Chapter 16

Receptor Tyrosine Kinases and Drug Resistance: Development and Characterization of In Vitro Models of Resistance to RTK Inhibitors

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Abstract

Aberrant expression of receptor tyrosine kinases (RTKs) has been extensively associated with alterations in the physiological activities of cells. These include cell growth and differentiation, cell death/survival, and the motility of cells which can subsequently lead to emergence of various diseases including cancer. Recent advances in the treatment of cancer have involved using RTKs as therapeutic targets. Unfortunately, the clinical use of receptor tyrosine kinase inhibitors (RTKIs) for the treatment of cancer has been hindered by innate or acquired resistance among some patients, as also experienced with classical chemotherapy. It has become apparent that the deregulated expression of RTKs may play a significant part in driving this resistance. In order to fully elucidate the role of RTKs in drug resistance, the use of preclinical models has helped to mimic this clinical problem. In this chapter, we describe the methods associated with establishing and characterizing cell line models of drug resistance to the dual RTKI, lapatinib. These methods include the assessment of lapatinib resistance; cross-resistance to other RTKIs; the alteration of RTK expression; and other associated phenotypic changes such as cellular migration, invasion, and *anoikis* sensitivity/resistance.

Key words RTKIs, Drug resistance, Cell line models, Phenotypic changes, Cross-resistance, Motility, Invasion, *Anoikis*, RTK protein expression

1 Introduction

Receptor tyrosine kinases (RTKs) have been implicated in regulating many cellular functions, including differentiation, cell cycle, proliferation, cell motility, and cell death/survival. Theses cellular processes are instigated by the vital role of RTKs in signal transduction and subsequent affect on gene transcription in the nucleus [1]. RTKs are subdivided into 20 different families based on their specific structural features and homologous domains [2, 3]. One of the most widely documented and well-characterized subfamily, the epidermal growth factor receptor (EGFR) family has been utilized by pharmaceutical companies as a therapeutic target for some current and emerging anticancer therapies. For example, trastu-

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zumab (a monoclonal antibody targeting HER2) and lapatinib (small-molecule receptor tyrosine kinase inhibitor (RTKI) targeting EGFR/HER1 and HER2) are currently used in the treatment of HER2 over-expressing breast cancer [4]. In recent years the treatment of cancer by targeted therapies or classical chemotherapy has been hindered with the emergence of drug resistance among some cancer patients and recent evidence suggests that the deregulated expression of RTKs may be casually involved in this resistance [5, 6]. Many RTK subfamily members have been implicated in regulating drug resistance. These include IGF1R, whose aberrant expression has been associated with regulating response to classical chemotherapy [7–11]. EGFR [7], HER2 [12], FGFR [13, 14], and cMET [15] are among many other RTKs whose deregulated expression can alter drug sensitivity/resistance.

The use of in vitro models of drug resistance as a means of investigating the mechanisms underlying the clinical situation has been widely reported [13, 16–19]. In order to investigate how resistance to RTKIs may affect the cellular phenotype and relevant RTK expression, here we describe a method of generating and characterizing a cell line model of resistance to an RTKI. As previously reported, the acquisition of resistance to certain RTKIs may be associated with cross-resistance to other RTKIs [13, 19]; thus in addition to generating the in vitro models of resistance, we describe the method of assessing the level of resistance induced using the acid phosphatase method which can be applied to assessing potential cross-resistance to other TKIs. As outlined above, the expression of many RTKs may be altered with the onset of drug resistance. For the purpose of assessing the alteration of RTK expression in drug resistance, we explain the appropriate procedure for sample collection and preparation for immunoblotting (as described in Chapter 1 of this volume). A correlation between intrinsic or acquired drug resistance and an alteration in the aggressive phenotype of cells (in terms of metastatic ability) has also been reported [20]; thus in this chapter we explain some common methods of assessing cellular motility and invasion as well as cell response to cell death induced by anchorage-independent conditions (*anoikis*).

