Published in final edited form as:
*Bioorg Med Chem Lett*. 2009 November 1; 19(21): 6233–6236. doi:10.1016/j.bmcl.2009.08.090.

# Inhibition of ErbB2(Her2) expression with small molecule transcription factor mimics

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## Abstract

Small molecules that mimic the transcriptional activation domain of eukaryotic transcriptional activators have the potential to serve as effective inhibitors of transcriptional processes. Here we show that one class of transcriptional activation domain mimics, amphipathic isoxazolidines, can be converted into inhibitors of gene expression mediated by the transcriptional activator ESX through small structural modifications. Addition of the small molecules leads to decreased expression of the cell surface growth receptor ErbB2(Her2) in ErbB2-positive cancer cells and, correspondingly, decreased proliferation.

Many human cancers are characterized by elevated levels of proteins that regulate cell cycle progression and proliferation. In approximately one-third of breast cancers, for example, the cell surface growth receptor ErbB2(Her2) is overexpressed, and this is correlated with increased metastasis and resistance to chemotherapeutic agents.[1] Such proteins are promising drug targets, with sales of the ErbB2(Her2) antibody/inhibitor Herceptin (Trastuzumab) reaching $750 million in 2006.[2] An emerging alternative strategy is to directly down-regulate the expression of relevant proteins through blocking specific transcriptional activator-coactivator interactions that are critical for activation of the encoded gene (Figure 1a).[3, 4] The challenges of this strategy are considerable, in part because many transcriptional activators exhibit poorly characterized, multi-partner binding profiles that are difficult to reconstitute with a small molecule.[5] We have previously described a class of small molecules that serve as generic mimics of amphipathic transcriptional activators.[6-9] Here we show that this same scaffold can be converted to a transcriptional inhibitor, a molecule that effectively abrogates the expression of the growth receptor ErbB2 at low micromolar concentrations and, correspondingly, inhibits the proliferation of ErbB2-overexpressing cancer cells.

To block the interaction of an activator with its target in the transcriptional machinery (the coactivator), a small molecule must bind to either protein partner with sufficiently high affinity to block the binding of the second protein. In the course of developing small molecule-based transcriptional activators, we identified several amphipathic isoxazolidines that mimic the

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transcriptional activation domain (TAD) of endogenous amphipathic activators, the domain that interacts directly with the transcriptional machinery (Figure 1b).[6-8] When localized to DNA, the isoxazolidine TADs up-regulate transcription in human cell culture up to 80-fold.[9] Thus, in the absence of a DNA-targeting moiety, we reasoned that this molecule could serve as a competitive inhibitor of activator-coactivator interactions (Figure 1a). Supporting this idea, the isoxazolidine TADs display a multi-partner binding profile consistent with their natural counterparts; in addition, small structural changes alter the binding pattern of the TAD mimics.[6, 12] We hypothesized that this molecular scaffold would be an excellent starting point for inhibitor development.

The activator chosen as a target for this study is ESX (ESE-1/ELF-3/ERT/Jen), an epithelial-specific transcriptional activator that has been shown to regulate expression of the ErbB2 oncogene.[13, 14] ESX interacts with multiple co-activator proteins; the most well-characterized of these interactions is with Med23(Sur2/DRIP130/CRSP130), a coactivator located in the mammalian mediator complex.[13] Several lines of evidence suggest that the ESX-Med23 interaction is a key regulator of ErbB2 expression. Furthermore, partial inhibition of ESX-Med23 complex formation has an inhibitory effect on the proliferation of ErbB2-overexpressing cells.[11, 13] Within the minimal region of ESX(137-SWIIELLE-144) that binds to Med23, tryptophan 138 is essential for the ESX-Med23 interaction. NMR spectroscopic studies suggest that this residue along with Ile139, Ile140, Leu142 and Leu143 form a hydrophobic surface along an amphipathic helix that interacts with Med23.[13]

A fluorescein-tagged variant of **1** (**1b**) was assessed for its ability to interact with a region of Med23 (residues 352-625) that contains the binding site for ESX by fluorescence polarization, and binding was observed with a $K_D$ of 5.9 ± 0.1 μM (Figure S2 in Supporting Information). Although low micromolar dissociation constants are sufficient for function as a transcriptional activator, tighter binding is likely necessary to inhibit the formation of a complex l between a DNA-bound transcriptional activator and the transcriptional machinery. Isoxazolidine **1** does not contain a large hydrophobic substituent similar to Trp138 in ESX; when this residue is mutated to Phe, binding and ESX activity are attenuated.[13] We thus sought to enhance the affinity for Med23(352-625) and increase the resemblance of the molecules to ESX by replacing the N2 benzyl substituent with larger hydrophobic aryl groups ranging from *p*-$CF_3$Phe (**2a**) to biphenyl (**4a**) (Figure 2).

