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## **Tamoxifen resistance and epigenetic modifications in breast cancer cell lines**

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## ABSTRACT

Epigenetic mechanisms play crucial roles in many processes, including neoplasia, genomic imprinting, gene silencing, differentiation, embryogenesis and X chromosome inactivation. Their relevance in human disease and therapy has grown rapidly with the recent emergence of drugs that target for example DNA methylation or histone acetylation. Epigenetic effects were also recently highlighted by the deciphering of the mechanism of action of steroid hormones and anti-hormones acting through nuclear receptors. In this review, we focus on the epigenetic effects associated with long-term treatment of breast cancer cells with the antiestrogen (AE) tamoxifen, in the context of resistance appearance. We summarize the data obtained with a model cell line developed in our laboratory supporting a role for HP1 proteins in the irreversible inactivation of gene expression by long-term treatment with AE.

**Key Words :** epigenetic, antiestrogen, tamoxifen, MCF-7, silencing, HP1.

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## **1-AE and resistance: facts and models**

The antiestrogen tamoxifen is the drug most often used for the long-term treatment of early breast cancer [1]. However, a serious limitation of this endocrine therapy is the inevitable appearance of resistance, which either occurs *de novo* or is acquired after several months of treatment. The main feature of *de novo* resistance is the lack of estrogen receptor (ER) alpha expression. However, about 70% of breast tumors express ER $\alpha$ , and among them 70-80% respond positively to tamoxifen treatment [2]. Acquired resistance to tamoxifen may be due to either a simple selection of cells from an already heterogeneous population or a two-stage process of cell alteration followed by cell selection due to the drug's cytostatic effect.

Several recent general reviews have focused on the different mechanisms that underlie this phenomenon, [3-7]. The hypotheses that have been investigated to date are presented in Figure 1 and principally concern: a) ER modifications (by mutation, alternative splicing or post-translational modifications like phosphorylation or acetylation) [3, 8]; b) coregulator dysfunction, (by alteration of their synthesis rate, or by post-translational modifications) [9]; c) interference with growth factor pathways by either phosphorylation induced by growth factor-stimulated kinases (which results in ligand-independent activation of ER), or non-genomic action of the ER itself on growth factor pathways [10-12]; and d) competition with intra-tumoral estrogens synthesized by either breast, ovary or peripheral adipose tissues [7, 13].; Other hypotheses concerning e) the hepatic metabolism (which generates estrogenic metabolites) and the biodisponibility (involving binding proteins like AEBS, or the mdr-1 channel) of tamoxifen were also investigated, but the results were conflicting and no conclusions about their contribution to resistance mechanisms could be drawn [7].

In most cases, these hypotheses were proposed on the basis of results obtained using different cellular models established after long-term culture in either estrogen-deprived conditions or the presence of AE.

### ***AE-resistant cell lines***

The resistant cell lines described in the literature are generally E2-independent and either tamoxifen and ICI<sub>182,780</sub> cross-resistant (LY2, ZR75-LCC3, MCF-7/LCC9, ZR-75-9a1, T47Dco), tamoxifen-stimulated and ICI<sub>182,780</sub>-resistant (MCF-7-WES), or tamoxifen resistant and ICI<sub>182,780</sub> responsive (MCF-7/LCC2) [7]. As an example, a series of resistant cell lines (T47D-r, MCF7-r, ZR-75-1-r) developed by Sommer was obtained after long-term cultivation of parent ER $\alpha$ (+) cell lines (T47D, MCF-7, ZR-75-1) in the pure antiestrogen ICI<sub>182,780</sub> (Faslodex) [14]. ER $\alpha$  protein expression was lost in the three cell lines with both estrogen-independent and ICI<sub>182,780</sub>-resistant behaviors. Concerning the cellular phenotypes of tamoxifen resistance, most were found to be simply refractory (no longer growth inhibited) to the drug. However, some of them like MCF-WES [15] and MCF/TOT [16] were found to be growth stimulated by tamoxifen, the former being cross-resistant to ICI<sub>182,780</sub> whereas the latter was not. However, such growth-stimulated phenotypes seem to occur in only a minority of patients [7]. Last, the diversity of the resistant phenotypes suggests that the regulation of many cellular pathways may be affected during the establishment of resistance.

Ten years ago, our laboratory developed the MVLN cell line [17, 18] which derives from MCF-7 cells. This stably transfected cell line contains the luciferase gene under the control of the palindromic estrogen response element (ERE) from the 5' flanking region of the *Xenopus* vitellogenin A2 gene, inserted in front of the *Herpes simplex* virus thymidine kinase promoter [17, 18]. Since their description, these cells have been used by many other laboratories (at present, about 50 references) for various studies requiring easy detection of transcriptional responses to E2. A major field of study concerns the detection of the endocrine disrupting activity of environmental contaminants with the following representative references : [19-25]. However, MVLN cells have also been used for more fundamental endocrine studies that will

be further developed in detail [26-31]. In particular, long-term AE treatment studies of MVLN led to the generation of clonal cell lines resistant to 4-hydroxytamoxifen (OHTam) obtained after either 3 or 6 months (Cl6.8 and Cl6.32) of AE treatment (Cl6.8 and Cl632 were slightly growth-stimulated by OHTam (data not shown)) [32, 33].

## **2- Modification of gene expression associated with AE resistance**

Different phenotypes have emerged from the studies on particular gene expressions in AE-resistant cell lines. This may reflect the fact that only a small number of genes has been analyzed, or it may indicate that multiple mechanisms can lead to resistance. Global gene expression analysis using genomic or proteomic approaches has established molecular portraits of human breast tumors [34] as well as ER $\alpha$ (-) or ER(+) cell lines [35, 36]. A recent profiling study of ER $\alpha$ -positive primary breast carcinomas, identified a 44 genes signature that discriminated tumors that were responsive to first line tamoxifen therapy from those that were resistant [37]. The genes included in the signature were related to the extracellular matrix, apoptosis or the immune system and they appeared to be more efficient than the commonly used traditional predictive factors. Another study compare the antiestrogen-responsive MCF-7/LCC1 and the cross-resistant (tamoxifen and ICI<sub>182,780</sub>) MCF-7/LCC9 cell lines using a novel algorithm for microarrays gene analysis. The authors found that changes had occurred in another signaling pathway (involving interferon regulatory factor-1, nucleophosmin, NF $\kappa$ B, and CRE binding), after ICI<sub>182,780</sub> treatment [38]. All these studies point to the diversity and complexity of the signaling pathways that can be altered after such prolonged treatments.