2 Materials

2.1 Generation of In Vitro Models of RTKI Resistance

For the purpose of this chapter we will use the HER2+ breast cancer cell line SKBR3 and the dual RTKI, lapatinib, as a means of generating an in vitro model of RTKI resistance. Lapatinib is currently used in the clinic for the treatment of HER2-positive breast cancer; however evidence of innate or acquired resistance to this drug has been reported [21, 22].

1. SKBR3 cells (American Type Tissue Collection, Rockville, MD, USA).

2. Culture medium: RPMI-1640 medium supplemented with 10 % fetal bovine serum and 2 mM L-glutamine.
3. General tissue culture equipment: 25 cm^2 and 75 cm^2 vented flasks, 15 mL centrifuge tubes.
4. Hemocytometer.
5. Trypsin-EDTA solution: (0.5 g/l porcine trypsin and 0.2 g/l EDTA·4Na in Hank's Balanced Salt Solution with phenol red).
6. Lapatinib (or another RTKI as relevant to the study in question): dissolved in sterile dimethyl sulfoxide (DMSO).

2.2 Toxicity Assays and Cross Resistance

The evaluation and quantification of toxicity assays described here are based on the acid phosphatase method.

1. 96-Well plates.
2. Lapatinib or other RTKIs (e.g., trastuzumab, neratinib, afatinib).
3. Phosphate-buffered saline (PBS).
4. Sodium acetate buffer: 4.1 g sodium acetate or 6.8 g sodium acetate trihydrate, 500 µL Triton X-100, 500 mL dH_2O. Adjust the pH to 5.5 with HCl.
5. Acid Phosphatase substrate: 0.027 g of 10 mM *para*-nitrophenol phosphate in 10 mL sodium acetate buffer.
6. Aluminum foil.
7. 1 M sodium hydroxide (NaOH): dissolved in distilled H_2O.
8. Multichannel pipette.
9. Microplate reader capable of detecting absorbance at 405 nm.

2.3 Sample Preparation for Immunoblotting

1. Cell scrapers.
2. Benchtop centrifuge and 1.5 mL centrifuge tubes.
3. Vortex.
4. Radioimmune precipitation buffer (RIPA): 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5 % sodium deoxycholate, 1 % Triton X-100, 0.1 % SDS.
5. Protease and phosphatase inhibitor cocktail: 10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 2 mM sodium fluoride.

2.4 Motility, Migration, Invasion, and Anoikis Assays

1. 24-Well plates and 6-well plates.
2. Hemocytometer.
3. Sterile 200 µL pipette tips.
4. 8.0 µM pore cell culture inserts.

5. Extracellular matrix (ECM) gel (Sigma-Aldrich, St Louis, MO, USA).
6. 95 % ethanol solution: 95 mL ethanol and 5 mL deionized H_2O.
7. Poly-HEMA (Sigma-Aldrich, St Louis, MO, USA): 12 mg/mL solution. Add 1.2 g of poly-HEMA to 100 mL 95 % ethanol.
8. Crystal violet: 0.25 % solution in distilled H_2O.
9. Acetic acid: 33 % solution in distilled H_2O.
10. Alamar blue (Bio-Rad, Hercules, CA, USA).
11. Aluminum foil.
12. Microplate reader capable of detecting absorbance at 570 nm.

3 Methods

3.1 Generation of In Vitro Models of RTKI Resistance

1. Seed SKBR3 cells into two 25 cm^2 tissue culture flasks.
2. Dissolve lapatinib in sterile DMSO to a concentration of 10 mM.
3. Prepare a working stock of 10 μM (1:1,000 dilution of the 10 mM stock) using RPMI medium. Further dilutions may be required if initial treatments for drug selection are low.
4. When cells have reached sub-confluency (approximately 60–70 % confluency) drug selection can begin.
5. One flask of SKBR3 cells is treated with a low dose of lapatinib, (we began selection with 5 nM lapatinib; it is important however to determine the IC_{50} of the cell line in question before deciding an appropriate initial treatment concentration—*see* **Note 1**) and the other flask will be remain untreated to be used as an age-matched control. Drug treatment should be prepared in a universal container and mixed before adding to the flask.
6. Cells should be maintained at the same drug concentration and medium changed every 48–72 h until the cells begin to grow and become confluent.
7. Confluent cells should be subcultured using 2–3 mL of trypsin incubating at 37 °C for 3–5 min to detach cells. Trypsinized cells are neutralized in equal volumes of culture medium and centrifuged for at 200 × *g* for 5 min. The cell pellet is resuspended in 1 mL of medium and 1:2 is reseeded into a new flask.
8. The following day, when cells have attached, medium may be changed and drug added as appropriate. At this stage the drug concentration used for selection may be increased in a stepwise fashion, generally doubling the concentration after each passage (*see* **Note 2**).

