be concentration dependent. These results show that the observed effects are due to a specific interaction of the LXXLL motif with the ERα in combination with the localization properties of the nona-arginine tag. The fact that the nucleoli recruitment of ERα was observed for all the nona-arginine LXXLL probes underlines the affinity of all these peptides for the full-length ERα in a cellular context. The absence of this interaction in a biochemical context for some of these peptides on the isolated ERα LBD shows that cellular evaluation, as presented here, provides a more physiologically relevant view on the binding interaction. The binding affinity of the peptides for the ERα can be evaluated by quantifying the ERα-nucleoli recruitment. This shows that certain peptide sequences, such as SRC-1 Box2, Tif-2 Box1, and Tif-2 Box2, show a higher affinity than others. This is in line with observations that the ERα has distinct preferences for the different NR boxes of coactivators (Voegel et al., 1998).

The LXXLL probes maintain the property to recruit the ERα into the nucleoli even in the presence of overexpressed SRC-1 and Tif-2 coactivators, normally found bound to ERα in the nucleoplasm. The probes thus can compete with full-length coactivators in living cells. Quantification of ERα-nucleoli recruitment upon LXXLL-peptide treatment therefore provides, to our knowledge, a new, simple, and efficient method to assess the potency of molecular probes to act as ERα-coactivator inhibitors under physiological conditions. Among the panel of peptides tested, the Tif-2 Box2-R_9 and SRC-1 Box2-R_9 peptides were found as the most potent inhibitors of SRC-1 and Tif-2 coactivator binding.

The specific physical inhibition of the ERα-coactivator interaction could be correlated with a decrease in ERα transcriptional activity when perturbed with these peptides, both in a luciferase reporter assay and for activity of the endogenous *pS2* gene in MCF-7 cells. Again, all LXXLL probes were functional and decreased ERα gene-mediated luciferase transcription, whereas the control nona-arginine peptide and the AXXAA probes did not. In line with the nucleoli recruitment results, the Tif-2 Box2 and SRC-1 Box2 showed the strongest inhibition. The luciferase gene transcription is inhibited to around 50% (Figure 5A). This value is also obtained when a similar peptide is overexpressed in U2OS cells from a transfected DNA construct and in accordance with literature data on overexpressed peptides (Norris et al., 1999; Hall et al., 2000). The Tif-2 Box2-R_9 showed a full inhibition of the *pS2* gene transcription in MCF-7 cells. The extent of the transcriptional inhibition may depend on the cell type, assay format, and ligand-exposure time.

It was previously shown that interaction of Tif-2 with ERα is strongly and selectively affected by mutations in NR-Box 2 (Ding et al., 1998). In a similar manner, it was shown that estradiol-bound full-length ERα displays unambiguous SRC-1 box preferences for NR-Box 2, while the recruitment of boxes 1 and 3 of SRC-1 was not detected in vitro (Bramlett et al., 2001). The 75% homology found between the SRC-1 Box2 and Tif-2 Box2 sequences provides a good explanation for the common features, both in terms of ERα-binding capacity and ERα-mediated transcription inhibition that were observed in cells for these two peptides. Overall, there is a clear relationship between the inhibitory effect on ERα-mediated gene transcription and ERα translocation into the nucleoli by the molecular probes.

The mechanism of ERα inhibition by ICBs typically is via a direct inhibition of the ERα-coativator interaction (Rodriguez et al., 2004; Vaz et al., 2008). The nona-arginine-based inhibitors reported here feature the same mechanism, but additionally displace the ERα after inhibition of the ERα-coactivator interaction. The mechanism of action is thus most likely a combination of inhibition and sequestration of the receptor. The method of cellular evaluation of inhibitory capacity on the full-length ERα as presented here could therefore be applied to evaluate coactivator binding inhibitors in general. These inhibitors could be either provided themselves with localization and visualization tags or be used in competition assays with the molecular peptide probes presented here. Also, this method can provide a basis to address NR selectivity for LXXLL-based inhibitors in a cellular context, when applied to a panel of different NRs.

The receptor's conformation is postulated to be a better indicator of the antagonist activity of different ERα ligands than cell-based reporter gene assays (Larson et al., 2005; Michalides et al., 2004; Zwart et al., 2007b). The molecular probes presented here and derivatives thereof could provide a valuable entry to

diagnose the antagonist capacity of specific ligands in different cell types. In the presence of specific ERα antagonists, the receptor adopts a conformation that prevents coactivators from binding (Shiau et al., 1998). It can be speculated that the molecular probes presented here will not interact with this antagonist-liganded ERα. However, in the case of antagonist resistance, the ligand frequently acquires an agonistic function (Osborne et al., 2003; Girault et al., 2003) and the molecular probes might interact again with the ERα and thus induce nucleoli recruitment. The method presented here will thus provide ways to probe the surface of the ERα and determine its conformation in different cell-types, dependent on ligand, or with specific posttranslational modifications or point mutations.

