Selective estrogen receptor modulators (SERMs) show differential effects upon ERα activation function 1 (AF-1). Tamoxifen allows strong ERα AF-1 activity, whereas raloxifene allows less and ICI 182,780 (ICI) allows none. Here, we show that blockade of corepressor histone deacetylase (HDAC) activity reverses the differential inhibitory effect of SERMs upon AF-1 activity in MCF-7 cells. This suggests that differential SERM repression of AF-1 involves HDAC-dependent corepressors. Consistent with this, ICI and raloxifene are more potent than tamoxifen in promoting ERα-dependent sequestration of progesterone receptor-associated corepressors. Moreover, ICI and raloxifene are more efficient than tamoxifen in promoting ERα binding to the corepressor N-CoR in vivo and in vitro. An ERα mutation (L379R) that decreases N-CoR binding increases AF-1 activity in the presence of ICI and raloxifene and reverses the effect of the L37X mutation. The L37X and L379R mutations also alter the ligand preference of ERα action at AP-1 sites and C3 complement, an action that also involves AF-1. Together, our results suggest that differential SERM effects on corepressor binding can explain differences in SERM effects on ERα activity. We propose a model for differential effects of SERMS on N-CoR binding.

Estrogen signaling is mediated by two estrogen receptors (ERα and ERβ), which are conditional transcription factors (1–3). In the best understood pathway of ER action, the ERs bind specific estrogen response elements (EREs) in the promoter of estrogen-regulated genes and activate transcription by recruiting a large coactivator complex composed of p160 coactivators such as GRIP1 and SRC-1 and the histone acetyltransferases p300/CREB-binding protein and pCAF (4). Like other nuclear receptors, the ERs are comprised of an N-terminal domain (NTD), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). The DBD mediates ERE recognition, and the NTD and LBD contain distinct activation functions (AF-1 and AF-2, respectively) that mediate coactivator recruitment. The ERs also modulate expression of genes with alternate estrogen response elements, such as AP-1 sites and SF-1 sites (5–7). ERs usually function as antagonists in breast, but generally act as pure agonists in other tissues. Tamoxifen shows agonist effects, including prevention of osteoporosis, and harmful agonist effects, including emergence of tamoxifen-stimulated breast cancers and increased rates of uterine cancer (9). Raloxifene and GW5638 show agonist effects in bone and other tissues, but lack harmful uterotrophic effects (20). ICI shows agonist effects in bone (21), but generally acts as a pure antiestrogen (22). Understanding why SERMs exhibit differential estrogen-like activities will help in identification of new SERMs with favorable profiles of activity.

Some SERM estrogen-like effects stem from AF-1 (2, 23). ERα and ERβ AF-1 usually exhibit weak activity at genes with classical EREs, and consequently, SERMs exhibit little agonist activity at this type of gene. However, ERα AF-1 is strong in some cells (23–25), in the presence of high levels of AF-1 coactivators (26), in conditions of MAP and JNK kinase activation (27–29), and at certain promoters (23, 25, 30). In each of these cases, tamoxifen-ligated ERα exhibits activity that is equal to isolated AF-1, but raloxifene, GW5638, and GW7604-ligated ERα show less activity (20, 31), and ICI-ligated ERα shows no activity at all (23). Thus, SERMs show differential effects on
AF-1 activity at classical EREs.

Other estrogen-like effects of SERMs stem from ER action at genes with alternate response elements (5–7). ERα enhances AF-1 activity in the presence of tamoxifen but shows weaker activity in the presence of raloxifene. In contrast, raloxifene and tamoxifen show greater than 90% (31, 36) of the estrogenic activity at classical EREs (35, 10). Moreover, SERMs differ in their ability to promote N-CoR recruitment to estrogen-regulated promoters in vivo (10, 43). Several lines of evidence indicate that corepressors inhibit the activity of the SERM-ERα complexes (reviewed in Ref. 4). Moreover, it has been shown that tamoxifen is less efficient than raloxifene at recruiting N-CoR to estrogen-regulated genes with alternate response elements in uterine cells. Here, we describe how tamoxifen behaves as an agonist in this context when raloxifene does not (10, 26, 32).