9. Stocks of the final selected concentration should be frozen down along with stocks of the aged matched untreated cells. All subsequent assays, as described below should be performed on stocks within ten passages of each other, to avoid variability due to ageing of cells.

3.2 Assessment of RTKI Resistance

After cells have been growing at the desired concentration of the RTKI for several months, it is important to determine the approximate IC_{50} concentration of the resistant cells to determine the level of resistance induced compared to the age-matched parent cells. Furthermore, the potential development of a multidrug resistance phenotype of the cells should be assessed to determine whether or not cross resistance may occur to other similar RTKIs.

In this section we describe the acid phosphatase assay as a means of performing these toxicity assays for lapatinib, as well as other RTKIs that may be assessed for cross-resistance.

Cells should be fed with fresh culture medium the day before seeding assays and should be no more than 70–80 % confluent. We describe seeding densities and conditions for the SKBR3 lapatinib-resistant cells (SKBR3-LR) and their age-matched controls (SKBR3-Ag). Seeding densities/conditions may need to be optimised for other cell lines.

1. Detach the cells with trypsin-EDTA solution and centrifuge the cell suspension to obtain a pellet. The resulting pellet of cells should be resuspended in 5 mL of fresh medium. Count the cells using a hemocytometer and prepare a dilution of cells to a final concentration of 5×10^4/mL.
2. To seed the lapatinib-resistant cells (SKBR3-LR) and age-matched parent cells (SKBR3-Ag), dispense 100 μL of the 5×10^4/mL cell suspension using a multichannel pipette into each well of a 96-well plate, leaving the first lane empty (this will be used as a blank lane).
3. Gently shake the plate to ensure that the cells have spread evenly in each well and then place the cells in the incubator at 37 °C/ 5 % CO_2 overnight to allow the cells to attach.
4. The following day, examine the plated cells under the microscope to ensure that they have attached to the base of the well. Then proceed to making up a range of dilutions of your drug of interest (in this case lapatinib) by serial dilution. In the first lane after the blank, place 100 μL of medium into each well (*see* **Note 3**). In the next lane, add the drug dilutions beginning with the lowest and working up to the highest at the final lane in the plate.
5. Replace the plate back in the incubator for 5 days.
6. After 5 days of incubation, examine the cells under the microscope and then perform the acid phosphatase assay.

7. Remove the medium from all the wells and wash the wells twice with 100 μL PBS.
8. Add 100 μL of freshly prepared p-nitrophenol phosphate to each well.
9. Wrap the plates in aluminium foil and place back in the incubator for 2 h.
10. After incubation the reaction is stopped by adding 1 M NaOH and the wells turn to yellow. Read the plate at 405 nm, with 620 nm as reference wavelength (*see* **Note 4**).

3.3 Sample Preparation for Immunoblotting

The expression of some RTKs themselves may be altered with the onset of drug resistance. Using the cells with acquired resistance to lapatinib, (as mentioned in Subheading 3.1) we describe the preparation of samples for immunoblot analysis of the RTK targets of lapatinib, HER2, and EGFR.