Isoxazolidines **2a**-**4a** were prepared via straightforward manipulations of a previously reported isoxazoline (see Supporting Information for details).[8] For binding experiments with Med23 (352-625), the azide handle present in each of the structures was reduced under Staudinger conditions and the resulting amine was conjugated to FITC. Fluorescence polarization binding experiments with each of the fluorescein-labeled isoxazolidines revealed that increasing the size of the N2 substituent produced compounds that bound an order of magnitude more strongly to the fragment of Med23 that interacts with ESX (**2b**: 0.59 ± 0.06 μM; **3b**: 1.0 ± 0.2 μM; **4b**: 0.62 ± 0.08 μM; Figure S2 in Supporting Information).

Isoxazolidines **1a**-**4a** were tested for their ability to down-regulate ErbB2 expression in BT-474 cells, an ErbB2-overexpressing breast cancer cell line. Consistent with binding data, isoxazolidine **1a** did not significantly impact ErbB2 expression at concentrations up to 50 μM (Figure 3a) as compared to isoxazolidines **2a**-**4a**, all of which produced a dose-dependent reduction in ErbB2 expression after a 24-hour treatment. Of the three modified isoxazolidines, compound **4a** was the most effective at downregulating ErbB2 expression, with an $EC_{50}$ in the low micromolar concentration range. We subsequently evaluated the effects of compound **4a** at the transcriptional level. ErbB2 mRNA transcript levels were assessed via quantitative real-time polymerase chain reaction (qPCR) experiments. BT-474 cells treated for 6 hours with compound **4a** exhibit reduced ErbB2 mRNA levels as compared to those treated with

compound **1a** or vehicle (Figure 3b). This is consistent with isoxazolidine **4a** impacting ErbB2 expression at the transcriptional level.

The activity of isoxazolidine **4a** was further examined in a second ErbB2-dependent breast cancer cell line, SkBr3. Analogous to the activity observed in BT-474 cells, low micromolar concentrations of **4a** were sufficient to down-regulate ErbB2 expression (Figure 4a). Isoxazolidine **1a** showed no activity in that concentration range, although at concentrations above 25 μM reduced ErbB2 expression was observed. Previous studies have correlated lowered ErbB2 activity with attenuated cell proliferation;[15] therefore we examined growth effects of isoxazolidine **4a** in SkBr3 cells. Consistent with the results of Figure 4a, isoxazolidine **4a** inhibited cell growth with an $IC_{50}$ of 14 ± 1 μM, in contrast to isoxazolidine **1a** which did not affect growth (Figure 4b). Furthermore, the effects of isoxazolidine **4a** in the non-ErbB2 over-expressing cell line MCF-7 are reduced ($IC_{50}$ 27 ± 4 μM; Figure S3 in Supporting Information), in agreement with previous findings.[13, 16] In addition to its role in certain breast cancers, ErbB2 over-expression is a hallmark of head and neck cancers.[17] Towards this end, we assessed the ability of biphenyl **4a** to inhibit the growth of CAL27, SCC-25, and SCC-15 cell lines and found that while isoxazolidine **1a** had no impact on cell proliferation, **4a** dose-dependently inhibited cell growth (Figure S4 in Supporting Information). In the course of these experiments, we observed that increasing serum concentrations lead to attenuated effects of the small molecule. This may be attributable to serum binding, a well-known phenomenon for lipophilic molecules[18]; an additional contributor may be serum-dependent effects on cell signaling.[19] Differentiating these contributions will be the subject of future investigations.