While SERMs differ in their ability to promote N-CoR recruitment at one type of gene, it is not clear why. SERM-ERα complexes interact relatively strongly with the co-repressor complex in vivo (10, 40, 44, 45) but, at best, only weakly bind to ERα and N-CoR to estrogen-regulated genes with alternate response elements in Ishikawa uterine cells (10), and this effect may explain why tamoxifen behaves as an agonist in this context when raloxifene does not (10, 26, 32).

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Why do SERMs show differential effects on ERα activity? One possible mechanism involves effects upon ERα turnover. Tamoxifen increases ERα steady state levels, raloxifene and GW7604 reduce ERα levels, and ICI reduces ERα levels by more than 90% (31, 36). These effects correlate well with effects on ERα AF-1 activity, but in vivo competition assays reveal that the amount of ERα that occupies the ERE is relatively unaffected by SERMs (31, 37, 38). Moreover, ICI and raloxifene act as ERα agonists at genes with AP-1 sites (6). Thus, raloxifene and ICI inhibition of ERα activity is not simply a consequence of elimination of functional ERs from the cell. Another possible mechanism involves differences in corepressor recruitment. SERMs promote ERα interactions with the corepressors N-CoR and SMRT (39–42) and N-CoR recruitment to estrogen-regulated promoters. The following plasmids were previously described. Receptors were ERα/TR-Luc, TR-Luc, Col73-Luc, and actin β-galactosidase (56). TAT-Luc was a gift from K. Yamamoto (University of California, San Francisco) and C3 Complement-Luc was a gift from D. McDonnell (Duke University, North Carolina). Mammalian expression/en vitro transcription-translation vectors were ERα, ERαHinge (also known as HE241G), DBD-LBD, AB-DBD, AB, LBD, LBDS37X, G400V, K362A, V376R, E542K, PR, GR1P1, and p300 (12, 46). Bacterial expression vectors were GST-fused to the N-CoR C terminus (amino acids 1987–2453) and GRIP1 NR box region (563–706) and cloned into pGEX-5X-1.

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primary ERα antibodies that were used in this study were HC-20 (Santa Cruz antibodies) directed against the ERα C terminus, or number 1600024 (Geneka, Montreal, Canada) directed against the ERα NTD. The primary N-CoR antibody was N-19 (Santa Cruz antibodies). Primary antibody was diluted 1:2000 in PBS-T and incubated with the membrane for 1 h, followed by PBS-T washes, 1 × 15 min and then 2 × 5 min. The membrane was incubated for 45 min with horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG (Santa Cruz antibodies) diluted 1:2000 in PBS-T, followed by PBS-T washes (1 × 15 min and 4 × 5 min). After the last wash, the membrane was developed with a standard ECL kit (Amersham Biosciences), covered with Saran wrap, and exposed to x-ray film. Westerns were quantified as described for GST pulldown assays.

RESULTS

A HDAC Inhibitor Eliminates Differential SERM AF-1 Inhibition at an ERE-responsive Promoter in MCF-7 Cells—The nuclear receptor corepressor complex inhibits transcription by recruiting histone deacetylases, or HDACs (4). To begin this study, we determined whether inhibition of HDACs would affect SERM inhibition of ERα action. We chose tamoxifen, raloxifene, and ICI as representative SERMs that permit, partially inhibit, and completely inhibit AF-1 activity, respectively.

Fig. 1A shows that, as often noted, an ERE-responsive reporter (ERE-TK-LUC) exhibited weak constitutive activity in MCF-7 breast tumor cells and that this activity was enhanced by estrogens and reduced by SERMs, especially by ICI. A similar reporter (TK-LUC), which lacked an ERE, was not affected by ligand. Addition of HDAC inhibitor (trichostatin A, TSA) enhanced basal activity of both reporters. However, TSA also specifically blocked the ability of SERMs to inhibit ERE-dependent transcription, with the result that fairly equivalent levels of ERE-dependent transcription were observed in the absence of hormone and in the presence of each ligand. Similar results were also obtained in T47D breast cancer cells and GHFT1–5 pituitary cells (not shown).