Another compelling study performed on T47D breast cancer cells sensitive to ICI<sub>182,780</sub>, and on the T47D-r-resistant cell line, used DNA chip hybridization in parallel with a proteomic approach [39]. Although clear differences were evidenced between the reproducible variations

of gene expression found with the two techniques, several genes such as cathepsin D (lysosomal aspartic protease), Rab11a (small GTPase), MxA (belonging to the dynamin superfamily of large GTPases) and hAG-2 (human homologue of the *Xenopus laevis* anterior gradient-2-protein) were found to vary in the same way (in T47D-r : down-regulation for cathepsin D, hAG-2, and Mx-A, and up-regulation for Rab11a) [39].

Gene expression was also analyzed by large scale measurement using nylon cDNA arrays in our tamoxifen-resistant cell lines (Cl6.8 and Cl6.32 mentioned above) [33]. The OHTam-resistant cells developed an increased sensitivity to growth stimulation by estradiol, which correlated with a dysregulation of gene expression. Indeed, the genes involved in cell cycle and proliferation control, DNA replication, genomic stability and DNA repair were up regulated by estradiol much more in the Cl6.8 and Cl6.32 resistant cell lines than in the parental MVLN sensitive cell line. Overall, 39 to 61% of gene expression variations (in Cl6.32 and Cl6.8 cell lines respectively) displayed a hyper-response to estradiol compared with the MVLN parent cell line. Interestingly, a high percentage of deregulated genes (86%) was found to be common to the two resistant cell lines.

More recently, a study using promoter CpG island microarrays for high-throughput analysis, highlighted the relationship between epigenetic modifications and tamoxifen or ICI<sub>182,780</sub> resistance [40]. In AE-resistant cells, hypomethylation of promoters regulating growth-stimulatory pathways was more frequent than hypermethylation-based effects, with clear differences between tamoxifen- and ICI<sub>182,780</sub>-resistant sublines.

Altogether, these global analyses showed that the expression of many genes is altered upon establishment of AE-resistant cell lines. Further studies are undoubtedly needed to achieve a pertinent classification of all these genes and to identify the clusters of genes most relevant to resistant tumors.



### **3-Antihormone treatment and epigenetic modifications**

Recent developments have linked epigenetic mechanisms to ER action (for recent reviews see [41, 42]. Concerning AE treatment, a link between epigenetic alterations (which influence the possibly heritable phenotype without altering the genotype) and resistance appearance was reported 12 years ago by Van Agthoven *et al.* [43]. In this study, ZR-75-1 cells were first treated for 3 days with 5-azacytidine, a DNA methylation inhibitor, to induce epigenetic modifications. Cells were then grown for 3 weeks in the presence of OHTam or the absence of estrogen. The number of resistant cell colonies was found to be very reproducible and increased along with the 5-azacytidine dose. More importantly, cells were both completely AE-resistant and estradiol-independent for proliferation. This pioneer study was supported by DNA-methylation analysis of AE-resistant MCF-7 cell lines [40]. Recently, a similar strategy was used to restore the tamoxifen sensitivity of ER $\alpha$ -negative MDA-MB-231 breast cancer cells [44]. Treatment with a combination of a methylation inhibitor (5-aza-dC), and a histone deacetylase inhibitor (trichostatin A) for short periods led to re-expression of the estrogen receptor and enhanced responsiveness to OHTam. Since the loss of estrogen receptor expression appears to be the dominant mechanism accounting for *de novo* resistance to tamoxifen (ER $\alpha$ -negative tumors behaving more aggressively), in principle, therapies based on ER-sensitization should be beneficial.

In addition, it has been well documented that long-term exposure to tamoxifen lead to genotoxic effects, a finding underlined by the interaction between DNA (adducts) and some AE metabolites. The chemical reactions involved in such modifications are still being investigated using different approaches [45, 46]. Among the different AEs that have been studied (toremifen, raloxifen, tamoxifen, OHTam, ICI<sub>182,780</sub>), tamoxifen was the most efficient to form bulky DNA adducts [47]. Concerning the biological effects of these DNA

modifications, their involvement in the emergence of endometrial cancer is not fully accepted [48, 49] and liver seems to be the preferential tissue for formation of the DNA adducts [50].

Interestingly, two recent studies by Tryndyack et al. pointed out that long-term exposure to tamoxifen led to both genotoxic (DNA adducts) and epigenetic (DNA hypomethylation) effects in rat liver [51, 52]. These effects may play a role in hepatic carcinogenesis, since the level of proteins involved in genomic stability (such as Rad51, Ku70 or DNA pol) was altered. These changes were associated with the progressive loss of promoter activity by CpG methylation for some repetitive elements of the line-1 family and for the c-myc proto oncogene which resulted in increased gene transcription.

### ***Recruitment of chromatin modification enzymes by liganded ER $\alpha$***

Transcription regulation by ER results from a dynamic interplay between the liganded receptor and multiple cofactors (for recent reviews [9, 53-56]). The dominant model holds that gene activation/inactivation promoted by ER is the result of a defined and complex sequential program initiated by recruitment of coactivators and corepressors [56, 57]. Agonist-bound ER mainly recruits cofactors that activate transcription such as members of the p160 family and p300/CBP. More surprisingly, cofactors with a repressive activity such as RIP140 [58] also interact with agonist-bound ER $\alpha$ . Similarly, OHTam-bound ER $\alpha$  recruit either corepressors like NCOR1 and SMRT or coactivators like SRC-1 *via* their AF1 domain [59]. The balance in these recruitments is the basis for the tissue-specific agonist activity of OHTam [60, 61] and has also been suspected to be associated with OHTam resistance. Among all the cofactors whose expression has been compared in OHTam-sensitive and resistant cells, SRC-1, SUG1, NCOR1 and AIB1 have shown differences in the level of protein expression [9]. Another level of complexity was highlight by the recent studies of Carrol and Lin who mapped all the ER and RNA polymerase II binding sites using genome-wide ChiP analysis [62, 63]. They found

that most estrogen binding sites were located very far from the start sites of transcription. Although the functional significance of these distant sites is unknown at present, they may be involved in the specificity and activity of antiestrogens, in combination with other cis or trans-acting elements.

The complex program of gene regulation initiated by cofactor recruitment results in many enzymatic modifications that can basically be divided into two classes: ATP-dependent nucleosome remodeling and histone tail modification. The latter proceeds from enzymes involved in acetylation (HATs) and deacetylation (HDACs), methylation (HMTs) and demethylation (HDMs), and ubiquitin- and SUMO- ligase, kinase, phosphatase and poly(ADP)ribosylase activities [56]. Some of these enzymatic activities such as HAT and kinases were considered to introduce transient and fully reversible marks due to the existence of opposite enzymatic activities (*i.e.*, HDAC and phosphatases). Conversely, protein methylation (particularly on lysine) was initially considered as a permanent modification that could result in a more stable alteration of gene expression [64]. However, recent data showing the existence of histone demethylase activity (HMD) has dramatically changed this concept [65-68]. Moreover, the compelling study of Garcia-Basset et al. recently demonstrated that the HMD LSD1 was needed for the activation of most ER $\alpha$  target genes in MCF-7 cells [69]. This mechanism physiologically used to prevent constitutive gene activation by unliganded nuclear receptors, has challenged our vision of the hypothesized histone code for long-term epigenetic memory (reviewed in [70]).

