Lung Cancer 108 (2017) 96–102

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Synergy between next generation EGFR tyrosine kinase inhibitors and miR-34a in the inhibition of non-small cell lung cancer

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ARTICLE INFO

Article history:
Received 11 November 2016
Received in revised form 7 February 2017
Accepted 25 February 2017

Keywords:
MicroRNA
miR-34a
Erlotinib
Afatinib
Osimertinib
Rociletinib

ABSTRACT

Objectives: EGFR tyrosine kinase inhibitors (TKIs) are widely used to treat NSCLC, primarily patients with activating mutations, with more limited response in wild-type disease. However, even with EGFR-mutated disease, many patients fail to respond, most who initially respond fail to respond completely, and almost all develop resistance and inevitably progress. New therapeutic options that improve these outcomes could provide substantial clinical benefit. We previously demonstrated strong synergistic effects between erlotinib and the tumor suppressor microRNA miR-34a, sensitizing NSCLC cells with primary resistance (EGFR wild-type) and restoring sensitivity in cells with acquired resistance. Here, we report results of further research combining miR-34a with newer generation EGFR-TKIs in similar experiments.
Materials and methods: Human NSCLC cell lines with varying degrees of primary and acquired resistance to erlotinib were assessed for sensitivity to a broad set of combined doses of miR-34a mimic and afatinib, rociletinib or osimertinib. Multiple analytical approaches were used to characterize effects on cancer cell proliferation as additive, antagonistic or synergistic.
Results: Mimics of miR-34a synergized with afatinib, rociletinib or osimertinib in all EFGR-mutant cells tested. Best and consistently strong synergy was observed in cell models with acquired resistance. Synergy was also evident in most EGFR wild-type cells with miR-34a combined with rociletinib and osimertinib, but not with afatinib. The effects were observed across a broad range of dose levels and drug ratios, with maximal synergy at doses yielding high levels of inhibition beyond those possible to be induced by the single agents alone.
Conclusion: Combined miR-34a and EGFR-TKIs synergistically sensitize both EGFR wild-type and mutant NSCLC cells, supporting clinical investigation of these combinations as a strategy to overcome both primary and acquired resistance to EGFR-TKIs in NSCLC, possibly with an improved therapeutic index.

1. Introduction

Tyrosine kinase inhibitors (TKIs) blocking epidermal growth factor receptor (EGFR) are widely used as monotherapies to treat patients with advanced (Stage IV) non-small cell lung cancer (NSCLC), primarily patients with adenocarcinoma and EGFR activating mutations, with additional indications as maintenance therapy and in previously-treated patients regardless of EGFR mutation status [1]. Currently approved EGFR-TKIs include the first-generation compounds erlotinib (Tarceva®) and gefitinib (Iressa®), the second-generation, irreversibly-binding afatinib (Gilotrif®), and most recently, the third-generation osimertinib (Tagrisso™/AZD9291), which is effective against EGFR-TKI inhibitor-sensitizing mutations and the T790 M resistance mutation that underlies most cases (~50–60%) of acquired resistance to earlier generation EGFR-TKIs [2–4]. Another third-generation compound, rociletinib (CO-1686), has shown promising efficacy and safety in patients with T790M-associated disease in a phase I/II trial but has recently been withdrawn from an ongoing randomized phase III trial [5]. As with most targeted agents, even with EGFR-mutated disease and the later-generation agents, many patients fail to respond, most who initially respond fail to respond completely, and almost all develop resistance and inevitably progress [3,6–8]. Therefore, new therapeutic strategies that improve upon these outcomes could provide substantial clinical benefit.

MicroRNAs (miRNAs) are naturally occurring, short non-coding RNAs that modulate the expression of hundreds of genes across dis-

Abbreviations: EGFR:, epidermal growth factor receptor; TKI:, tyrosine kinase inhibitor; miRNA:, microRNA; DRI:, dose reduction index; CI:, combination index; NSCLC:, non-small cell lung cancer.

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http://dx.doi.org/10.1016/j.lungcan.2017.02.020
0169-5002/

tinct cellular pathways, governing a plethora of cellular processes including tumor suppression [9–11]. The tumor suppressor miR-34a represses the expression of >30 oncogenes as well as genes involved in tumor immune evasion (e.g. PD-L1, DGKζ) [12–15]. The potential ability to simultaneously repress genes across multiple oncogenic pathways makes miR-34a and other tumor suppressor miRNAs interesting drug candidates [16]. The broad effects also support the rationale for combining miR-34a with standard anticancer therapies, such as TKIs which are frequently subject to primary and acquired resistance in the clinic. Previously, we performed studies that demonstrated strong synergistic effects between miR-34a and the first-generation EGFR-TKI erlotinib, sensitizing NSCLC cell lines with both primary (EGFR wild-type) and acquired erlotinib resistance [17]. Our results also suggested an improved therapeutic index, showing far greater activity for the combined agents than could be achieved by either agent alone. Here, we report the results of further in vitro research combining miR-34a with the newer generation EGFR-TKIs afatinib, rociletinib and osimertinib in similar experiments.