1. To compare RTK expression in lapatinib-resistant compared to parent-sensitive cells; flasks for both cell line variants should be seeded on the same day and be of a similar passage number.
2. Remove medium from a sub-confluent flask and rinse twice in ice-cold PBS.
3. Pipette 1 mL of ice-cold PBS into the flask and using a cell scrapper, detach the cells into the PBS.
4. Remove the cell suspension in PBS, place in a 1.5 mL microcentrifuge tube, centrifuge at 7,500 × *g* for 5 min to pellet cells, and remove the supernatant.
5. Resuspend the resulting pellet in 100–1,000 μL of RIPA buffer containing appropriate protease and phosphatase inhibitors (*see* **Note 5**).
6. Place the suspension on ice for 30 min, vortexing every 10 min.
7. Centrifuge the lysates at 13,000 × *g* for 15 min at 4 °C and transfer the resulting supernatant into a fresh tube which can be stored for long term at −80 °C.
8. Proceed to quantify protein, run samples for gel electrophoresis and subsequent immunoblotting. For assessing the expression of HER2 (~185 kDa) and EGFR (~175 kDa), samples should be run on 7.5 % gels.

3.4 Characterization of RTK-Resistant Cell Line Models

In addition to the development of drug resistance, altered RTK expression may in turn alter other phenotypic characteristics of cells. An aggressive cancer phenotype has been associated with increased cellular migration and invasion and the ability to survive in suspension enabling cells to metastasize though the bloodstream [23]. In order to fully understand the role of RTKs with drug resistance, it is important to characterize the subsequent effects on cells that may be induced as a result of the development of drug resistance. In this

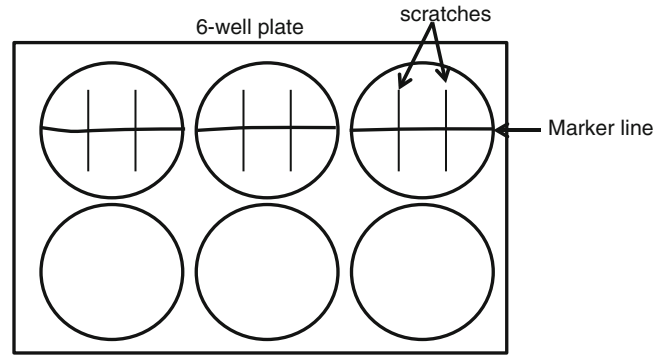

Fig. 1 Schematic layout of wound healing assay in a 6-well plate

section we discuss some in vitro methods of characterizing cells in terms of motility (by wound healing), migration and invasion (through cell culture inserts), and *anoikis*. For purpose of these assays we describe seeding densities and conditions for the SKBR3 lapatinib-resistant cells and their age-matched controls. Seeding densities/conditions may need to be optimised for other cell lines.

3.4.1 Wound Healing Assay

1. Seed the cells into 6-well plates, allowing 3 wells for each cell line variant. Prior to seeding, draw a line horizontally across the bottom of each well on the outside of the plate.
2. When cells have reached at least 90 % confluence, cells should be washed twice with PBS and then low serum (0.1–1 %) containing medium is added to each well (*see* **Note 6**).
3. The following day, using a sterile 200 μL pipette tip, make a scratch across the monolayer of cells moving from one side of the well to the other crossing the marker line (Fig. 1)
4. Wash cells (very gently so as not to remove the cells) with PBS and replace with 1.5 mL of low-serum medium.
5. Photograph the cells using phase-contrast microscopy at 10× field of view, at each wound made just above and just below the marker line; this will be time 0 (0 h) (*see* **Note 7**).
6. Resulting wounds may be measured using an arbitrary distance function available on programs such as Image J and Cell A software. The measurements may be calculated as a percentage of that taken at 0 h.

3.4.2 Migration and Invasion Assays

For migration and invasion assays the general protocol is the same; however for migration, the cells are seeded into inserts that have not been coated with ECM.

1. On the day prior to seeding invasion assays, place cell culture inserts into a 24-well plate. Wrap the plate in parafilm and incubate at −20 °C for at least 1 h. Sterile 200 μL pipette tips should also be incubated at −20 °C.

