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Chemical-Based Translational Induction of Luciferase Expression: An Efficient Tool for in Vivo Screening of Protein Farnesylation Inhibitors

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ABSTRACT

We describe the development of a cell system for in vivo screening of inhibitors of the mevalonate pathway. To this aim, we have constructed a bicistronic mRNA, transcribed from a constitutive cytomegalovirus promoter, containing the *Renilla reniformis* luciferase RNA open reading frame sequence as first cistron and the *Firefly* luciferase RNA sequence as a second cistron. The intercistronic space is made of the R17 binding sequence of the bacteriophage R17 protein. A chimeric protein able to bind to a specific sequence in the hairpin and to induce internal ribosome entry in the RNA switches on translation of the second cistron. 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 that are able to inhibit protein farnesylation rescue the cytoplasmic location of the R17-eIF-4G-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 seems 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 do so.

In the last 10 years of drug development, the mevalonate pathway has become an important target for pharmacological research. This pathway seems 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 treatments for

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. In this report, 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. In brief, when a chimeric protein made up with the C-terminal region of eIF4G1,

ABBREVIATIONS: CAAX, isoprenylation box of a protein (C, cysteine; A, aliphatic amino acid; X, serine or methionine); eIF-4G, eukaryotic initiation factor-4G; FTI, farnesyl transferase inhibitor; PBPE, 1-benzyl-4(2-pyrrolidinylethoxy)benzene; AEBS, antiestrogen binding site; ICI 182,780, faslodex.

the RNA binding domain of the bacteriophage R17 coat protein, and the carboxyl-terminal region of H-Ras is addressed to the plasma membrane after farnesylation, it is 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 2). 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-eIF-4G-CAAX chimeric protein was permanently transfected into HeLa cells, the clone best responding 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.

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 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, Mannheim, Germany) according to the manufacturer's instructions. Clones were checked for their efficacy after 3 weeks under G418 selection.
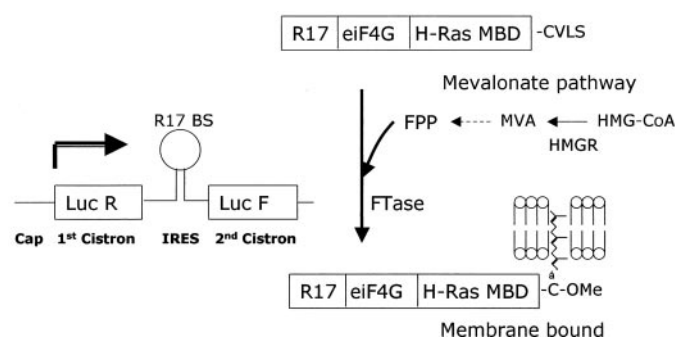
Scheme 1. Basal conditions. Regulation of the translation of the second cistron by farnesylation inhibitor. The farnesylated R17-eIF4G chimeric protein is located in the plasma membrane and is unable to promote internal ribosome entry involved in the second cistron translation.
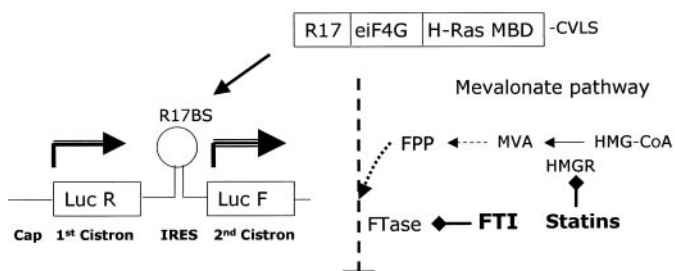
Scheme 2. Inhibitor treatments. In the presence of inhibitor of the mevalonate pathway, R17-eIF4G is bound to the R17 RNA domain and allows translation of the second cistron (Luc F) by internal ribosome entry.