SIGNIFICANCE

The ERα-coactivator interaction, which is based on the molecular recognition between consensus helical LXXLL motifs of the coactivator and a hydrophobic pocket at the surface of the receptor, is an essential step for the ERα transcriptional activation process to take place. This interaction is an important target for the development of anti–breast cancers drugs. Here, a new, to our knowledge, chemical biology approach is reported in which synthetic LXXLL-peptide probes provided with specific visualization and localization tags interact directly with ERα in cells and perturb its localization and transcriptional activity. These probes monitor the affinity of specific peptide sequences for the full-length ERα in cells. This approach represents a significant advancement over biochemical interaction studies, which frequently focus on the isolated ERα LBD alone. The molecular probes feature a nona-arginine tag that facilitates cellular entry and also accounts for specific subcompartmental localization at the nucleoli. This specific feature allows these probes to dislocate the ERα from the DNA to the nucleoli upon binding and thus perturb the assembly of the receptor transcription complex. This molecular displacement of the ERα is accompanied by an inhibition of its transcriptional activity. Quantification of the ERα-nucleoli recruitment is a simple and rapid method to assess the affinity of peptide sequences for ERα in the physiological context of a cell. Moreover, since the timing of addition and concentration are easily controlled and their amino acid sequences can be fully modified, these arginine-tagged probes promise to be useful molecular tools to elucidate and modulate ERα functioning.

EXPERIMENTAL PROCEDURES

Peptide Synthesis

The synthesis of all peptides was performed on solid support, using the Fmoc strategy. Biotin-PEG Nova Tag™ resin (0.48 mmol/g, Novabiochem) was allowed to swell in dimethylforamide (DMF) at room temperature for 30 min. After DMF was drained, the beads were incubated with 40% piperidine in DMF for the deprotection of the Fmoc group, and subsequently the amino acids (AA) were introduced using the following coupling conditions: AA (3 eq), 2-(6-chloro-1H-benzotriazole-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (3 eq), and N-methylmorpholine (6 eq) in DMF for 40 min. The Fmoc deprotection and introduction of amino acids was repeated until the complete sequence was assembled.

The resin was washed 5 times with dichloromethane and ether successively and dried under vacuum for at least 1 hr. Cleavage of side chain protecting groups and of the peptides from the resin was achieved by shaking the resin in a mixture of trifluoroacetic acid (TFA), triisopropyl silane, ethane-1,2-dithiol, and H_2O (95/1/2.5/1.5) for 2 hr. Then, the resin was removed by filtration and washed twice with TFA. Most of the TFA was evaporated from the combined filtrates, and a 10-fold volume excess of cold diethyl ether was added dropwise to achieve peptide precipitation. The crude peptides were isolated by centrifugation (3500 rpm, 10 min), washed with cold ether, taken up in water and lyophilized, and finally purified by reverse-phase high-performance liquid chromatography on a nucleodur C_{18} Gravity column (125 × 21 mm, Macherey-Nagel) with a linear 60 min gradient of A (0.1% TFA in H_2O) and B (0.1% TFA in MeCN) from 10% B to 100% B and a flow rate of 20 mL/min^{-1}. After purification, all the synthesized peptides were lyophilized and stored at −80°C. The purity of the peptides was assessed by analytical liquid chromatography–mass spectrometry at 210 nm, 254 nm, and 280 nm using a diode array ultraviolet/visible detector. All the nona-arginine peptides were obtained pure with yields ranging from 13% to 41%.

In Vitro Competitive Fluorescence Polarization Assay Expression and Purification of ERα-LBD Protein