Taken together, these data suggest that isoxazolidine **4a** is a much improved mimic of the transcriptional activation domain of ESX relative to the generic TAD mimic **1a**, validating our strategy for using an artificial TAD as a scaffold for the design of transcriptional inhibitors. Treatment of ErbB2-positive cell lines with isoxazolidine **4a** attenuated ErbB2 protein levels as well as ErbB2 mRNA transcripts. Additionally, isoxazolidine **4a** inhibits the growth of several ErbB2-overexpressing cell lines. This improved ESX mimicry does not, however, correspond to improved activity as a transcriptional activation domain. When localized to DNA, isoxazolidine **1a** activates transcription 80-fold at 1 μM whereas isoxazolidine **4a** shows quite modest activity (4-fold),[6] emphasizing the distinct requirements for an activator versus an inhibitor of transcription. Further pharmacological investigations of isoxazolidine **4a** in a broader range of ErbB2 over-expressing cancers as well as application of this strategy to the development of a repertoire of transcriptional inhibitors will be reported in due course.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

A.K.M. is grateful to the NIH (CA140667), Novartis (Novartis Young Investigator Award), and the NSF (PECASE) for support of this work. J.-P.D. thanks the American Cancer Society for a postdoctoral fellowship (PF-06-116-01). We would like to thank Taocong Jin at the Molecular Biology Core Laboratory (School of Dentistry, University of Michigan) for helpful discussions and qPCR assistance and Kenneth Guire at the Center for Statistical Consultation and Research (CSCAR, University of Michigan) for statistical analysis of qPCR data.

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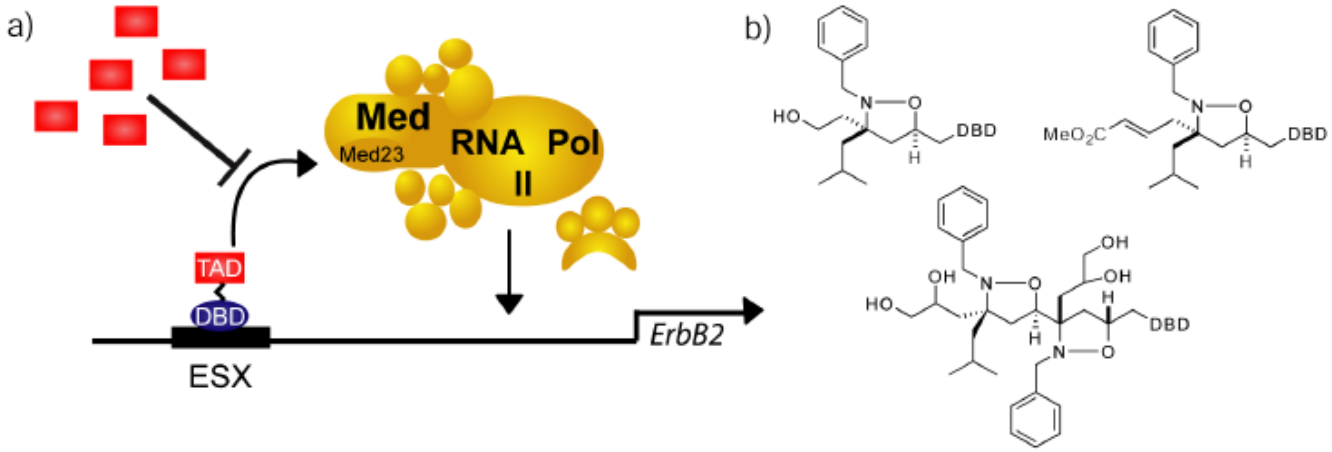


**Figure 1.**

(a) Down-regulation of ErbB2(Her2) expression can be accomplished by blocking the activator-coactivator interactions responsible for initiating gene expression with transcriptional activation domain (TAD) mimics.[4, 10, 11] (b) Amphipathic isoxazolidines that mimic the function and mechanism of transcriptional activation domains when attached to a DNA binding domain ('DBD').[6-9]