Another major actor of transcription silencing is heterochromatin protein 1 (HP1), a strong suppressor of position effect variegation in *Drosophila*, that plays an active role in the establishment of heterochromatin [71, 72]. A model of heterochromatin spreading suggests the recruitment of HP1 by methylated H3K9, followed by HP1-mediated recruitment of SU(VAR)3-9, a histone methylase that modifies adjacent nucleosomes. This, in turn, leads to

the spread of HP1 proteins along the chromatin fiber, forming a protein matrix that hampers gene activation (Fig. (2A)) [73]. This initial model could explain how long-term silencing of gene expression occurs in long domains of heterochromatin fibers. However, silencing at short distances involving a self-propagation of HP1 that is not SU(VAR)3-9 dependent, has also been proposed [73, 74]. More recent studies have shown that the role of HP1 is not restricted to inhibition of the heterochromatin fiber, but instead, that HP1 is a multi-functional protein that could act as a key regulator of some of the euchromatic genes regulating the cell cycle [71, 75, 76]. Although no direct links have been found between HP1-based mechanisms and the repressive activity of nuclear receptors, HP1 isoforms were found to interact *in vitro* or *in vivo* with some of the cofactors involved in receptor signaling [77, 78]. Interestingly, the TIF1 $\beta$  cofactor (termed KAP-1 in mammalian cells), found in an NCoR protein complex directly recruited by the OHTam-liganded ER $\alpha$  [60, 79], was found to interact both *in vivo* and *in vitro* with HP1 (Fig. (2B)) [80, 81].

#### **4-Epigenetic modification in the MVLN cell model**

While many studies have focused on gene expression changes observed in established AE-resistant cell lines, our laboratory developed a strong interest in the early changes in gene regulation that occur after short-term tamoxifen treatment of estrogen-sensitive breast cancer cell lines. Our hypothesis was that short-term AE treatment would affect a defined gene network by epigenetic modifications. Once established, these early modifications might facilitate further progression of tamoxifen resistance.

Initially, the MVLN cell line was created in an attempt to find cellular clones susceptible to acquire a luminous phenotype after mid or long-term OHTam treatment. However, such clones were never observed. Instead, we noted that the transgene expression was rapidly ( $t_{1/2}$  = 7-15 days) (Fig. (3A)) and irreversibly inactivated by OHTam treatment [28]. A fixed residual

expression level (less than 2% of the initial value) remained after inactivation by OHTam and could not be raised by estradiol treatment, regardless of the stimulation time. It should be stressed that in the absence of AE treatment, the transgene expression was perfectly stable. The important point to be noted concerns the time course of this irreversible inactivation process, which was clearly incompatible with a selection process and revealed a new facet of AE action linking transcriptional silencing to long-term tamoxifen effects. After various durations of OHTam treatment, cells were dispersed and individual clones grown for 1 month in medium without AE (Fig. (3B)). The number of luminous clones decreased as a function of treatment time but, importantly, their activity was either comparable with that of parental MVLN cells (*i.e.*, switched on) or fully inhibited (*i.e.*, switched off), with no intermediate states.

We also demonstrated that the magnitude of inactivation was dependent on the structure of the AE used [28]. For example, the total AE ICI<sub>164,384</sub> was clearly less efficient than OHTam and among the triphenylethylenic series (LN643, LN2839, toremifene, clomifene, OHTam and tamoxifen), OHTam and tamoxifen were the most efficient. As a control, when similar incubation of the cells was performed in estrogen-deprived medium without antihormone, luciferase expression was inhibited but this inhibition was totally reversible.

Another important feature of the phenomenon was the fact that when the same luciferase reporter plasmid was exogenously transfected in inactivated MVLN cells, its expression was unaltered, indicating that the estrogenic signaling was not defective and that chromatin alteration of the endogenous transgene was probably involved in the irreversible inactivation process.

What about natural genes? We showed that the endogenous pS2 gene was not inactivated by OHTam treatment of MVLN cells, even after several months of treatment [32]. By contrast, the endogenous progesterone receptor (PR) gene expression also gradually declined in an

irreversible fashion. It should be noted that 6 months of treatment were necessary to obtain an average inactivation higher than 70%.

### ***Transgene localization***

Analysis of the luciferase transgene promoter by PCR cloning and sequencing did not reveal any mutation suggesting that the inactivating effect of OHTam was not a consequence of the genotoxic effect of the drug. By Southern blot analysis, we showed that three complete copies of the Vit-tk-luc transgene were integrated at a single site [29]. Interestingly, the integration site appeared to be of importance since other clones bearing the same transgene inserted at different sites were not inactivated by OHTam treatment [40]. Finally, using FISH analysis experiments, we localized the transgene in a sub-telomeric position, consistent with a heterochromatic based inactivation process [31]. Indeed, telomeres that are composed of short repeat sequences adopt a particular heterochromatin conformation [82]. Epigenetic silencing near telomeres (termed the telomeric position effect) has been extensively studied in *Saccharomyces cerevisiae* [83] and was recently investigated in vertebrates [84-86]. The inactivation of genes placed in close proximity to telomeres might involve both the spreading of the heterochromatic structure along chromatin fiber, and DNA or histone modifications [72, 85].

### ***DNA methylation***

We next investigated CpG methylation in the luciferase and PR promoters. Using methylation-sensitive restriction enzymes, we found that inactivation of the luciferase transgene correlated with the methylation of a single *Not I* site located in the integrated transgene [29]. However, when global methylation status was investigated using the classical Frommer's bisulfite method, no CpG was found to be methylated in any copy of the tk

promoter or in the PR promoter [32]. Two promoters (PRA and PRB) control the expression of progesterone receptor isoforms (A and B). It has been documented that PRB is often methylated and isoform B expression repressed, in uterine endometrial carcinomas [87, 88]. Reexpression of this isoform by aza-deoxycytidine and/or trichostatin, indicates that methylation could be the key player of inactivation in that case [89, 90]. However, in the ER $\alpha$ (-) MDA-MB-231 cell line, Ferguson showed that promoter methylation status was not always correlated with the PR expression. Indeed, receptor reexpression could be achieved without concomitant demethylation of the promoter [91]. The interpretation of these data was complicated by the finding that even methylation of the exon part of the promoter gene could be involved in gene expression regulation [89]. Concerning our results, it is possible that the methylation status of PR (or the tk promoter) was not the key player in the inactivation process. However, one cannot exclude the possibility that methylation occurred in a DNA region outside of the PR promoter (or the transgene vit-tk-luc) that was not analyzed, the *Not I* methylation site being a vestige of this hypothetical modification pattern.