2. Dilute the ECM to 1 g/L using culture medium.
3. Pipette 100 μL of the 1 g/L ECM into each of the inserts (*see* **Note 8**).
4. The coated inserts in the 24-well plates should be placed at 4 °C overnight.
5. The following day, prior to seeding cells, incubate the plate (containing coated inserts) at 37 °C for at least 1 h.
6. Remove the excess ECM from the coated inserts and wash the inserts three times with serum-free medium or PBS.
7. Trypsinize and count cells and prepare a dilution of cells to the concentration of 1×10^6 cells/mL. Seed cells in a volume of 500 μL in each insert and place 500 μL of medium containing 10 % FBS in the well beneath the insert (*see* **Note 9**).
8. Allow the cells to migrate or invade for 72 h. Time may vary according to the cell line used.
9. Gently wash the inserts and plates three times with PBS. Using a PBS-soaked cotton swab, wipe the inside of the insert to remove any cells that have not migrated or invaded, taking care not to disturb the cells on the outside of the insert.
10. Place the inserts back into the washed plate and add 250 μL of 0.25 % crystal violet into each well beneath the insert.
11. Incubate the inserts in the crystal violet for 10 min on a rocker.
12. Wash the inserts three times with PBS and then allow to air-dry overnight.
13. Photograph the stained insert under a microscope in 10× field of view (Fig. 2).
14. To quantify the level of migration/ invasion, elute the crystal violet. Place 250 μL of 33 % acetic acid into each well with the corresponding insert for that well. Incubate the plates for approx. 10 min. Transfer 50 μL of each well into 3 wells of a 96-well plate, and read plates at 570 nm.

3.4.3 Anoikis Assay

Anoikis is a form of apoptosis induced by the loss of cell anchorage. Resistance to *anoikis* encourages anchorage-independent growth of cells, which inevitably promotes tumorigenesis and in particular the ability of cells to survive in and metastasize through the bloodstream [24].

1. Two days prior to seeding for the *anoikis* assay, coat half a 24-well plate with poly-HEMA made up to a concentration of 12 g/L in 95 % ethanol (200 μL/ well). The other half of the plate should be coated with 95 % ethanol only which will be used as a control.
2. Leave the coated plate to air-dry in a sterile laminar flow cabinet overnight.

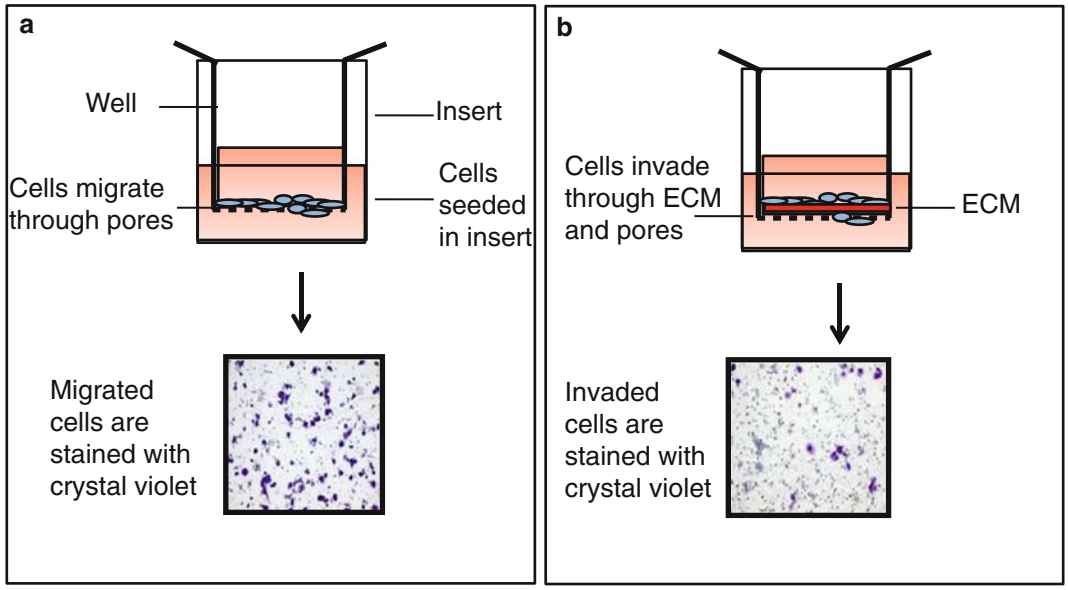

Fig. 2 Schematic layout of migration (**a**) and invasion (**b**) assays and the images of migrated/invaded cells stained with crystal violet