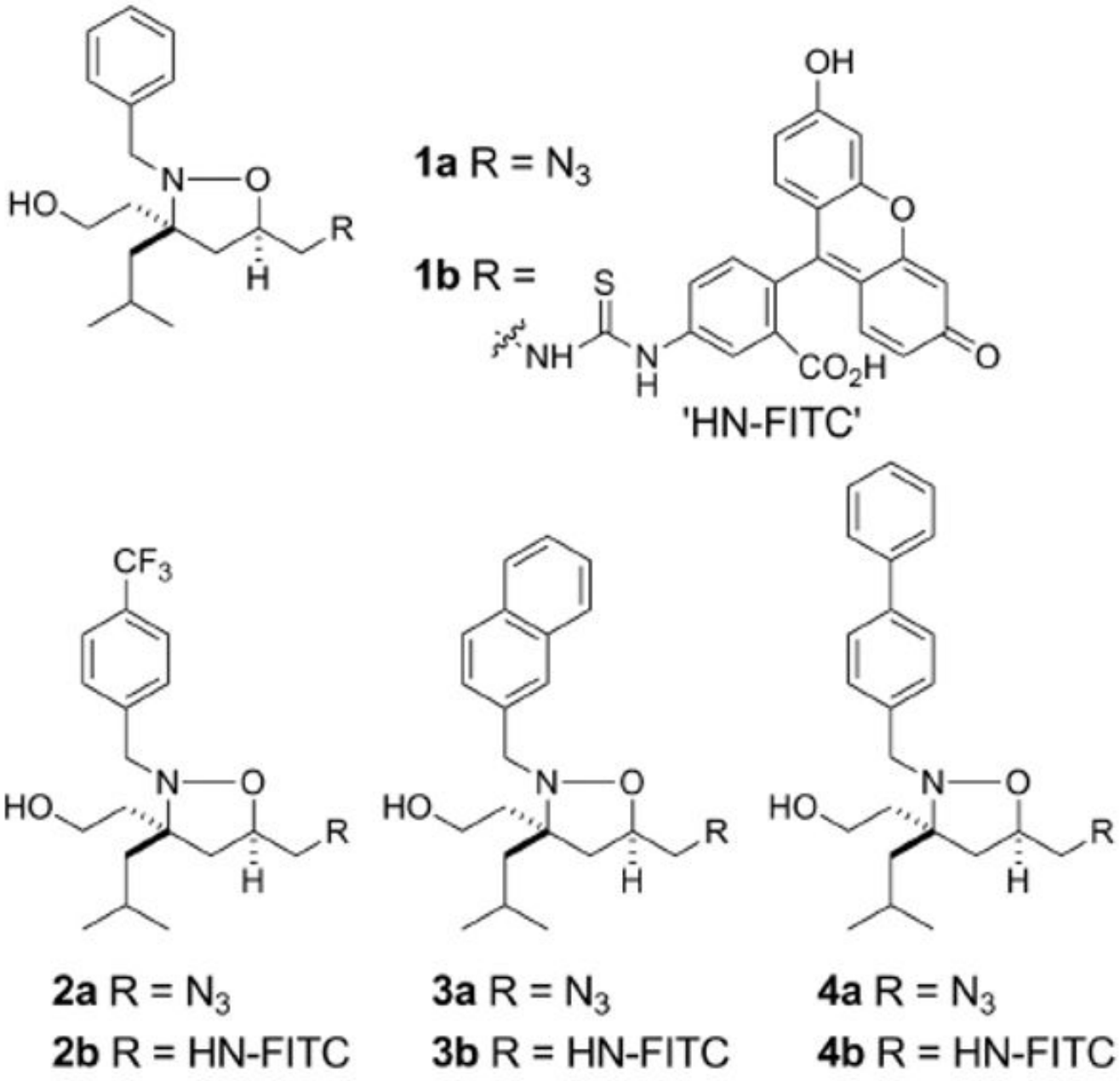


**Figure 2.**

Isoxazolidines **1a**-**4a** were synthesized, and their fluorescein labeled derivatives **1b**-**4b** were evaluated for binding to MBP-Med23(352-625). FITC=2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-methanethioamidobenzoic acid. See Supporting Information for details.