### ***Role of histone deacetylase activity***

Treatment of OHTam-inactivated clones with trichostatin A, a histone deacetylase (HDAC) inhibitor, partially restored the luciferase transgene expression (4-fold increase in the residual expression). In order to mimic the irreversible inactivation of the luciferase transgene by OHTam liganded-ER $\alpha$ , we engineered a fusion protein (termed HDAC-ER-GR) which contained human HDAC1 fused at its C-terminal end to the ER $\alpha$  DNA-binding domain (DBD), and the glucocorticoid receptor ligand-binding domain (LBD) [30] (Fig. (4A)). This chimeric protein was targeted to estrogen-response elements (ERE) in the presence of glucocorticoid agonists, such as dexamethasone or bimedrazol (Bim), or the RU 486 antagonist. A double transfectant MVLN cell line stably expressing the HDAC-ER-GR

protein was then developed (MELN-HEG). In these cells, the liganded HDAC-ER-GR protein produced an antiestrogenic effect on cell growth and the expression of several natural E2-regulated genes (pS2, PR, cathepsin D) when it translocated to the nucleus upon treatment by a glucocorticoid agonist. This effect, which probably occurred through competition between the liganded HDAC-ER-GR and liganded ER $\alpha$ , was not observed with control chimeric proteins bearing, for instance, a mutant enzymatically deficient HDAC. However, long-term targeting of HDAC-ER-GR to ERE did not mimic the inactivating effect of OHTam because the inhibition obtained was clearly reversible, but expression of HDAC-ER-GR significantly accelerated the OHTam-driven phenomenon (Fig. (4B)). Altogether, these results showed that HDAC1 activity might have participated in irreversible silencing but was not sufficient to trigger it on its own.

#### ***HP1- and chromatin-based mechanisms***

In order to test an HP1-based mechanism in our MVLN model of inactivation, we first investigated, by ChIP experiment, the presence of HP1 isoforms on the promoter of the luciferase transgene during the inactivation process. We compared MVLN cells inactivated or not by 45-days of treatment with OHTam and found HP1 $\alpha$  to be associated only with the inactivated transgene, with a concomitant slight increase in H3K9 dimethylation [31].

The involvement of HP1 $\alpha$  was reinforced by the finding that the OHTam inactivating effect was mimicked by its direct or indirect targeting to ERE. This was achieved by fusing either the KRAB (Krupple-associated box) domain of the KOX-1 repressor (known to repress gene expression by recruitment of HP1 proteins) or HP1 $\alpha$  itself to the ER $\alpha$  DNA-binding domain (ER-DBD) and the androgen receptor ligand-binding domain (AR-LBD) (Fig. (5A)). In the corresponding MVLN cell lines, stably expressing either KRAB-ER-AR (K-MVLN) or



HP1 $\alpha$ -ER-AR (H-MVLN), treatment with the R1881 androgen agonist used to target the chimeric proteins to EREs led to irreversible inactivation of the luciferase transgene.

The extent of inactivation was higher for the K-MVLN (bearing the KRAB module) than for the H-MVLN cell line. Interestingly, the effect of the KRAB module was prevented by the simultaneous addition of estradiol, as was the case for the inactivation induced by OHTam. However, the length of treatment required to reach maximal inhibition was longer with R1881 as compared with the inactivation by OHTam (Fig. (5B)). These data collectively suggested that HP1 was a key player in AE-induced transcriptional silencing although still not as efficient as OHTam (at least in terms of kinetics). Very interestingly, an elegant study performed in NIH3T3 cells confirmed that targeting of a KRAB domain induced the stable and heritable silencing of a transgene [92]. This effect involved the KAP1-mediated recruitment of HP1 $\alpha/\gamma$  isoforms and the spatial relocation of the transgene to condensed chromatin.

Altogether, our data obtained using the MVLN cells allow us to propose a hypothetical model of irreversible inactivation of the luciferase transgene which is presented in Figure 6. Chimeric receptors are targeted to EREs by the ER- DNA- binding domain part of the protein. The HP1 $\alpha$  module of the HP1 $\alpha$ -ER-AR chimeric protein could induce the self-propagation of free HP1, either directly or mediated by H3K9 methylation (Fig. (6A)). In the case of the KRAB-ER-AR chimeric receptor (Fig. (6B)), the KRAB module could first recruit cofactors such as the KAP1 cofactor which is a platform to assemble HP1 and other enzymatic activities like histone methyl transferases (HMT), leading to a short-range and heritable silencing of euchromatic targets [92]. In the case of OHTam, it is thus conceivable to hypothesize a model in which the interaction of ER $\alpha$  with a corepressor such as NCoR1 could recruit similar silencing machinery to operate the same type of silencing (Fig. (6C)).

## **5- Conclusions and perspectives.**

In conclusion, the OHTam-induced inactivation process that we described in MVLN cells is situated between the long-term effects of tamoxifen and the more general problem of transcriptional silencing. Along with other studies cited in this review, our investigations present evidence of the epigenetic effects of AE action. Epigenetic processes are actively studied in the cancer field and “epigenetic therapy”, which appears to be very promising might offer a highly beneficial solution to the problem of AE resistance. For instance, HDAC inhibitors have shown promising results in blocking the growth of AE-resistant cells [93-95]. However, more thorough analysis of the early epigenetic changes that occur during OHTam treatment is required. For instance, high-throughput promoter CpG island microarray analysis during the cytostatic phase of AE treatment (outside of the selection phase process) might reveal new targets that are primarily and irreversibly epigenetically affected during resistance appearance.

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## Figure legends

### **Figure 1. Overview of the working hypotheses concerning tamoxifen resistance.**

Estrogen receptor (ER) modifications may involve: mutations, alternative splicing or post-translational modifications. Coregulator dysfunction may involve variations of their cellular content as well as post-translational modifications. Interference with growth factor pathways may involve phosphorylation of the ER by growth factor-activated kinases [mitogen-activated protein kinase (MAPK), p90 ribosomal S6 kinase (RSK), serine threonine protein kinase B (AKT)], or the non-genomic action of ER on growth factor receptors. Intra-tumoral estrogens (synthesized either by breast, ovary or peripheral tissues) may compete with the binding of tamoxifen at the level of the ER. Tamoxifen metabolism and biodisponibility, involving liver metabolism and intracellular sequestration, were also investigated but with no clear indication that they could be involved in tamoxifen resistance.

