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Chemical-based translational induction of luciferase expression: an efficient tool for the *in vivo* screening of protein farnesylation inhibitors

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running title: *luciferase translation managed by protein farnesylation*

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ABBREVIATIONS ; CAAX : isoprenylation boxe of a protein (C :cystein ; A : aliphatic amino-acid ; X : serine or methionine). eIF-4G : eukariotic initiation factor-4G. FTI ; farnesyl transferase inhibitor. PBPE : 1-benzyl-4(2-pyrrolidinylethoxy)benzene. AEBS : antiestrogen binding site

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Abstract

We describe the development of a cell system for the *in vivo* screening of inhibitors of the mevalonate pathway. To this aim we have constructed a bicistronic mRNA, transcribed from a constitutive CMV promoter, containing the *Renilla* luciferase RNA open reading frame sequence as first cistron and the *Firefly* luciferase RNA sequence as a second cistron. The inter cistronic space is made of the R17 binding sequence of the bacteriophage R17 protein. Translation of the second cistron is switched on by a chimeric protein able to bind to a specific sequence in the hairpin and to induce internal ribosome entry in the RNA. This chimeric protein is made up of the bacteriophage RNA binding domain (R17) fused to the ribosome recruitment core of the eIF-4G1 eukaryotic translation initiation factor and to the CAAX box of H-Ras addressing the protein to the plasma membrane where it is not efficient.. Internal ribosome entry upstream of the *Firefly* cistron is therefore under the dependence of the mevalonate pathway inhibitors Indeed products able to inhibit protein farnesylation, rescue the cytoplasmic location of the R17-eIF4G-CAAX protein which once more becomes a translation factor for the expression of the second cistron. To exemplify the system the present work checks the ability of various antiestrogens to interfere with the mevalonate pathway. It appears that pure antiestrogen, able to selectively bind the estrogen receptor, is unable to switch on the second *Firefly* cistron although selective antiestrogen-binding-site ligands are able to.

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In the last ten years of drug development, the mevalonate pathway has become an important target for pharmacological research. This pathway appears to play a key role in cellular proliferation and transformation, providing cells with a number of essential products including sterols, steroids, ubiquinone, isoprenoids, etc. Adjustment of this pathway has provided various efficient drugs including some to treat cardiac disease with the statin family of drugs and cancer with farnesyl transferase inhibitors (Karp et al., 2001; Yamamoto et al., 2003). Until now the drugs acting on this pathway had been first screened by enzymatic *in vitro* studies. Here, an *in vivo* screening is described for the determination of drugs hindering protein isoprenylation. It is based on the fact that isoprenylation occurs post-translationally regulating proteins' biological activities by governing their cellular location (Choy et al., 1999). This work uses a previously described system (Boutonnet et al., 2004) involving inhibition of farnesyl-transferase induced translational control of luciferase gene expression. Briefly, a chimeric protein made up with the C-terminal region of eIF4G1, the RNA binding domain of the bacteriophage R17 coat protein and the carboxy terminal region of H-Ras is, when addressed to the plasma membrane after farnesylation, unable to activate translation of the second cistron reporter gene of a bicistronic RNA containing an R17 binding sequence (scheme 1A). In the presence of farnesyl transferase inhibitor the chimeric protein becomes cytoplasmic and its translation initiation property is rescued (Boutonnet et al., 2004)(scheme 1B). The screening system developed here will allow the detection of drugs hindering the mevalonate pathway. Removal of the drug's effect by introduction of the lacking metabolite will reveal which enzyme is inhibited by the drug along the mevalonate pathway. The gene encoding the R17-eIF4G-CAAX chimeric protein was permanently transfected into HeLa cells, the best responding clone to lovastatin was selected with transient transfection of the bicistronic mRNA and characteristic activities of the farnesyl transferase inhibitor (FTI 277)

were determined. We used this clone to evaluate the potential cross-talk between various classes of antiestrogens with the mevalonate pathway.

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Materials and Methods

Plasmids have been previously detailed (Boutonnet et al., 2004)

Cell culture, construction of permanent cell line. Human cervical epithelial cells (HeLa) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1 mg/ml G418 (for stable cell clones) in a 10% CO_2 incubator at 37°C. Transfections were performed with the Fugene 6 reagent (Roche) according to the manufacturer's instructions. Clones were checked for their efficacy after three weeks under G418 selection

Chemical. FTI-277 was purchased from Calbiochem, lovastatin, mevalonate (mevalolactone), tamoxifen were from Sigma, ICI 182,780 from Tocris, PBPE was synthesized in our lab (Poirot et al., 2000) estradiol (E_2) was from Steraloid.