To highlight TSA effects upon ERα ligand preference, we calculated fold TSA induction in the presence of each ligand (Fig. 1B). 6-fold TSA induction was obtained in the absence of ligand; 22-fold induction was obtained with ICI, which does not allow AF-1 activity, 12-fold with raloxifene, which allows intermediate AF-1 activity, and 7.5-fold with tamoxifen, which allows the most AF-1 activity. These differential TSA effects were not seen at the core TK promoter, and TSA did not alter the overall profile of SERM effects on ERα steady state levels (Fig. 1C). Thus, TSA enhances ERE-dependent transcription more potently in the presence of SERMs. This suggests that SERMs increase TSA-sensitive HDAC-dependent inhibition at the ERE-responsive promoter but not at the parental TK core promoter. Moreover, the fact that TSA induction is larger in the presence of ICI and raloxifene relative to tamoxifen shows that the magnitude of this HDAC-dependent effect varies with individual SERMs.

SERMs Show Differential Ability to Sequester Corepressors from Progesterone Receptor—Next, we determined whether SERMs would differentially influence ERα interactions with the corepressor complex in vivo. To perform this experiment, we investigated whether SERMs would enhance the ability of ERα to potentiate the activity of DNA-bound antiprogestin-PR complexes using the system developed by Bagchi and coworkers (41). This assay measures the ability of ERα to sequester unspecified N-CoR containing corepressor complexes and therefore gives an indication of overall ERα interactions with shared corepressor complexes rather than a direct indication of ERα interactions with particular corepressor complex components.

In agreement with previous results, ERα potentiated the activity of a PR-RU486 complex bound to a progestin-responsive reporter gene (TAT3-LUC) in the presence of tamoxifen but not estradiol (Fig. 2). However, ERα also potentiated PR activity more potently in the presence of raloxifene and most strongly with ICI. We conclude that ICI and raloxifene are more efficient at promoting sequestration of corepressors from the PR-RU486 complex in vivo.

Differential Ability of SERMs to Promote ERα Interactions with N-CoR—N-CoR consists of separable N-terminal repression domains that interact with HDACs and other components of the corepressor complex and a C-terminal domain that contains the nuclear receptor-interacting region (4). SERMs pro-
SERMs Promote Corepressor Recruitment

Fig. 3. SERMs differentially promote ERα interactions with N-CoR in vivo. A, HeLa cells were transfected with 10 μg of ERα expression vector or 10 μg of empty vector (mock) along with 10 μg of expression vector for a GAL-N-CoR fusion protein (amino acids 1944–2453) and treated with ligand for 24 h. The upper panel shows anti-ERα Westerns blot of transfected HeLa cell extracts after immunoprecipitation with anti-GAL4 antibody. The lower panel shows a Western blot of ERα in cell extracts prior to immunoprecipitation. B, the side panel shows average results of three separate experiments. Data are expressed as the ratio of the relative intensity of the ERα band in the IP (immunoprecipitate, corrected for background and multiplied by a factor of ten) over the intensity of the ERα band in Western blot prior to immunoprecipitation (corrected for background).

Fig. 4. SERMs differentially promote ERα interactions with N-CoR in vivo. A, labeled ERα was bound to GST fusions (shown in schematic at top) in the presence of vehicle or ligand, separated by SDS-PAGE, and probed for autoradiography. The panels show binding to N-CoR C terminus (amino acids 1944–2453), SMRT C terminus (amino acids 987–1491), and GRIP1 NR box region (amino acids 563–1121), which contain nuclear receptor-interacting regions (black stripes). 10% input and ERα retained by GST are shown as controls. B, the side panel shows average results from 15 separate experiments involving GST-N-CoR. Data are expressed as percentage of input ERα retained on GST-corepressor beads after correction for background binding to GST control beads.
SERMs in vitro cleft and the DBD-hinge. ER determinations. Data are the average of five separate determinations.

N-CoR suggesting that other regions of ERα contribute to N-CoR binding. The ERα DBD-LBD region did bind N-CoR with a ligand preference that resembled full-length ERα, but an ERα deletion mutant that lacked the hinge domain (ERα hinge) and the isolated AB-DBD and AB regions did not bind N-CoR. Thus, ERα interactions with N-CoR require the hydrophobic cleft and the DBD-hinge.