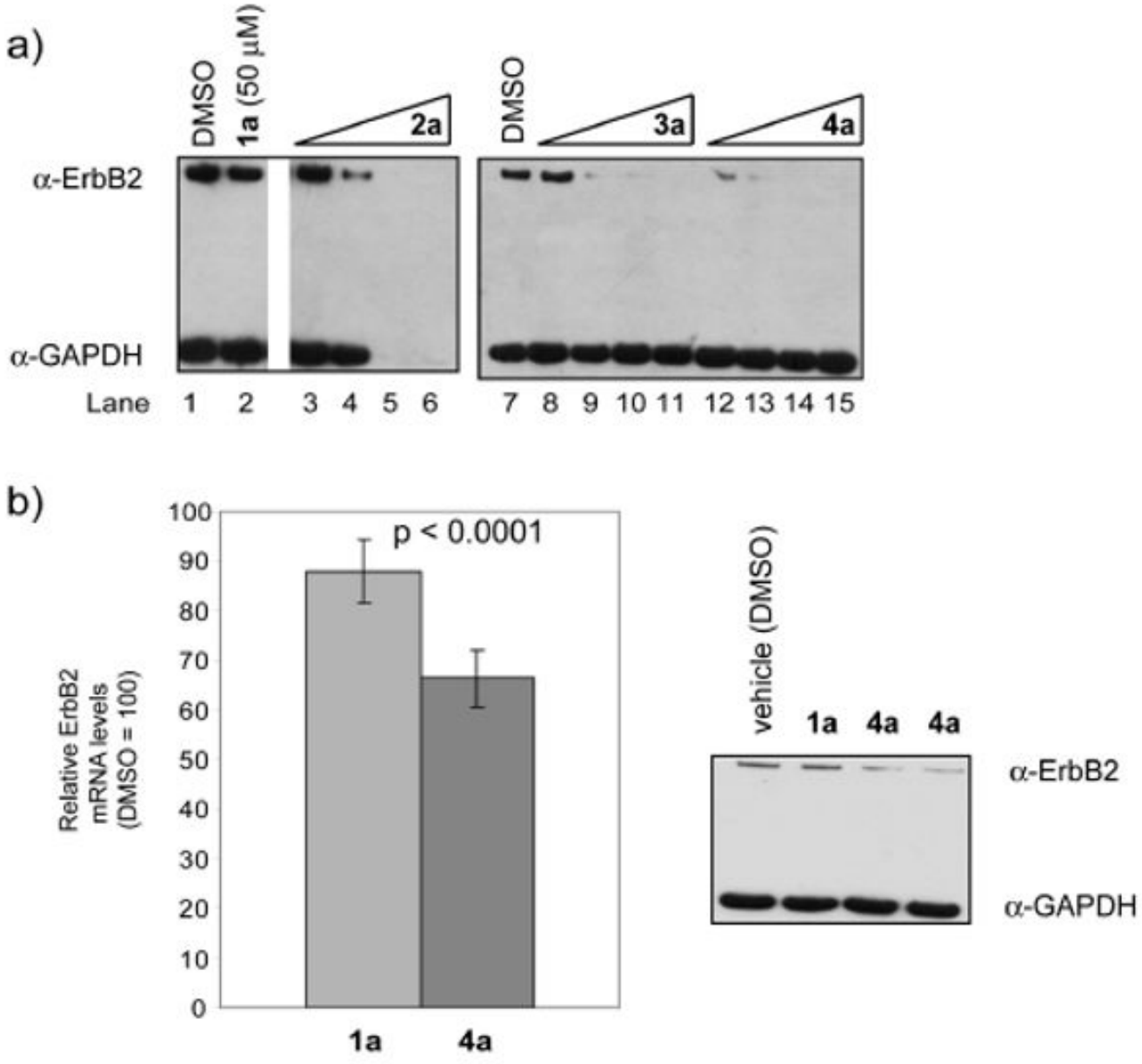


**Figure 3.**

(a) Effect of isoxazolidines **1a**-**4a** on ErbB2 expression in BT-474 breast cancer cells as assessed by Western blot analysis. Cells were treated with compounds dissolved in DMSO for 24h before analysis. Lane 1: DMSO; lane 2: 50 μM **1a**; lanes 3-6: 3.2, 6.25, 12.5, 25 μM of **2a**; lane 7: DMSO; lanes 8-11: 3.2, 6.25, 12.5, 25 μM of **3a**; lane 12-15: 3.2, 6.25, 12.5, 25 μM of **4a**. (b) Effect of isoxazolidines **1a** and **4a** on ErbB2 transcript levels as assessed by quantitative real-time polymerase chain reaction. Cells were treated with compounds dissolved in DMSO (10 μM) for 24h before analysis. Graph shows the average (16 experiments done in quadruplicate) of ErbB2 mRNA levels relative to DMSO and normalized to GAPDH. Error represents s.e.m. and p value obtained from Student's t-test; *right:* Western blot analysis of BT-474 cells from qPCR experiments. See Supporting Information for details.