Transient transfections and treatments. G418 selected clones (producing R17-eIF4G-CVLS) were transiently transfected with plasmid, encoding the bicistronic mRNA (pCRL), using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were treated with the various drugs (FTI 277, lovastatin, antiestrogens) 24h after transient transfection. Cells were then harvested and luciferase assays were performed with the Dual Luciferase assay kit (Promega) as recommended by the manufacturer. The statistical Z' value was determined as described by Zhang (Zhang et al., 1999)

Western blots. On completion of the experiments, HeLa cell monolayers were washed and scraped into 100 µl of ice-cold phosphate-buffered saline (Biowittaker) and pelleted at 1800x g for 10 min. The pellets were then supplied with 100µl of lysis buffer (tris HCl 20mM (pH 7.4) SDS 2%) at 90°C. After sonication, protein concentrations were determined and loading buffer (containing SDS, glycerol, Coomassie blue) was added and equal amounts of protein were submitted to SDS-PAGE (10%) analyses. After electro-transfer onto nitrocellulose membrane, proteins were visualized using the ECL+ detection system (Amersham Pharmacia

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Biotech) after incubation overnight at 4°C for the primary antibody anti HDJ 2 (mouse HDJ-2 Ab1 Neo Markers) and 1 h at room temperature for the secondary anti-mouse horseradish peroxidase antibody (Santa Cruz)

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Results

In a recent publication we described stably transfected HeLa cells with the artificial R17-4G-CAAX translational factor that would be an efficient biological recipient to test the activities and specificity of isoprenyl transferase inhibitors (Boutonnet et al., 2004). The advantage of our system is that R17-4G protein is composed of artificial peptidic domains that recognized artificial RNA sequence, not found in eucaryotic cells, avoiding interference in the cellular functions.

Choice of the recipient clone.

A high number of G418 resistant clones were obtained: we selected cell clones with low expression of the second cistron (high ratio LucR/LucF) in the absence of FTI 277 and high expression (high ratio LucF/LucR) in its presence (Fig 1). From these criteria we chose clone 12, obtained with R17-4G-CVLS able to respond to inhibition of the farnesylation of the translational factor when transiently transfected with the plasmid producing the bicistronic RNA reporter. Clone 12 gave homogeneous and reproducible translation response up to 20 passages after stable transfection. In this clone, expression of the artificial R17-4G-CVLS chimeric protein is so low that it cannot be detected by a current anti HA Western blot with 50µg of total protein loaded (data not shown). We controlled that R17-4G-CVLS cellular expression did not interfer with the prenylation of farnesylated protein HDJ-2 (see Fig 6) and Ras (data not shown) or geranylgeranylated proteins such as RhoA or Rap1A (data not shown) . The transient transfection efficiency of the bicistronic mRNA (pCRL) was monitored by the first cistron (lucR) expression. Studies were followed at completion when lucR was comprised between 120,000 and 240,000 RLU.

Kinetic studies.

Inhibition of the farnesylation of HRas or HDJ2 are actually commonly used reporters for checking the *in vivo* efficiency of farnesyl-transferase inhibitors: treatment lasts at least 24

hours and then Western blot assay is required. Here, only after four hours treatment a value of the activity is accurately determined (Fig. 2). Maximum activity is obtained after 8 hours of treatment in the presence of FTI 277, although an increase of the LucF/LucR ratio is detected from two hours treatment. This observation suggests that the efficiency of FTI-277 on the neofarnesylation of protein is faster than previously thought suggesting that the transcriptional estrogen like activities of FTI we previously observed (Doisneau-Sixou et al., 2003a), and appearing at least 30 hours after treatment, do not result from a direct activation of the transcription but presumably from lifting an inhibition which depends on the half-life of farnesylated protein(s).

Dose effect and reversibility

Dose effects were determined at 8 hours treatment. The maximum effect of FTI was obtained at 0.5 μM and a measurable activity was seen from 0.1μM (Fig3) although its in-use concentrations are between 5 to 20 μM. lovastatatin activity appeared from 2.5 μM and was maximum at 20 μM, as shown Fig3 . Its activity was completely inhibited by mevalonate at 100 μM which had no effect on FTI-277. These results validated the cell system and showed that low concentrations of inhibitor exhibited detectable activity in short time. Moreover the inhibitory power of the molecule was easily quantifiable.