The pET15b-ERα-LBD plasmid (gift from Bayer-Schering Pharma AG) encoding the LBD of the human ERα was used as template for PCR amplification using the primers 5′-CCGGATGGATCCATGGGCAGCAGCCATCATCAT-3′ and 5′-CCGGATCTCGAGTTAAGTGGGCGCATGTAGGCGG-3′. The PCR product (ERα-LBD residues 302–553) was digested with BamHI and XhoI enzymes (Fermentas) and subcloned into a pHT401 plasmid to introduce a Strep-tag and His-tag at the N terminus of the ERα-LBD construct. This plasmid (pHT503) was transformed into *Escherichia coli* Rosetta 2-DE3-placI (Novagen) cells that were then grown in Luria Bertani medium with 100 mg/L ampicilline and 34 mg/L chloramphenicol at 37°C. At OD_{600nm} ~0.8, protein expression was induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside for 16–18 hr at 16°C. Bacteria were then concentrated by centrifugation for 10 min at 5,000 rpm and were kept at −80°C until purification. Cells lysate chromatography was performed at 4°C on an ÄKTA™ purifier using a HisTrap™ HP 5 ml column (GE Healthcare) pre-equilibrated with the protein extract buffer: 300 mM NaCl, 50 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 20 mM imidazole (pH 8.0). Protein elution was obtained with 500 mM NaCl, 50 mM Na_2HPO_4, 2 mM NaH_2PO_4, 500 mM imidazole, and 10% (v/v) glycerol (pH 8.0); fractions containing the ERα-LBD protein were then desalted on a Sephadex G25 PD-10 column (Amersham Biosciences) pre-equilibrated with 25 mM NaCl, 20 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, and 1 mM tris (2-carboxyethyl) phosphine.

Fluorescence Polarization Measurements

A reaction mixture containing a fluorescein-labeled coactivator (SRC-1 Box2) peptide FL-CQLLTERHKILHRLLQEGSPSD (10^{-8} M), the ERα-LBD protein (10^{-6} M), and estradiol (10^{-5} M) was prepared. Fluorescence polarization inhibition experiments were performed in 384-well plates (Optiplate-384 F, Perkin Elmer) by adding 10 μl of the reaction mixture to 40 μl of inhibitor peptides at increasing concentrations. After 2 hr of incubation at 4°C, the fluorescence polarization of the labeled coactivator peptide was measured on a plate reader fluorometer (Safire^{2TM}, Tecan) with excitation at 470 nm and emission at 519 nm for fluorescein. The concentration of inhibitor peptide that resulted in a half-maximum decrease in the polarization value of the fluorescent coactivator peptide displayed from the purified ERα was defined as IC_{50} and was determined from three independent experiments with the ORIGIN 7 program (Scientific Graphing and Analysis Software, OriginLab Corp).

Cell-Viability Assay

U2OS cancer cells (human osteosarcoma; ATCC number HTB-96) were split at 25% confluence in 96-well plates and maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (v/v) antibiotics penicillin/streptomycin (GIBCO) and 10% heat-inactivated fetal calf serum (GIBCO). After 1 day, cells were incubated with 100 μl of increasing concentrations of peptides (range from 0.01 to 5 g/L) directly diluted in the complete medium, for 14 hr. Subsequently, 10 μl of the cell proliferation reagent WST-1 was added to each well, and cells were incubated again in

a humidified atmosphere for 2.5 hr before measurement. The absorbance of the samples was then measured using a microplate reader (Safire^{2TM}, Tecan) at 450 nm, and cell toxicity was expressed as the percentage of inhibition of WST-1 incorporation, compared with control cells not treated with peptides. All cell experiments were further performed at peptide concentrations that did not induce more than 20% of cell toxicity.

Cell Imaging by Confocal Laser Scanning Microscopy (CLSM): Cellular Localization of the Synthetic Peptides

U2OS cells were cultured on coverslips for 48 hr before imaging in the same complete medium as mentioned above. Fourteen hours before imaging, cells were incubated with peptides at the nontoxic peptide concentrations described above and then were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. A 10-min treatment of the fixed cells by PBS-NH_4Cl 50 mM and then PBS-Glycine 20 mM was used to avoid self-fluorescence bleaching. Cells were permeabilized with Triton X-100 0.1% for 10 min, washed 3 times, and incubated for 30 min with a blocking solution of PBS-BSA 0.1%. The cellular distribution of peptides was revealed after immunolabeling of the cells using a Cy5-conjugated monoclonal mouse anti-biotin antibody (Jackson ImmunoResearch) diluted 1:400 in PBS and their CLSM imaging (Leica TCS SP2), with the following settings: λex = 633 nm and λem = 650–700 nm.

Cellular Assessment of ERα-LXXLL-Peptides Binding

U2OS cells were cultured on coverslips in a 6-well plate for 48 hr before imaging in DMEM without phenol red supplemented with 1% penicillin/streptomycin and 10% hormone-depleted (charcoal/dextran treated) and heat-inactivated fetal calf serum (Thermo Scientific HyClone). At 50% of confluence, U2OS cells (neither expressing ERα nor ERβ) (Monroe et al., 2003) were transiently transfected by PEI (Polysciences) or Fugene® 6 (Roche) with 1 μg of pcDNA3.1 plasmid containing the HA-ERα-CFP construct (Zwart et al., 2007a). Cells were incubated overnight with the nona-arginine peptides dissolved in the culturing medium. Afterward, estradiol was added to the cultures at a final concentration of 10^{-6} M for 3 hr. Cells were then fixed and stained as described above and imaged by CLSM (objective X63) with the followed independent settings: λex = 458 nm and λem = 470–500 nm for CFP, and λex = 633 nm and λem = 650–700 nm for Cy5.