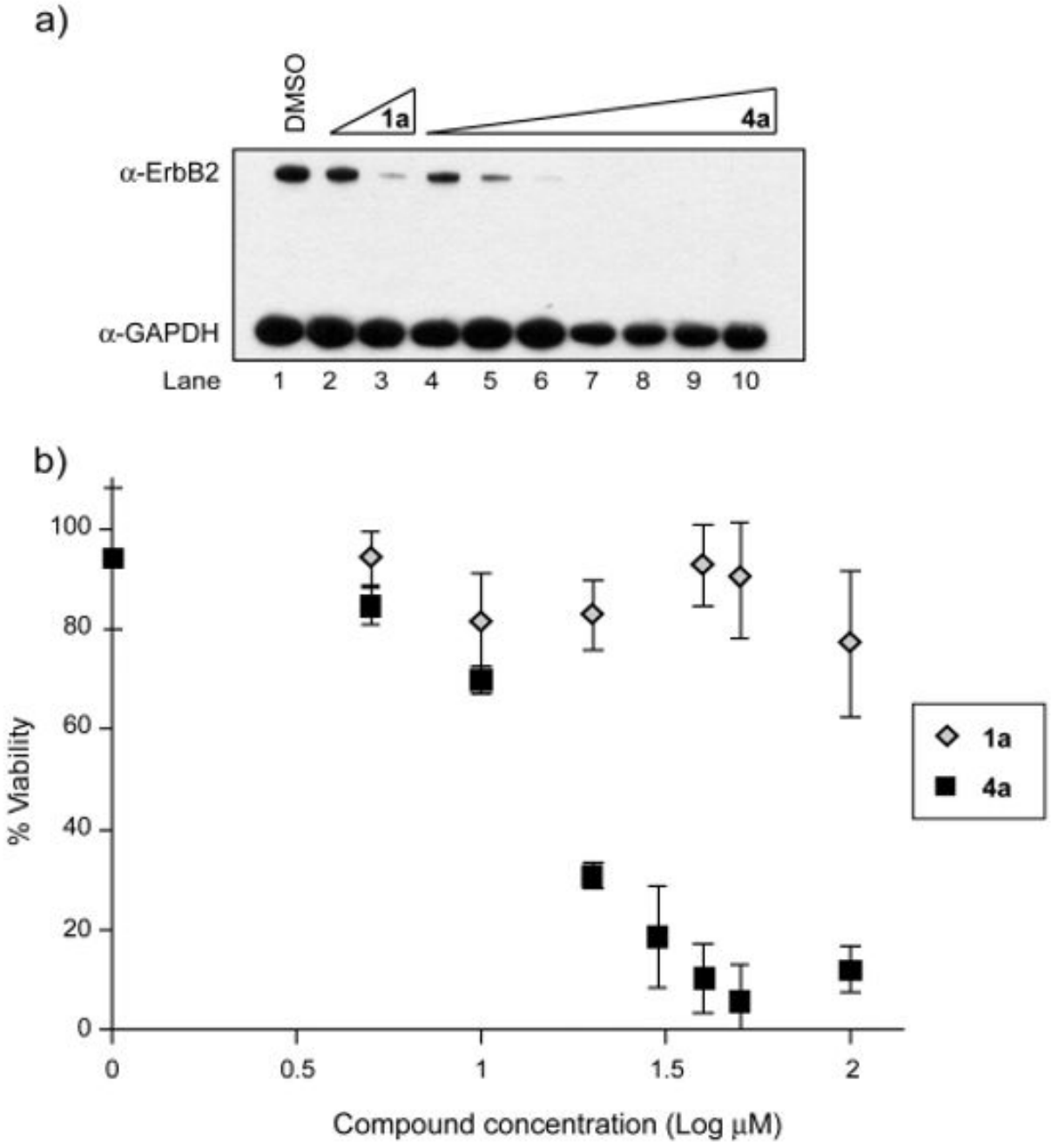


**Figure 4.**

(a) Effect of isoxazolidines **1a** and **4a** on ErbB2 expression in SkBr3 breast cancer cells as assessed by Western blot analysis. Cells were treated with compounds dissolved in DMSO for 6h before analysis. Lane 1: DMSO; lanes 2-3: 10, 50 μM of **1a**; lanes 4-10: 0.625, 1.25, 2.5, 5, 10, 25, 50 μM of **4a**. (b) Effect of compounds **1a** and **4a** on the viability of ErbB2-positive SkBr3 cells. Viability was measured 24h after dosage via WST-1 assay. Data points are median values of experiments done in triplicate with error bars representing standard deviation. See Supporting Information for details.