We decided to take advantage of the system to go further into the study of the mechanism of the “cross talk” we had already described between farnesyl transferase inhibitors and tamoxifen (Doisneau-Sixou et al., 2003b). Although it was previously reported that estradiol bound to the estrogen receptor enhances HMGR expression (Di Croce et al., 1999) we checked here its efficiency on HeLa cells which *a priori* do not possess estrogen receptors (Kuiper et al., 1997). It is shown in Fig 4 that the antiestrogen tamoxifen and PBPE a selective antiestrogen-binding-site (AEBS) ligand (Delarue et al., 1999) are able, after 18h treatment, to increase the LucF/LucR ratio. However kinetic studies showed that measurable

effects are obtained after 8 hours for PBPE and 18 hours for tamoxifen. As expected, estradiol had no effect and was unable to counteract the tamoxifen effect. Pure antiestrogen ICI 182,780 (estrogen receptor selective) was ineffective. Mevalonate added to the culture medium was not able to completely abolish tamoxifen or AEBS ligand activities (Fig 5) suggesting that part of the AEBS ligand activities is directed against HMGR. Moreover as seen in (Fig 5) tamoxifen is already active at 1 µM, a concentration below its antiproliferative efficiency on HeLa cells (around 1µM on estrogen receptor positive cells and 10µM on the others) (Delarue et al., 1999). The high Z' factor values (Z'> 0.59 from tamoxifen concentration >5µM) and low coefficients of variation evidence that tamoxifen can be included in the family of the mevalonate pathway inhibitors. Western blot analysis of HDJ2 farnesylation is shown in Fig 6, after 24 hours treatment: (lane 1) ICI 182,780 had no effect on HDJ2 farnesylation, (lanes 6-7) PBPE induced a low effect that was not reversed by estradiol and (lane 8) tamoxifen (1µM) had no significant effect on HDJ2 prenylation. These results are in good agreement with those obtained with ICI 182,780 in Fig.5 and we can conclude that the selective estrogen ligand does not interfere with the mevalonate pathway. However, for the selective AEBS ligand PBPE, the sensitivity of the bicistronic system is higher than the one obtained with Western blot, indicating that a slight effect on farnesylation producing a few unprenylated R17-eIF-4G-CAAX proteins would induce a strong *Firefly* response of the bicistronic system. Moreover, whereas tamoxifen at 10µM did not induce any modification of HDJ2 farnesylation (as seen by western blot), we were able to detect inhibition of the farnesylation (with our system) for a 48-hour teatment at 50µM (data not shown).

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Discussion

Although the mevalonate pathway has become an important target in pharmacological research only biochemical tools are available for the screening of any new drugs. Using our previous work (Boutonnet et al., 2004) we have developed an *in vivo* screening system for the determination of drugs interfering with the mevalonate pathway. Reversal of the drug effect by mevalonate metabolites could reveal the point at which the test molecule inhibits the pathway. In this work the translational control, under farnesyl protein inhibitor, of the second cistron of a bicistronic mRNA was used to define a new estrogen receptor independent activity of tamoxifen that can be related to its interaction with the AEBS complex (Kedjouar et al., 2004). It appears that the ER selective antiestrogen ICI 182,780 has no effect on the translation of the *Firefly* luciferase second cistron although AEBS ligand (PBPE) exhibits a strong effect. Tamoxifen (which binds ER and AEBS with equivalent K_D) is also able to induce the translation of the second cistron revealing its influence on the protein farnesylation processs, By comparison with HDJ2 western blot analysis it appears that this new system is highly sensitive and is able to detect an inhibiting effect of tamoxifen on the mevalonate pathway. This tamoxifen effect showing a new dissociation in the tamoxifen estrogen receptor dependent and independent activities (Reddel et al., 1985) was not clearly demonstrated by Western blot analysis needing drastic treatments to be detected at all. The system appears to be quickly adaptable to high throughput screening for molecules involved in the mevalonate pathway. Moreover, changes of the farnesylated CAAX (CVLS) box of R17-eIF4G to a geranyl-geranylated box (Boutonnet et al., 2004) would permit the screening of molecules able to inhibit protein geranyl-geranylation.