Because the 537X mutation allowed N-CoR binding with all SERMs in vitro, we examined the phenotype of ERα537X in vivo. As expected (24), wild type ERα enhanced transcription of an ERα-responsive reporter (ERE-TK-LUC) in the absence of ligand in MDA-MB-453 breast cells, and estradiol gave further stimulation (Fig. 6A). The SERMs tamoxifen and raloxifene allowed some residual activity that stems from AF-1 (24), but ICI-liganded ERα showed reduced estrogen response. This is consistent with the requirement for H12 in p160 binding (15). First, ERα537X failed to elicit a tamoxifen response. This lack of response persisted even in the presence of a 10-fold excess of ERα537X expression vector (not shown). Fig. 6B shows that ERα537X was expressed at comparable levels to wild type ERα in the presence of SERMs. Thus, the lack of tamoxifen response even in the presence of ERα537X is not a consequence of a large reduction in ERα levels. Moreover, our analysis of the amount of ERα and ERα537X transfection vectors that are required to elicit responses from the ERα-responsive reporter indicate that ERα537X has not become superactive at low levels of transfected receptor and is not squelching its own activity under these conditions (data not shown). We conclude that an ERα mutation (537X) that enhances corepressor binding also eliminates AF-1 activity.

An ERα Mutation That Reduces N-CoR Binding Allows Equal AF-1 Activity with SERMs—The nuclear receptor corepressor-binding surface consists of residues that overlap the hydrophobic cleft that comprises part of the AF-2 surface (48, 51–54). In particular, a Leu residue at the base of H5 is important for corepressor binding (51). We therefore next determined whether mutation of the equivalent ERα residue (Leu-379) would influence SERM effects on corepressor binding. Fig. 7 shows that N-CoR binding was reduced in all conditions in the presence of ERαL379R but that these effects were especially prominent in the presence of ICI and raloxifene (about 0.5% of ICI-liganded ERαL379R bound N-CoR relative to 4% of ICI-liganded wild type ERα, about 0.2% of raloxifene-liganded ERαL379R bound to N-CoR relative to more than 2% of wild type ERα). ERαL379R also reduced GRIP1 binding in the presence of estradiol (Ref. 15 and not shown). Mutations in nearby residues (K362A, T371R, V376R, and E542K) did not show large effect on the overall ligand preference of N-CoR binding, even though some (K362A, V376R, and E542K) did reduce GRIP1 binding (Ref. 12 and not shown). Thus, Leu-379 is important for SERM-dependent ERα interactions with N-CoR in vitro, and these interactions are deficient in the ERαL379R mutant, but not in other mutations in the AF-2 surface.

We then tested ERαL379R in vivo. Like other ERα AF-2 mutants, ERαL379R showed reduced estrogen response and constitutive activity (Fig. 8A). More importantly, ERαL379R showed increased raloxifene and ICI response. Other ERα AF-2 mutants (K362A, V376R, and E542K) did not show large effect on the overall ligand preference of N-CoR binding, even though some (K362A, V376R, and E542K) did reduce GRIP1 binding (Ref. 12 and not shown). Thus, Leu-379 is important for SERM-dependent ERα interactions with N-CoR in vitro, and these interactions are deficient in the ERαL379R mutant, but not in other mutations in the AF-2 surface.
SERMs Promote Corepressor Recruitment

Mutations That Affect N-CoR Binding Have Parallel Effects on ERα Action at AP-1 Sites—ERαs enhances AP-1 activity in the presence of estradiol and the partial agonist tamoxifen (5, 32), and these effects require ERα activation functions and p160s (6, 10). However, ERα also enhances AP-1 activity via a second AF-independent mechanism that is up-regulated in ERα deletions that lack AF-1 and strongly potentiated by ICI and raloxifene (6, 32, 34) and may involve coresspressors (6, 35). Thus, a mutation that reduces N-CoR binding would potentiate ERα AF-1 activity at AP-1 sites but should also inhibit the activity of ERα truncations that are committed to the AF-independent pathway. Moreover, mutations that enhance N-CoR binding may show increased ability to potentiate ERα activity in the AF-independent pathway.