Chemicals. FTI-277 was purchased from Calbiochem (San Diego, CA); lovastatin, mevalonate (mevalolactone), and tamoxifen were from Sigma-Aldrich (St. Louis, MO); ICI 182,780 was from Tocris Cookson Inc. (Bristol, UK); PBPE was synthesized in our lab (Poirot et al., 2000); and estradiol was from Steraloids (London, UK).

Transient Transfections and Treatments. G418 selected clones (producing R17-eIF-4G-CVLS) were transiently transfected with plasmid, encoding the bicistronic mRNA (pCRL), using LipofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were treated with the various drugs (FTI-277, lovastatin, antiestrogens) 24 h 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 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 (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) and pelleted at 1800g for 10 min. The pellets were then supplied with 100 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, and 2% SDS) at 90°C. After sonication, protein concentrations were determined and loading buffer (containing SDS, glycerol, and Coomassie blue) was added and equal amounts of protein were submitted to SDS-polyacrylamide gel electrophoresis (10%) analyses. After electro-transfer onto a nitrocellulose membrane, proteins were visualized using the ECL+ detection system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) after incubation overnight at 4°C for the primary antibody anti-HDJ 2 (mouse HDJ-2 Ab1; Neo Markers; Interchim, Montluçon, France) and 1 h at room temperature for the secondary anti-mouse horseradish peroxidase antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

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 of LucR to LucF) in the absence of FTI-277 and high expression (high ratio of LucF to LucR) in its presence (Fig. 1). From these criteria we chose clone 12, obtained with R17-4G-CVLS and 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 50 μg of total protein loaded (data not shown). We controlled that R17-4G-CVLS cellular expression did not interfere 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 comprised between 120,000 and 240,000 relative light units.

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 h and then Western blot assay is required. Here, after only 4 h treatment, a value of the activity was accurately determined (Fig. 2). Maximum activity is obtained after 8 h of treatment in the presence of FTI-277, although an increase of the LucF/LucR ratio is detected from 2-h treatment. This observation implies 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 observed previously (Doisneau-Sixou et al., 2003a), and appearing at least 30 h after treatment, do not result from a direct activation of the transcription but presumably from lifting an inhibition that depends on the half-life of farnesylated protein(s).