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Legends for Figures

Fig 1 - Selection of stable R17-EiF4G-CAAX transfected clones. G418 selected clones were transiently transfected with the LucR/LucF bicistronic reporter gene. White bars correspond to the LucR/LucF ratio 48 hours after transfection, black bars correspond to the increase of the LucF/LucR ratio in the presence of 5µM FTI 277(24 hours)

Fig 2 - Kinetic studies on clone 12. 24 hours after transient transfection of the LucR/LucF bicistronic reporter gene, drug was added to the medium and LucF/LucR ratio was determined at various times. FTI 277 results are expressed as induction of the second cistron (LucFT after FTI treatment/ $LucF_0$ without treatment) and are representative of six independent experiments in triplicate. Z'= 0.647 (8h), Z'= O.62 (16h), Z'= 0.6 (24h)

Fig 3 - Dose effect of FTI 277 and lovastatin. 8-hour treatments were performed with increasing concentrations of FTI 277 and lovastatin in the absence or presence of mevalonate 100µM. 0.25µM< FTI 277< 2µM : 0.64< Z'< 0.78

Fig 4 - Kinetic studies of antiestrogen effects. 24 hours after transient transfection, drugs were added and the LucF/LucR ratio was determined at various times. Tamoxifen and PBPE were used at 10 µM.

Fig 5 – Dose effect of antiestrogens. Pure antiestrogen ICI 182,780, tamoxifen and selective AEBS ligand PBPE were added to the culture medium for 18 hours in the presence or absence of estradiol (100 nM). 2µM< PBPE< 10µM: 0.2< Z'< 0.61

Fig 6 – Western blot of HDJ 2. HeLa cells were treated for 24 hours with various molecules, then lysed at 90°C in Tris buffer (10mM) containing 2% SDS. Lysates were sonicated (to break DNA) and 10 µg of protein were submitted to SDS-PAGE. The HDJ 2 protein was detected on nitrocellulose filter with specific antibody against both farnesylated and unprenylated HDJ 2.

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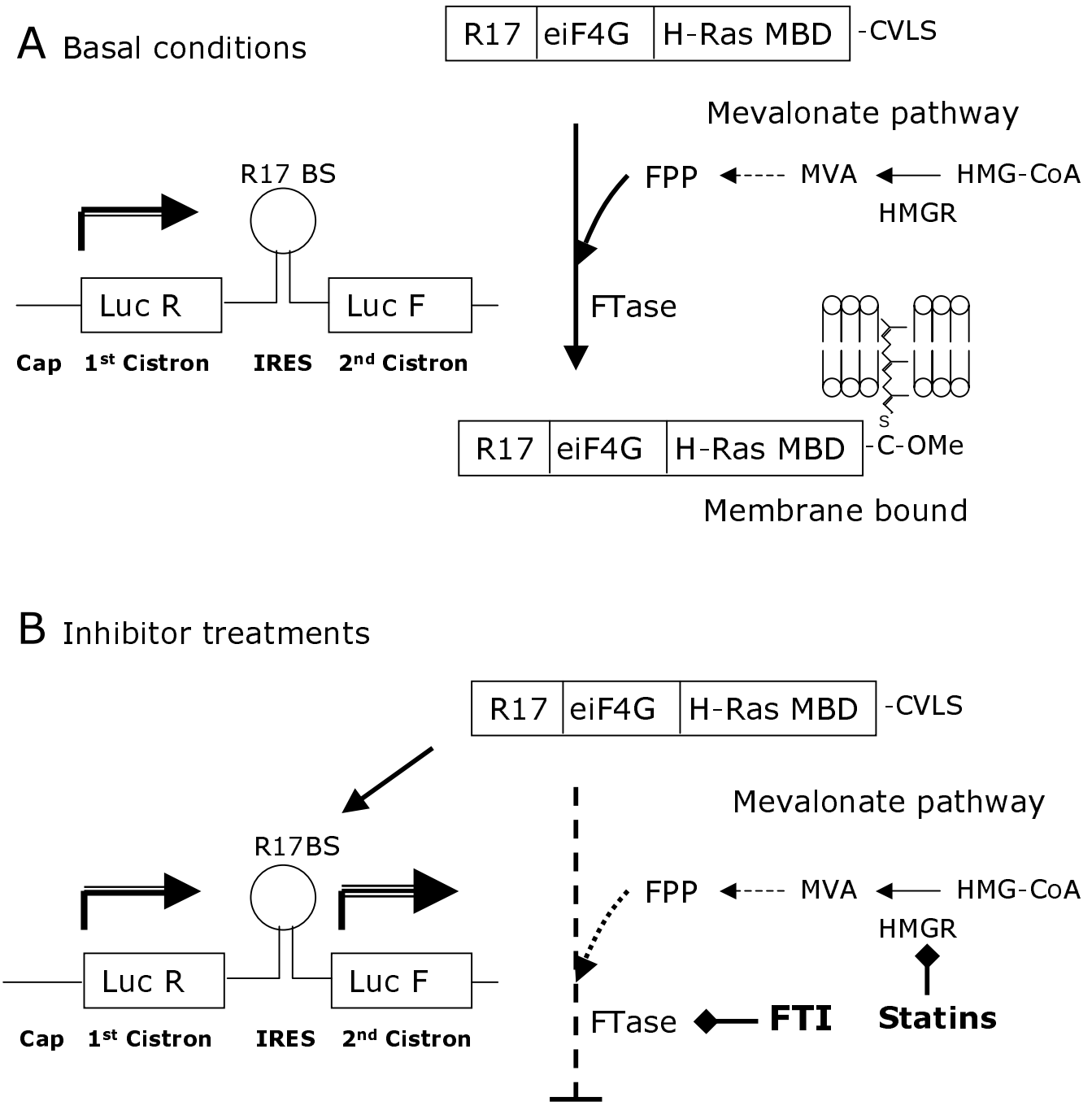