### **Figure 2. Recruitment of HP1 protein to histones.**

**A.** Initial methylation of lysine 9 (K9) of histone H3 creates a high affinity binding site for HP1. Bound HP1 next recruits (protein-protein interaction) the histone methyltransferase (HMT) SU(VAR)3-9 that methylates adjacent nucleosomes. This in turn leads to the spread of HP1 along the chromatin fiber, which is supposed to adopt a more condensed conformation.

**B.** Hypothetical model of recruitment of HP1 by tamoxifen-liganded estrogen receptor alpha bound on an estrogen response element (ERE). Corepressors NCoR and KAP-1 would be recruited next. The subsequent binding of HP1 to KAP-1 would allow the initiation of a series of cycles (as described in A) leading to the spreading of HP1 along the chromatin fiber and the inhibition of associated gene expression.

**Figure 3. Irreversible transgene inactivation by 4-hydroxytamoxifen treatment in MVLN cells.**

Adapted from [28]. (A) MVLN cells were cultured for various times in DCC medium containing 200 nM OHTam. After OHTam treatment, they were stimulated for 48h with 1nM estradiol, and luminescence (per mg protein) was then recorded. (B) MVLN cells were cultured in either 200 nM OHTam, FCS medium or DCC medium for 30 days. They were then dispersed in FCS medium to obtain, 1 month later, separate clones whose luciferase activity was analyzed with a camera, and the percentage of luminous clone was determined.

**Figure 4. Effect of HDAC-ER-GR chimeric receptor.**

**A.** Schematic representation of the chimeric construct : DNA- binding domain (DBD), ligand-binding domain (DBD), glucocorticoid receptor (GR), estrogen receptor (ER), histone deacetylase 1 (HDAC1).

**B.** Adapted from [30]. As indicated in the figure, cell lines, as well as MVLN(-) [i.e., containing the tet-on system but devoid of chimeric construct], were treated for various times with 200 nM OHTam or 200 nM OHTam + 100 nM Bim. Recovered luciferase expression was induced in the presence of 1 nM E2 for 48 h. The results are expressed as the mean  $\pm$  SD of triplicate values (RLU/mg protein) and as the percentage of luciferase activity under control condition at day 0.

**Figure 5. Irreversible inactivation kinetics of luciferase transgene in MVLN, H-MVLN and K-MVLN.**

**A.** Schematic representation of the chimeric construct: androgen receptor (AR), heterochromatin protein 1  $\alpha$  (HP1 $\alpha$ ), and Kruppel-associated box (KRAB) domain of the KOX-1 protein.

**B.** Adapted from [31]. As indicated in the figure, cell lines, as well as MVLN(-) [i.e., containing the tet-on system but devoid of chimeric construct], were treated for various times with OHTam 100 nM or R1881 10 nM. Recovered luciferase expression was obtained and expressed as described in Figure 3.

**Figure 6. Hypothetical models of irreversible inactivation of the luciferase transgene in MVLN cells.**

Recruitment of silencing complexes by: **A.** The chimeric receptor HP1 $\alpha$ -ER(DBD)-AR(LBD), which drives the HP1 module near an estrogen responsive element (ERE). This in turn leads to the recruitment of the methyltransferase (HMT) SU(VAR)3-9 and the subsequent spreading of HP1 (as depicted in Figure 2A). **B.** The chimeric receptor KRAB module-ER(DBD)-AR(LBD), which drives the KRAB module near an ERE. This in turn would lead to the recruitment of the corepressor KAP-1 and then the protein HP1, followed by a sequence similar to the one described in Figure 2A [HDAC1 (histone deacetylase 1) could be involved in inhibitory protein complexes depicted in the figure]. **C.** The wild type estrogen receptor, which would operate through the mechanism depicted in Figure 2B.