Dose Effect and Reversibility. Dose effects were determined at 8 h of treatment. The maximum effect of FTI was obtained at 0.5 μM, and a measurable activity was seen from 0.1 μM (Fig. 3), although its in-use concentrations are between 5 and 20 μM. Lovastatin activity appeared from 2.5 μM and was maximum at 20 μM, as shown Fig. 3. 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 a 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 18 h of treatment, to increase the LucF/LucR ratio. However, kinetic studies showed that measurable effects are obtained after 8 h for PBPE and 18 h for tamoxifen. As expected, estradiol had no effect and was unable to counteract the tamoxifen effect. Pure antiestrogen ICI
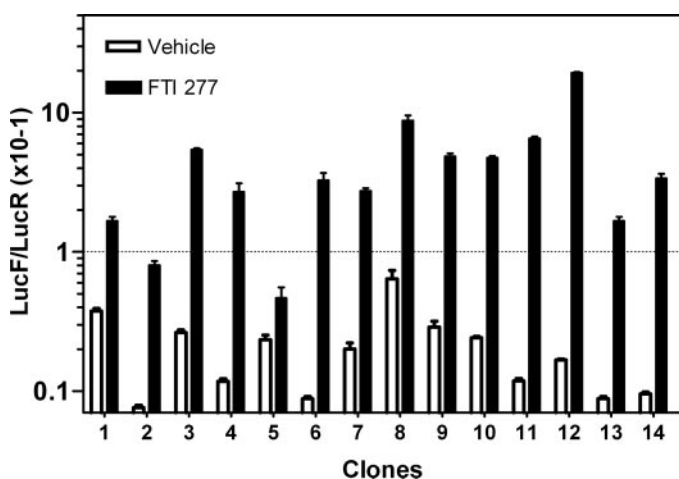
182,780 (estrogen receptor selective) was ineffective. Mevalonate added to the culture medium was not able to completely abolish tamoxifen or AEBS ligand activities (Fig. 5), suggesting that part of the AEBS ligand activities is directed against *HMGR*. Moreover, as seen in Fig. 5, tamoxifen is already active at 1 μM, a concentration below its antiproliferative efficiency on HeLa cells (around 1 μM on estrogen receptor positive cells and 10 μM on the others) (Delarue et al., 1999). The high Z' factor values (Z' > 0.59 from tamoxifen concentration > 5 μM) and low coefficients of variation provide evidence that tamoxifen can be included in the family of
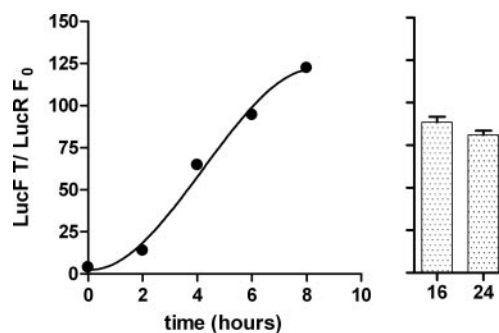
Fig. 2. Kinetic studies on clone 12. 24 h after transient transfection of the LucR/LucF bicistronic reporter gene, drug was added to the medium and LucF/LucR ratio was determined at various times. FTI-277 results are expressed as induction of the second cistron (LucFT after FTI treatment/ $LucF_0$ without treatment) and are representative of six independent experiments in triplicate. Z' = 0.647 (8 h), Z' = 0.62 (16 h), Z' = 0.6 (24 h).
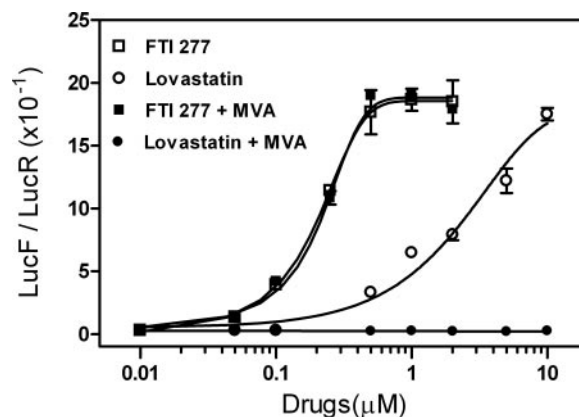
Fig. 3. Dose effect of FTI-277 and lovastatin. Eight-hour treatments were performed with increasing concentrations of FTI-277 and lovastatin in the absence or presence of mevalonate 100 μM. 0.25 μM < FTI-277 < 2 μM: 0.64 < Z' < 0.78.

Fig. 4. Kinetic studies of antiestrogen effects. 24 h after transient transfection, drugs were added and the LucF/LucR ratio was determined at various times. Tamoxifen and PBPE were used at 10 μM.
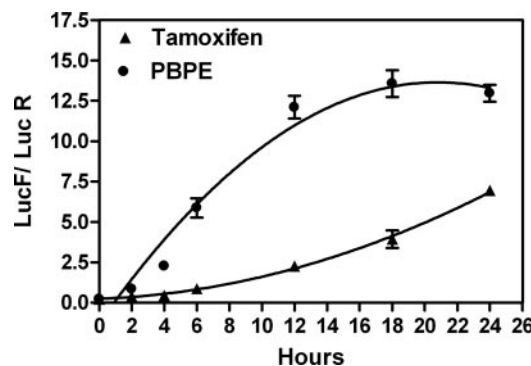
Fig. 1. Selection of stable R17-EiF4G-CAAX transfected clones. G418 selected clones were transiently transfected with the LucR/LucF bicistronic reporter gene. White bars correspond to the LucR/LucF ratio 48 h after transfection, black bars correspond to the increase of the LucF/LucR ratio in the presence of 5 μM FTI 277 (24 h).

the mevalonate pathway inhibitors. Western blot analysis of HDJ2 farnesylation is shown in Fig. 6; after 24-h treatment, ICI 182,780 had no effect on HDJ2 farnesylation (lane 1), PBPE induced a low effect that was not reversed by estradiol (lanes 6-7), and tamoxifen (1 μM) had no significant effect on HDJ2 prenylation (lane 8). 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 to the bicistronic system. Moreover, whereas 10 μM tamoxifen 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-h treatment at 50 μM (data not shown).