Scheme . Regulation of the translation of the second cistron by farnesylation inhibitor – A) The farnesylated R17-eIF4G chimeric protein is located in the plasma membrane and is unable to promote internal ribosome entry involved in the second cistron translation.-B) In the presence of inhibitor of the mevalonate pathway R17-eIF4G is bound to the R17 RNA domain and allows translation of the second cistron (Luc F) by internal ribosome entry.

Figure 1

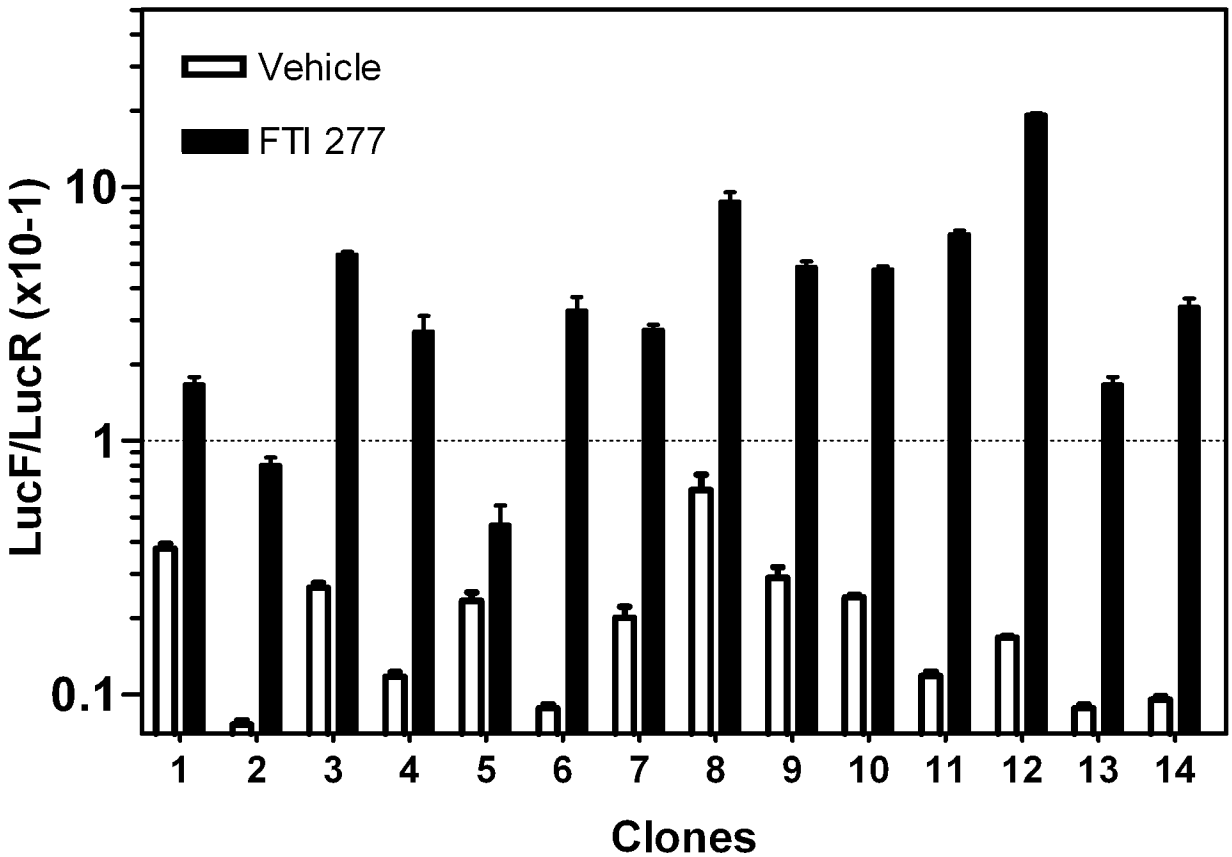

Vehicle
FTI 277
LucF/LucR (x10-1)
10
1
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Clones