To test these ideas, we examined the effect of the L379R mutation upon ERα action at AP-1 sites. Fig. 10A confirms that ERαs enhanced the activity of an AP-1-responsive promoter (ColI73-LUC) in the absence of ligand and in the presence of tamoxifen, estradiol, and DES in HeLa cells and that ERαG400V showed a similar profile. The same data show that ERαL379R exhibited equivalent activity at the AP-1-responsive reporter in the absence of ligand and in the presence of each SERM. The L379R mutation also enhanced estradiol and DES response at the AP-1 site. This is consistent with the suggestion that the corepressor complex restricts estrogen-liganded ERα activity in some contexts (44). Fig. 10B confirms that truncation of the ERαL379R NTD (DBD-LBDL379R) completely abolished all SERM effects. Thus, increased SERM activities that are observed with ERαL379R require AF-1 just as they do at ERαs. The same data confirm that the DBD-LBD enhances AP-1 activity in the presence of ICI and raloxifene but not tamoxifen or estradiol (6, 24) and that the L379R mutation abolishes these responses. Thus, the ICI and raloxifene response through the AF-independent pathway requires the N-CoR binding surface.

We then examined the effect of the 537X mutation, which increases and equalizes N-CoR binding in the presence of SERMs. Like ERαL379R, ERα537X showed equivalent activity at the AP-1-responsive reporter in the absence of ligand and in the presence of the three SERMs (Fig. 10A). However, unlike ERαL379R, truncation of the NTD did not abolish these SERM effects (Fig. 10B). Instead, ICI and raloxifene responses were preserved and tamoxifen and estradiol responses were increased. Thus, the 537X mutation equalizes the activity of ligands in the AF-independent pathway. We conclude that mutations that affect N-CoR binding alter the ligand preference of ERαs action at AP-1 sites through two mechanisms—An Exception to Parallels between N-CoR Binding and AF-1 Activity, the C3 Complement—ERαs shows potent AP-1 activity at the C3 complement promoter (C3) (25, 30). Fig. 11 confirms that ERαs and ERαG400V enhanced the C3 activity in the presence of estradiol and tamoxifen but not ICI or raloxifene in MDA-MB-453 cells. ERα537X failed to enhance C3 in the presence of any ligand, just as it did at a simple ERE. Moreover, ERαL379R showed enhanced ICI and raloxifene responses relative to wild type ERαs, just as it did at a simple ERE and the AP-1 site, and enhanced estradiol and DES response, just as did at the AP-1 site. ICI and raloxifene effects were dependent upon the presence of the NTD (not shown). Thus, a mutation (L379R) that reduces N-CoR binding enhances AP-1 activity at C3 in the presence of ICI and raloxifene.

Unlike its activity at ERαs and the AP-1 site, however, the L379R mutation did not preserve any tamoxifen response at C3 in MDA-MB-453 cells (shown here) and other cell types (includ-
ing HeLa and HepG2; data not shown). This finding, though not expected, is in line with previous observations that indicate that ERα tamoxifen activation of C3 involves an unspecified contribution from the LBD (25, 30). It is possible that this contribution is deficient in the L379R mutant. We conclude that N-CoR inhibits ICI and raloxifene activity at C3 but that low levels of N-CoR binding in the presence of tamoxifen do not fully explain why tamoxifen is a potent activator in this context.

DISCUSSION

Differential Corepressor recruitment underlies differential SERM effects at genes with alternate response elements in uterine cells (10). However, the structural basis for these differential SERM effects on corepressor recruitment is unclear, and it is controversial whether ERα shows meaningful direct interactions with N-CoR. It is also unclear whether similar mechanisms underlie differences in SERM activity in other contexts.

In this study, we showed that ICI and raloxifene promote HDAC repression at an ERE-responsive reporter in MCF-7 cells and ERα-dependent sequestration of PR-associated corepressor complexes, and that they do so more potently than tamoxifen. We also found that SERMs promote ERα interactions with the nuclear receptor interaction domain of N-CoR (C terminus) in immunoprecipitations, and estimates of ERα levels in the N-CoR-enriched fraction versus the cell extract suggested that the ICI and raloxifene are better than tamoxifen in promoting this interaction. Likewise, ICI and raloxifene are more potent than tamoxifen in promoting ERα association with the N-CoR C terminus in GST pulldown assays in vitro. Thus, SERMs show differential effects on ERα interactions with N-CoR and do so with a ligand preference that parallels their effects on suppression of AF-1.