3. The following day, coat the plates a second time, ensuring that the first coat has dried fully repeating the coating.
4. On day 3, rinse the plates twice with PBS (*see* **Note 10**).
5. Trypsinize and count cells and seed at a density of 1×10^4/mL/well onto the poly-HEMA and ethanol-coated plates.
6. Incubate cells for 48 h at 37 °C/5 % CO_2
7. Remove the plate from the incubator and add 100 μL of alamar blue directly on top of the 1 mL medium of each well (i.e., 1:10 dilution). Cover plates with aluminum foil and return them to the incubator for 5 h (*see* **Note 11**).
8. Remove plates, check for color change (*see* **Note 12**), and read plates at 570 nm, with a reference wavelength of 600 nm.

4 Notes

1. Previous reports have recommended starting at concentrations at least 10–20 % below the IC_{50} of the drug in question [25]. This will depend on the drug and cell line of use. It is important that both the untreated and drug-selected flasks are seeded from the same original flask of cells and maintained parallel in culture for the duration of generating the resistant cell line variant. Age-matched untreated cells act as a direct control for subsequent studies avoiding any differences that may be due to older versus younger passage numbers.

2. Depending on the drug, the development of a drug resistant cell line variant may take several months. In the case of lapatinib-selected SKBR3-resistant cells, approximately 6 months of sub-culturing in increased stepwise treatments of lapatinib (to a final concentration of 250 nM) was performed.
3. Adding 100 μL to each well will make a final volume of 200 μL per well, considering that 100 μL was used to seed the cells the previous day. When preparing drug dilutions for treating the plate, it is important to take into account the dilution affect from adding 100 μL of drug into each well that already contains 100 μL; in this case drug concentrations should be prepared twice that of the desired treatment.
4. Do not read the plate if there are bubbles in some of the wells, as results may be skewed. If this is the case, plates may be stored overnight at 4 °C. The following day the bubbles should be gone and the plate can then be read.
5. Depending on the size of the cell pellet it may be necessary to adjust the amount of RIPA buffer added to lyse the pellet. Larger cell pellets may require more buffer to perform lysis; however for smaller cell pellets it may be necessary to reduce the amount of lysis buffer so that the resulting protein is not too diluted.
6. The marker should be just at the edge of the photo; this will be a reference point for subsequent measurements of the wounds over the time points.
7. The number of time points you photograph will depend on the cell line; very motile cells will require photographing at 6, 12, and 24 h, whereas slower cells may be photographed at 24, 48 h, etc. Change the medium before every time point. When comparing two cell line variants (in this case a sensitive and resistant variant) all time points must be taken at the same time for both cell lines so that they are comparable.
8. It is necessary to work quickly when pipetting the ECM using ice cold pipette tips. The ECM tends to solidify quickly and it is important to avoid bubbles from forming when pipetting the ECM into the insert.
9. For cell lines that are not very motile it may be necessary to provide a migration/invasion stimulant. In this case, seeding the cells in low serum medium in the insert and having the regular 10 % FBS medium beneath the insert will suffice. Importantly, when comparing two cell line variants (e.g., sensitive versus resistant cells) the same conditions must be set up for both cell lines. If the proliferation rates of the two cell line variants are different then low-serum medium would help minimize the risk of differences observed as a result of altered proliferation.

10. Plates must be completely dry before rinsing in PBS.
11. Longer incubation time may be necessary depending on the cell line and cell number; this will need to be optimized as relevant.
12. Resazurin is the active ingredient of alamar blue which converts from an oxidized (blue color) to a reduced (red/pink color) in response to chemical reduction of growth medium resulting from cell growth.

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