Figure 2

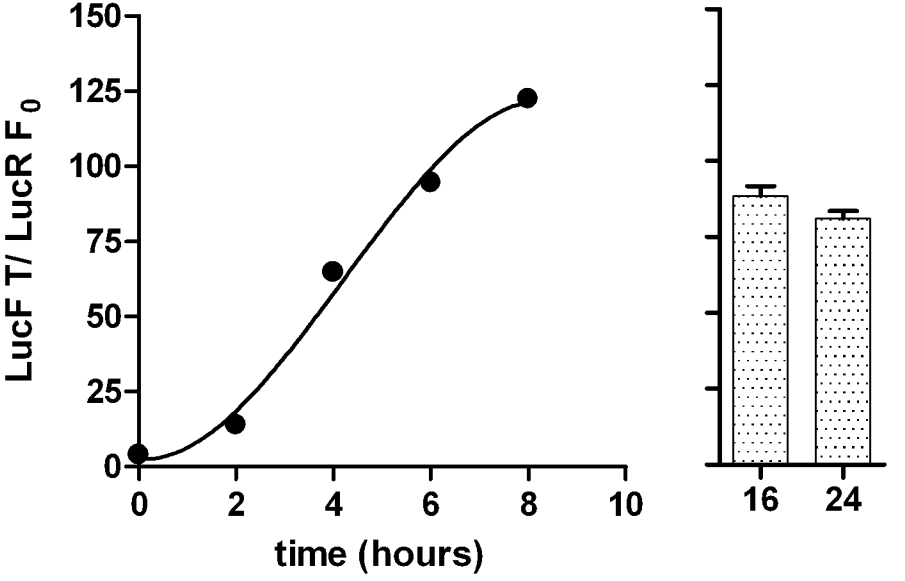


Figure 3

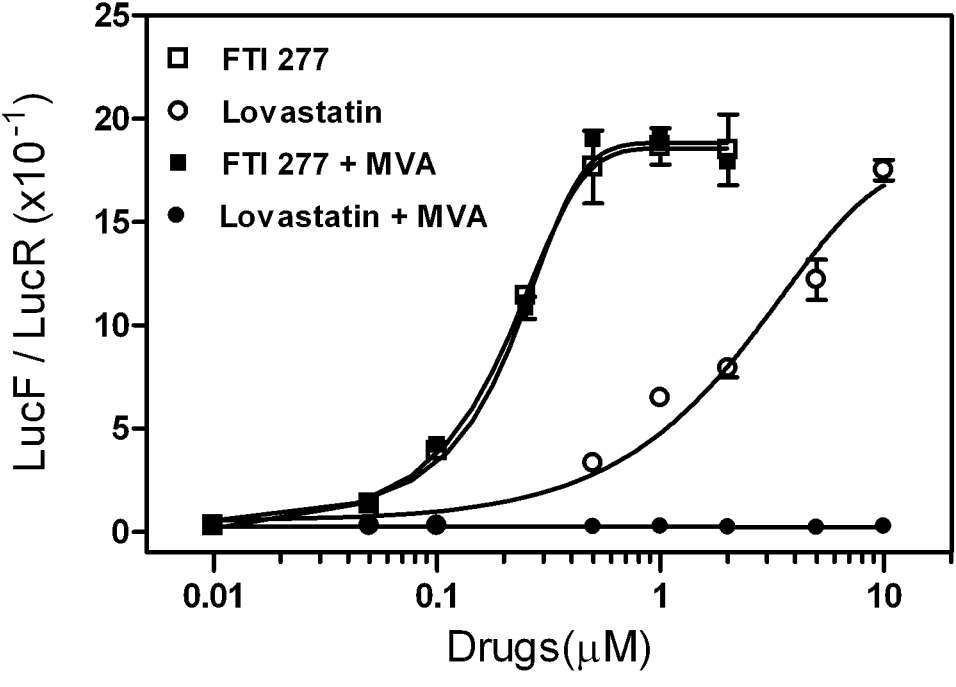

25
20
15
10
5
0
LucF / LucR (x10^{-1})
FTI 277
Lovastatin
FTI 277 + MVA
Lovastatin + MVA
0.01
0.1
1
10
Drugs(μM)