## 6- References

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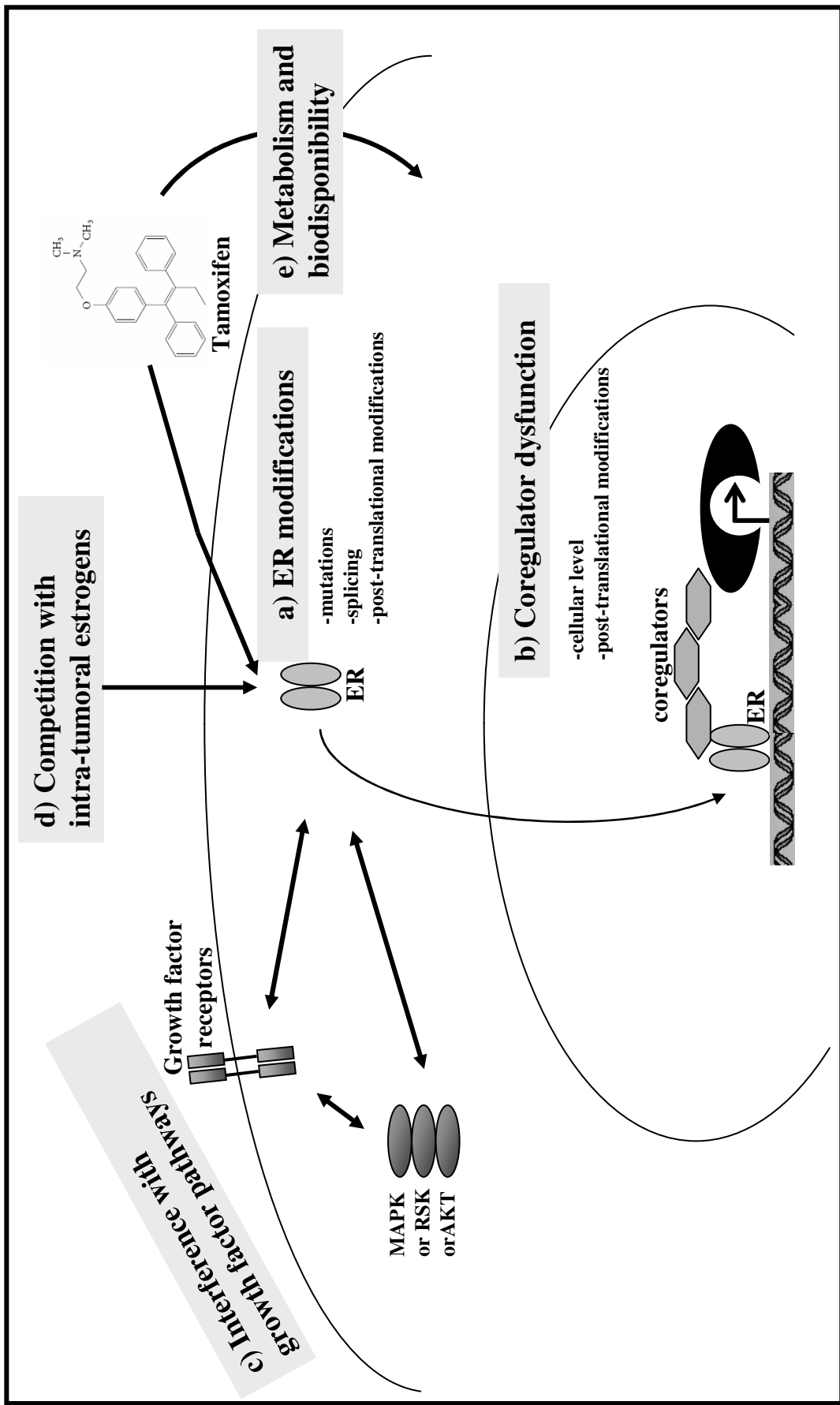
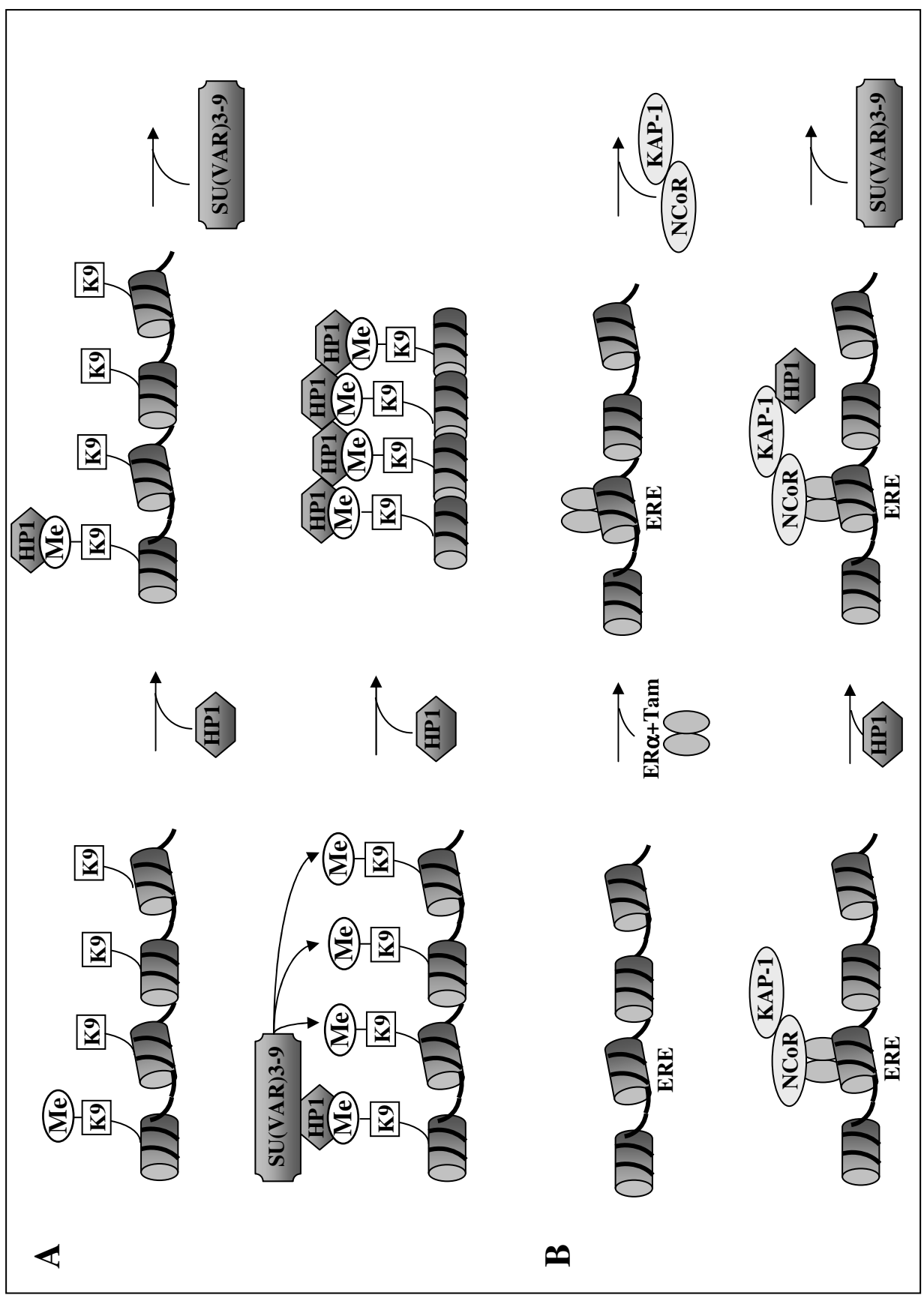
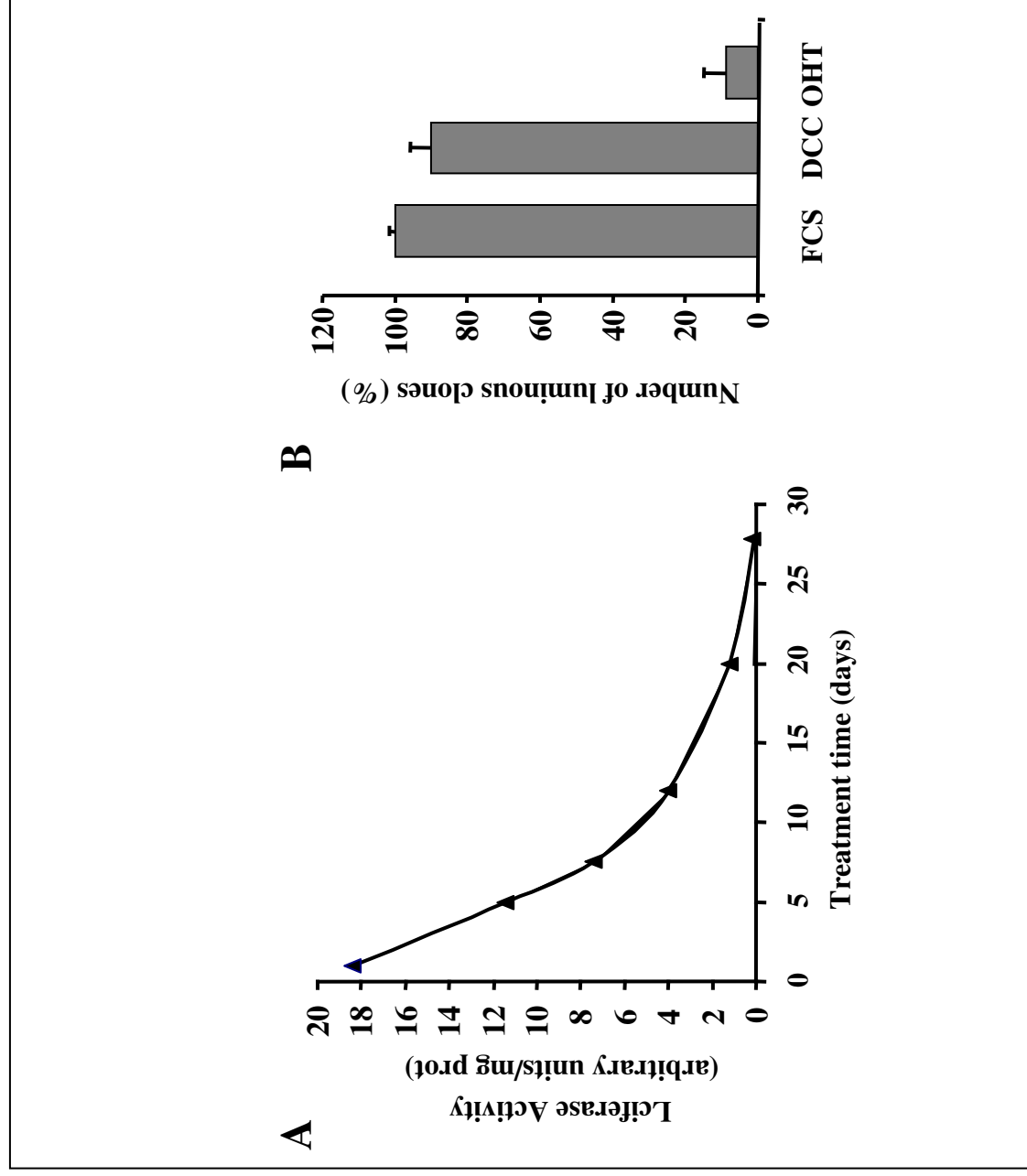