The quantification of ERα-CFP in the nucleoli was performed with the Quantify Histogram module within the CLSM Leica software. First, on the basis of Cy5 fluorescence, for each cell, regions of interest (ROIs) were designed, encompassing the nucleoli containing peptides and the nucleoplasm. Then, the average CFP fluorescence intensity per pixel in the ROIs were subtracted by the fluorescence intensity of the background to calculate the enrichment of ERα-CFP in the nucleoli compared to the nucleoplasm as the mean ratio (CFP intensity nucleoli/CFP intensity nucleoplasm). Results are presented as the ratios (nucleoli/nucleoplasm) found per peptide for two independent experiments, for a 10–20 cell population.

Cellular Assessment of LXXLL Peptides/Coactivators (SRC-1 and Tif-2) Competitive Binding for ERα

U2OS cells were cultivated and treated in the same conditions as above and transiently cotransfected with 1 μg of pcDNA3-ERα-CFP plasmid and 2 μg of pcDNA3-SRC-1-YFP plasmid or 2 μg of pcDNA3-YFP-Tif-2 plasmid. After cells were fixed and labeled with the Cy5-conjugated anti-biotin antibody, they were imaged by CLSM with the successive settings of CFP, Cy5, and YFP (λex = 514 nm and λem = 525–550 nm). The same method as described above was used to quantify ERα-CFP fluorescence into the nucleoli and was compared to the one found in the cytoplasm.

Inhibition of ERα-Mediated Gene Transcription ERα Luciferase Transactivation Assay

U2OS cells were split in 24-well plates and maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) without phenol red, supplemented with 1% (v/v) antibiotics penicillin/streptomycin and 10% hormone-depleted and heat-inactivated fetal calf serum (Thermo Scientific HyClone). After 1 day, cells were transfected using PEI with 10 ng of pCMV-HA-ERα full length, 200 ng of pERE-tK-firefly luciferase (Bindels et al., 2002), and 2 ng of SV40 *Renilla reniformis* luciferase plasmids. After DNA transfection, cells were maintained at 37°C under 5% CO_2 for 4 hr before treatment with synthetic peptides directly dissolved in the medium. For comparison, a pcDNA3.1 plasmid encoding for the SRC-1 Box2 peptide sequence (pSF5 vector) was also tested: cells were transfected with 20 ng of pSF5, 0.2 ng of pCMV-HA-ERα, 200 ng of pERE-tK-firefly luciferase, and 2 ng of SV40 *Renilla* luciferase plasmids. Four hours after adding the peptides, estradiol (10^{-8} M) was incubated with the cells for 16 hr before the reading of firefly luciferase expression by a dual-luciferase reporter assay system (Promega). Luminescent intensities were recorded on a Berthold Centro XS3 LB 960 (Berthold technologies). Firefly luciferase activity signal was first normalized with respect to the constitutive *R. reniformis* luciferase signal and second normalized to the luciferase activity found in control cells incubated with the R_9 tag alone. Results shown are representative of three independent experiments.

pS2 Gene Inhibition in MCF-7 Cells

For quantitative RT-PCR in MCF-7 cells, cells were cultured in phenol red–free DMEM containing 5% charcoal-treated serum for 72 hr prior to the onset of the experiment in order to deplete activated ER in the cells. Subsequently, cells were cultured overnight in the presence of the indicated peptides at a final concentration of 70 μM. After overnight treatment, 10 nM estradiol was added for 3 hr or the cells were left untreated. After exposure to hormones, cells were lysed, and RNA was extracted using Trizol (Invitrogen), according to the manufacturer's protocol. RNA was reverse-transcribed using SuperScript(tm) III Reverse Transcriptase (Invitrogen), on which QPCR was performed using CYBR Green (Applied Biosystems), according to the manufacturer's protocols. The *pS2* cDNA was amplified with the forward primer 5′ CATCGAC GTCCCTCCAGAAGA and the reverse primer 5′ CTCTGGGACTAATCACCG TGCT. As a control for equal loading, the observed signals were related to β-actin RNA levels, using a forward primer 5′ CCTGGCACCCAGCACAAT and reverse primer 5′ GGGCCGGACTCGTCATACT. Input levels were related to β-actin house keeping gene mRNA levels. Average values and SD were obtained for samples without peptide (duplicate) or with peptide (triplicate).

SUPPLEMENTAL DATA

Supplemental data include two tables and five figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00207-5.

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