Figure 4

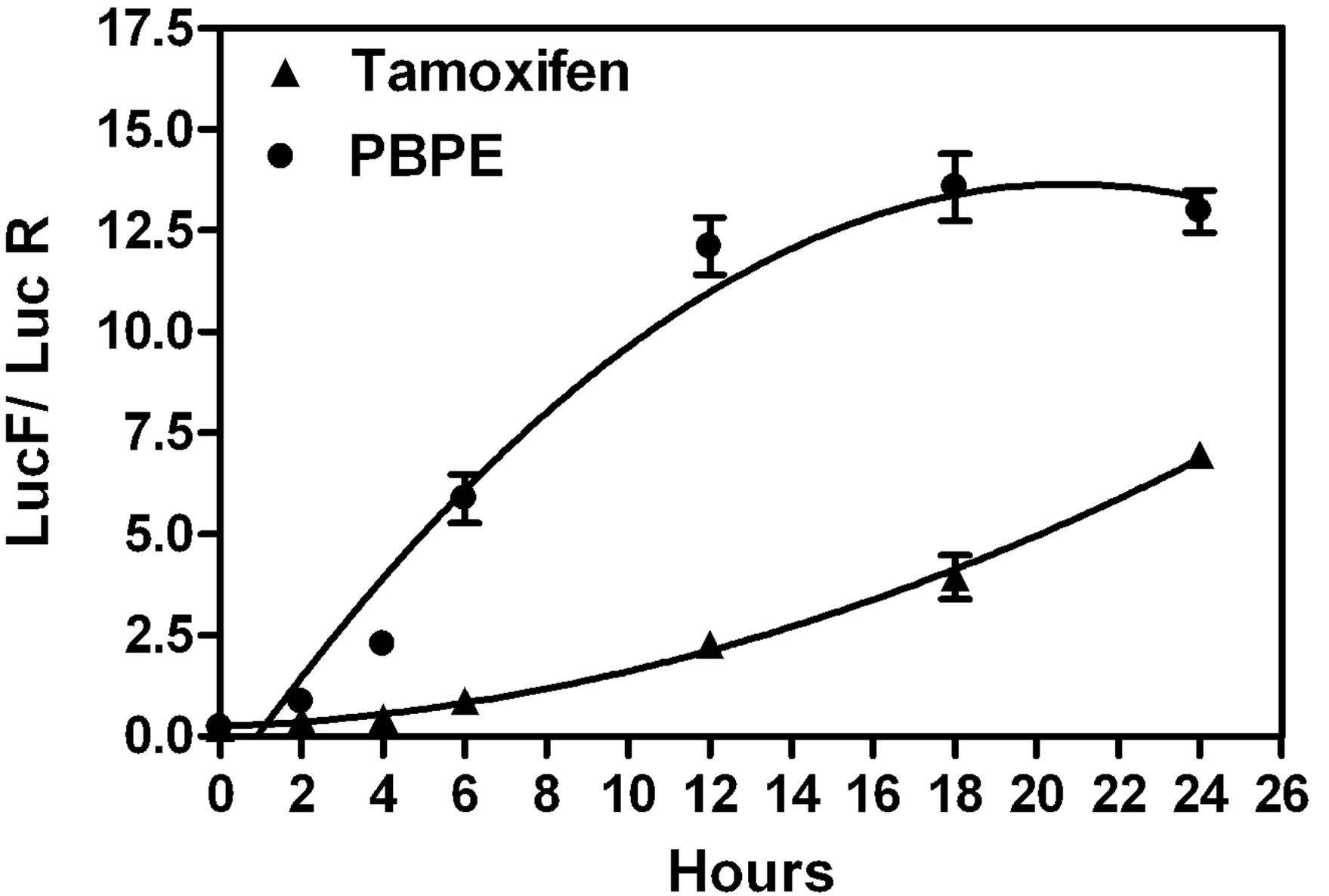
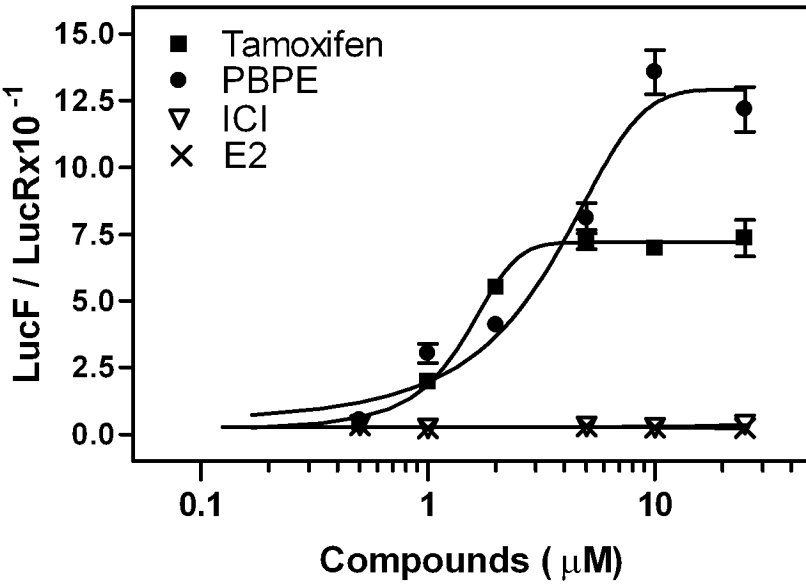