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
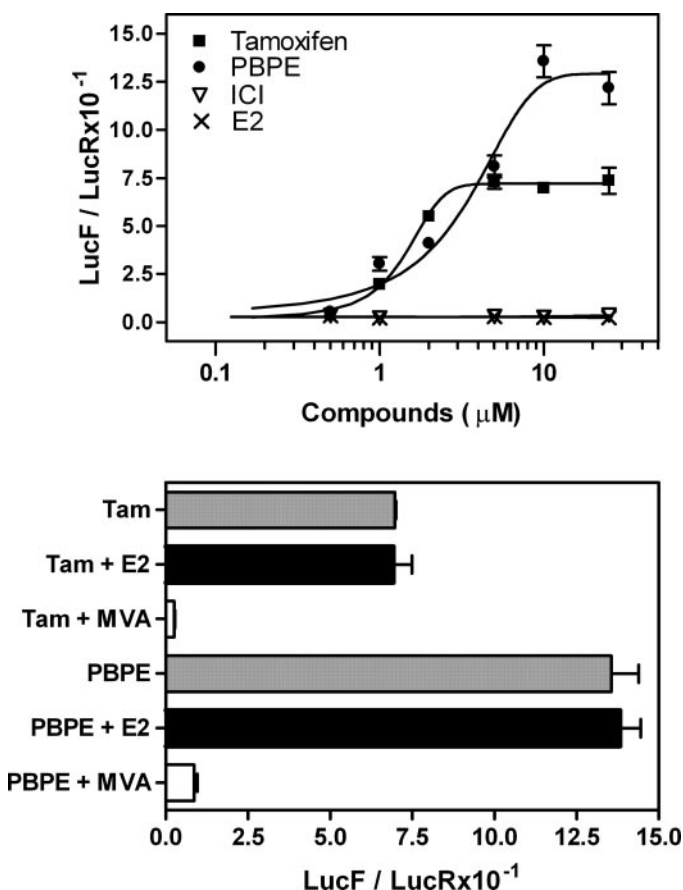
Fig. 5. Dose effect of antiestrogens. Pure antiestrogen ICI 182,780, tamoxifen, and selective AEBS ligand PBPE were added to the culture medium for 18 h in the presence or absence of estradiol (100 nM). 2 μM < PBPE < 10 μM: 0.2 < Z' < 0.61.
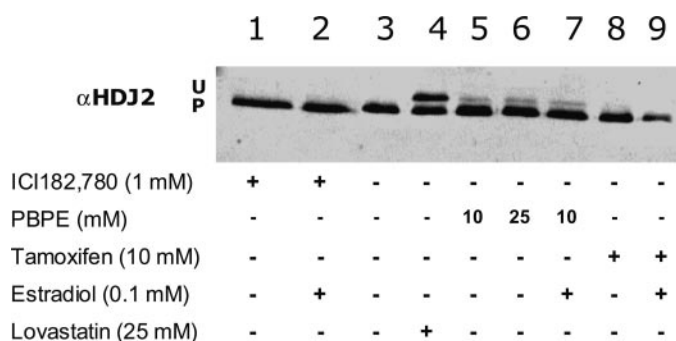
Fig. 6. Western blot of HDJ 2. HeLa cells were treated for 24 h with various molecules, then lysed at 90°C in Tris buffer (10 mM) containing 2% SDS. Lysates were sonicated (to break DNA), and 10 μg of protein were submitted to SDS-polyacrylamide gel electrophoresis. The HDJ 2 protein was detected on nitrocellulose filter with specific antibody against both farnesylated and unprenylated HDJ 2.

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 seems 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 seems 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 seems 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-eIF-4G 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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