Fig 1





**Fig 3**

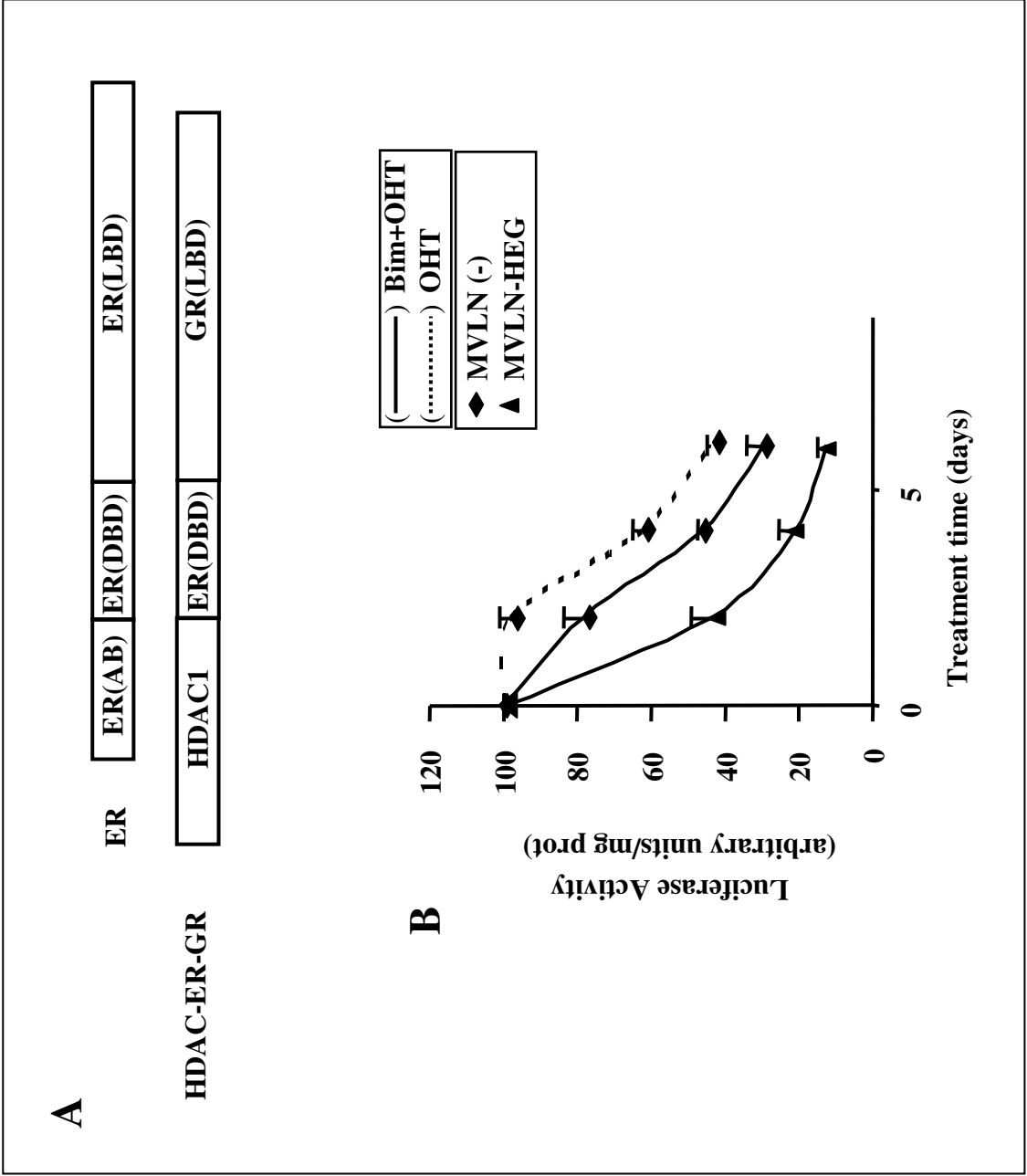


Fig 4