17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
LucF/ Luc R
Tamoxifen
PBPE
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Hours

Figure 5


Tamoxifen
PBPE
ICI
E2
LucF / LucRx10-1
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0.1
1
10
Compounds (μM)

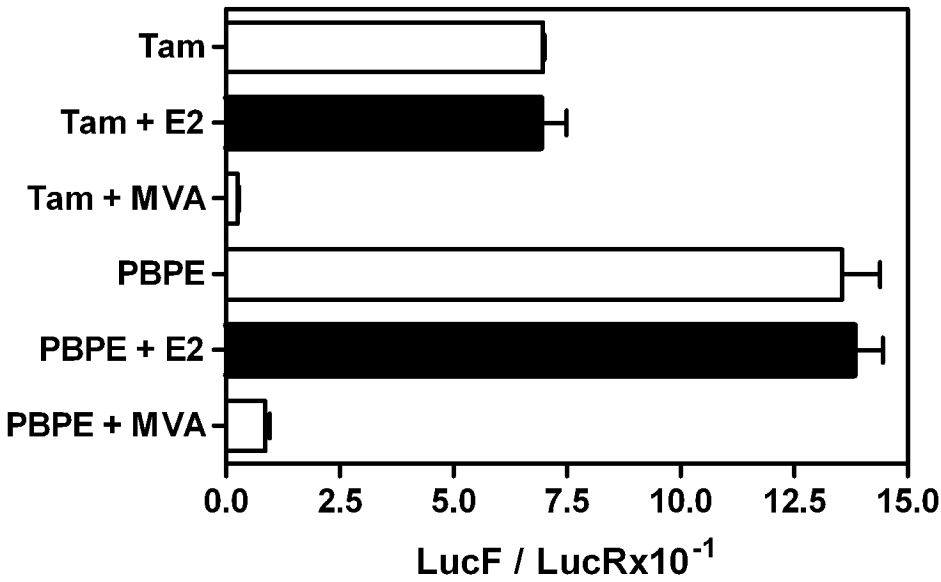
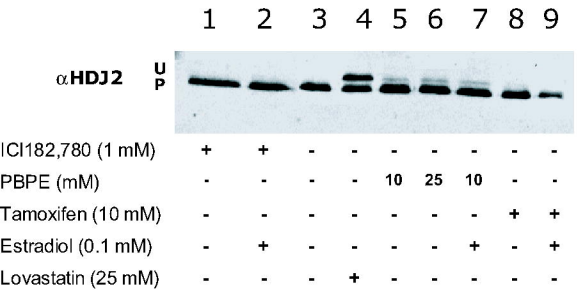

Tam
Tam + E2
Tam + MVA
PBPE
PBPE + E2
PBPE + MVA
0.0
2.5
5.0
7.5
10.0
12.5
15.0
LucF / LucRx10-1

Figure 6


1 2 3 4 5 6 7 8 9
αHDJ2
UP
ICI182,780 (1 mM) + + - - - - - - -
PBPE (mM) - - - - 10 25 10 - -
Tamoxifen (10 mM) - - - - - - - + +
Estradiol (0.1 mM) - + - - - - + - +
Lovastatin (25 mM) - - - + - - - - -