While SERMs promote N-CoR binding in vitro, the interac-
SERMs Promote Corepressor Recruitment

![Image](https://example.com/image.png)

**Fig. 10.** Mutations in ERα that affect N-CoR binding affect SERM activity at an AP-1-responsive reporter. **A**. HeLa transfection in which ERα activity was compared with ERαL379R and ERα537X at an AP-1-responsive reporter (Coll73-Luc). B. As in A except that the DBD-LBD truncation, missing AF-1, was used.

**Fig. 11.** The L379R mutation allows some AF-1 activity in the presence of ICI and raloxifene at the complement C3 promoter but decreases tamoxifen activation. Transfections were performed as in Fig. 5A except that the C3 complement promoter was used.

The diverse effects of SERMs on ERα interactions with N-CoR contribute to an emerging picture in which nuclear receptor antagonists exert diverse effects upon corepressor binding. The PPAR antagonist GW6471 promotes corepressor binding (58), whereas the TR antagonist NH-3 promotes corepressor release (59). PR antagonists show differential effects on corepressor binding (60). ERα residues that mediate corepressor binding (Leu-379 and others (42, 44, 45)) lie within the hydrophobic cleft. Estrogens permit docking of H12 against the lower part of the cleft and

N-CoR binding in vitro and examined their function in vivo. We found that a mutation (537X) that increases and equalizes N-CoR binding in the presence of SERMs eliminates AF-1 activity at simple EREs and AP-1 sites in vivo (42, 44). This idea is in line with observations that SERMs enhance ERα interactions with peptides corresponding to corepressor nuclear receptor-interacting domains in vitro (43, 44) and that mutations that influence ERα interactions with N-CoR or N-CoR-like peptides alter the ligand preference of ERα action in vivo (42, 44).

How can we reconcile the paradox that SERMs promote interactions with N-CoR in vivo, yet ERα only binds weakly to N-CoR in vitro? We suggest that ERα interactions with the N-CoR C terminus correspond to the ligand-dependent component of ERα/corepressor interactions, but that ERα also contacts other surfaces of the corepressor complex that augment corepressor recruitment. We also recognize that our binding assays may not faithfully recreate conditions that are required for maximal ERα/N-CoR interactions (perhaps ERα or N-CoR need specific modifications or cofactor interactions) and that our studies do not address the roles of N-CoR versus SMRT versus other repressors and certainly do not exclude the possibility that there are multiple repressors of ERα action. It will be interesting to ask whether ERα uses a similar surface to recruit other repressors, such as REA and HET/SAFb (56, 57).

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should prevent corepressor binding (Fig. 12, i). By contrast, SERMs displace H12. H12 can fold over in another position that exposes the H3-H5 region and promotes N-CoR binding (Fig. 12, iii). For this reason, the latter resembles the PPAR-SMRT structure (58) or the ERE-ICI structure (18). We therefore suggest that overall levels on N-CoR binding depend on equilibrium between H12 positions (ii and iii); tamoxifen would favor ii, ICI would favor iii, and raloxifene would permit both. Factors that might influence this equilibrium include SERM effects upon the length of the H11-H12 loop, which affect the ease of H12 docking in the cleft (14, 15), contacts between the SERM extension and either ERs or N-CoR, or unspecified effects of SERMs on core-LBD structure (see Fig. 5).

To what extent does differential corepressor binding account for SERM activity in vivo? The L379R mutation eliminates tamoxifen response at C3 (Fig. 11) and reduces tamoxifen response at EREs and AP-1 sites in HeLa cells (Figs. 9, 10). Thus, SERMs displace H12. H12 can fold over the cleft as in published ER-SERM crystal structures (Fig. 12, i). By contrast, SERMs displace H12. H12 can fold over the cleft as in published ER-SERM crystal structures (Fig. 12, i). By contrast, SERMs displace H12. H12 can fold over the cleft as in published ER-SERM crystal structures (Fig. 12, i). By contrast, SERMs displace H12. H12 can fold over the cleft as in published ER-SERM crystal structures (Fig. 12, i). By contrast, SERMs displace H12. H12 can fold over the cleft as in published ER-SERM crystal structures (Fig. 12, i).

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