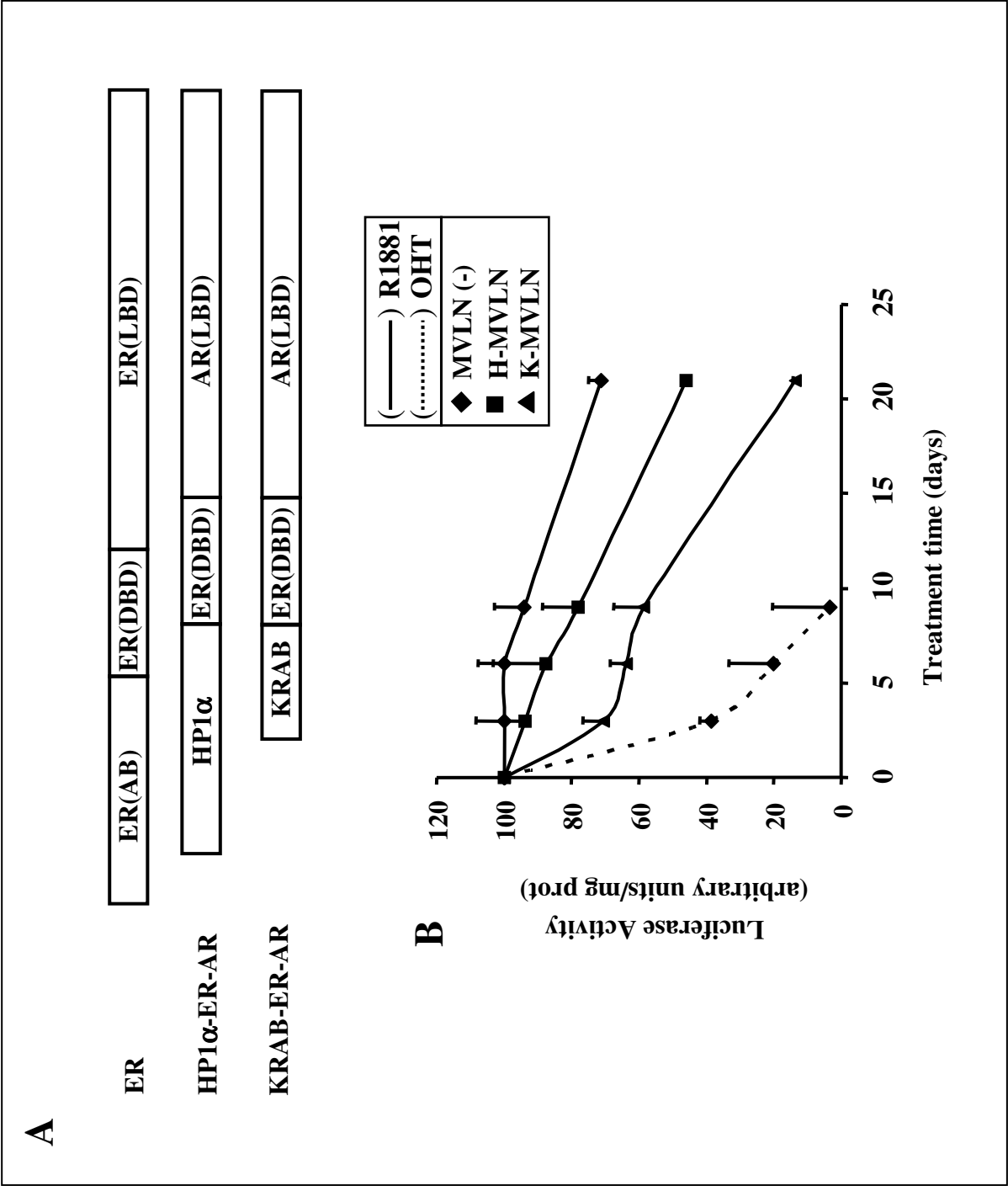


Fig 5

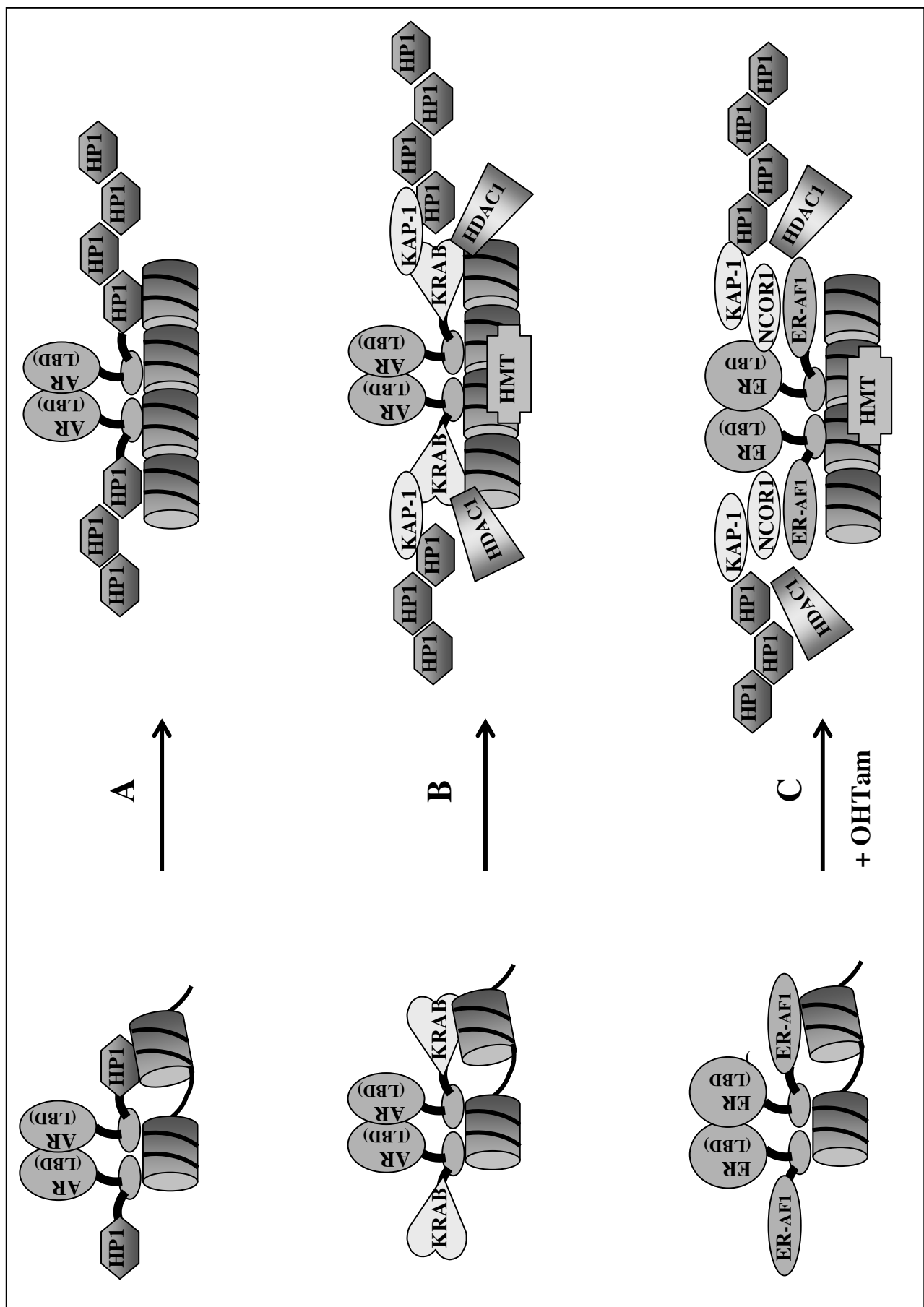


Fig 6