2. Materials and methods

2.1. Cell culture

Human non-small cell lung cancer (NSCLC) cell lines with varying EGFR gene status and sensitivities to erlotinib were used to assess the combinatorial effects of miR-34a with second and third generation EGFR-TKIs. These include cell lines with primary erlotinib resistance (A549, H460, H1299, H226; all EGFR-wild-type), a cell line with secondary erlotinib resistance (H1975, EGFR-L858R/T790M), and a cell line sensitive to erlotinib (HCC827, EGFR-delE746-A750). In addition, HCC827^{res} cells with acquired erlotinib resistance were used [17]. HCC827^{res} cells were further clonally expanded to generate the individual cell lines including HCC827-RC2. With the exception of HCC827-RC2, all cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All cells were maintained in RPMI-1640 medium with 10% fetal bovine serum at 37 °C in a humidified chamber containing 5% CO_2.

2.2. RNA isolation and qRT-PCR

Total RNA from clonal HCC827-RC cells was isolated from cell pellets using the mirVana PARIS RNA isolation kit (Ambion, Austin, TX). For the quantification of miRNA and mRNA by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), commercially available reagents were used. Total RNA was converted to cDNA using MMLV-RT (Invitrogen, Carlsbad, CA) and used in qRT-PCR as described in [17]. Expression levels of miRNA and mRNA were expressed as percent expression relative to levels in the HCC827 parental cell line. The following assays purchased from Thermo Fisher were used: hsa-miR-34a, 000426; AXL, Hs01064444_m1; EGFR, Hs01076090_m1; ERBB3, Hs00176538_m1; FGFR1, Hs00915142_m1; HGF, Hs00300159_m1; KRAS, Hs00364284_g1; PIK3CA, Hs00907957_m1; GAS6, Hs01090305_m1; MET, Hs01565576_m1; EGFR-T790 M, Hs00000106_mu; GAPDH, Hs99999905_m1; PPIA, Hs99999904_m1.

2.3. EGFR-T790M mutation analysis

DNA was isolated using the QIAamp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA was quantified using a NanoDrop-ND 1000 (Thermo Fisher Scientific Inc., Boston, MA) and diluted to 5 ng/μL. Primers and probes specific for the T790M mutation were as follows: forward primer, 5′-AGGCAGCCGAAGGGCA-3′; reverse primer, 5′-CCTCACCTCCACCGTGCA-3′; wild-type probe, 5′-VIC-CTCATCACGCAGCTCATG-MGB-3′ and mutant probe, 5′-FAM-CTCATCATGCAGCTCATG-MGB-3′ as previously described in [18]. Using 2 μL of diluted DNA template, amplification reaction size was 15 μL with primers, probes and PCR master mix concentrations as suggested by qPCR protocols for SNP genotyping (Applied Biosystems). Samples were incubated at 95 °C for 10 min, then 40 cycles of 15 sec at 95 °C and 1 min at 65 °C in an Applied Biosystems 7900 HT Fast Real-Time PCR System. As a positive control and to discriminate between the EGFR wild-type and the c.2369C>T (T790M) mutation, we used the H1975 cell line which encodes the EGFR-T790 M mutation.

2.4. miRNA and EGFR-TKIs treatment

Afatinib was purchased from LC Laboratories (Woburn, MA), rociletinib (CO-1686) and osimertinib (AZD9291) were purchased from Selleckchem (Houston, TX). Synthetic, double stranded versions of miR-34a were manufactured by Life Technologies (Ambion, Austin, TX). IC_{50} values of single agents were determined as previously published [17]. Briefly, miR-34a oligos were reverse-transfected in a serial dilution (0.03–30 nM) using RNAiMax lipofectamine (Life Technologies). As negative controls, cells were also transfected with RNAiMax alone (mock). Cells were incubated with AlamarBlue (Invitrogen) four days post transfection to determine cellular proliferation. AlamarBlue is a mitochondrial function stain that is converted by metabolically active and proliferating cells into a fluorescent product. Because data produced by this assay directly correlate with the number of viable cells, data are a direct read-out of proliferation and an indirect read-out of pathways that lead to cell killing such as apoptosis. AlamarBlue data were normalized to mock-transfected cells. Afatinib, rociletinib or osimertinib prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO) was added to cells one day after seeding at a final concentration ranging from 0.0001 to 100 μM. Solvent alone (1% final DMSO) was added to cells in separate wells as a negative control. Three days later, cellular proliferation was measured by AlamarBlue and normalized to the solvent control. Each data point was done in triplicates, and each experiment was independently performed 2–3 times. Combination studies were carried out using a fixed single drug ratio (ratio = $IC50_{TKI}$ / $IC50_{miR\text{-}34a}$) reflecting approximate IC_{50} concentrations of TKI and miR-34a, and as well as at multiple fixed ratios in H1975 and HCC827-RC2 cells as previously published [17]. Cells were reverse-transfected with miR-34a, and afatinib, rociletinib or osimertinib was added one day thereafter to the medium. Three days post drug treatment, cellular proliferation was determined by AlamarBlue. Each data point was performed in triplicates, and combination studies were performed at least two times in each cell line.

2.5. Combination data analysis

Combination index (CI) values, isobologram, and curve shift analysis were described in detail in our previous publication [17]. Briefly, CI values derived from non-linear regression trend lines indicate additive (CI = 1), antagonistic (CI > 1), or synergistic (CI < 1) drug combinations at any given effect level (Fa, fraction affected; inhibition of cancer cell proliferation). Isobolograms describe the dose-dependent relationship of TKIs and miR-34a for drug concentrations that led to 50% inhibition of cancer cell proliferation (IC_{50}). Data points above or below the line of additivity indicate antagonism or synergy, respectively. In addition, dose reduction index (DRI) values are calculated to indicate by how much the concentration of each drug in the combination can be reduced (compared to its use as a monotherapy) to produce the same effect (e.g. Fa = 50%).