

Extratumoral Insulin-Like Growth Factor-1 Promotes Resistance To HER2-Targeted Tyrosine Kinase Inhibitors And Macrophage Polarization Through Redox Signaling

Neha Dutta^{1,2}, Gargi Mukherjee^{1,2} and Anindita Chakrabarty^{1,2*}

¹ *Carcinogenesis and Cancer Therapeutics Laboratory*

² *Department of Life Sciences, Shiv Nadar Institution of Eminence, NH91, Greater Noida, Uttar Pradesh, 201314, India*

*Corresponding author: anindita.ac@gmail.com; anindita.chakrabarty@snu.edu.in

Content:

Figure S1A-B: Measurement of transcript levels of CYP4503A5 in MDA-MB-453 and HepG2 cells after lapatinib and tucatinib treatment
Figure S2A-B: Titration of lapatinib and tucatinib doses for ROS detection
Figure S3A-B: Titration of IGF-1 dose and time for neutralization of lapatinib-induced ROS
Figure S4A-B: Correlation analysis of mitochondrial ROS, IGF-1 and IGF-1R transcript levels after lapatinib and tucatinib treatment
Figure S5A-B: Dose optimization of BEZ-235 and Camptothecin in MDA-MB-453 cells
Figure S6A-B: Correlation analysis of mitochondrial ROS, transcript levels of IGF-1 and IGF-1R and nuclear NRF2 expression after lapatinib and tucatinib treatment
Figure S7: Measurement of Keap1 and NRF2 transcript levels
Figure S8: Assessment of M1 and M2 markers in RAW264.7 cells treated with conditioned media from MDA-MB-453 cells
Figure S9: Evaluation of M1 and M2 markers in RAW264.7 macrophages treated with canonical stimuli
Figure S10: Evaluation of RAW264.7 macrophage-mediated immune clearance of IGF-1/BEZ-235/CT/Linsitinib-treated cancer cells
Figure S11: Evaluation of M1-activated THP1-mediated immune clearance of IGF-1/BEZ-235/CT/Linsitinib-treated cancer cells
Figure S12: Assessment of ROS production by M1-activated THP1 macrophages following IGF-1 treatment
Figure S13: Evaluation of immune clearance of cancer cells by M1-activated THP1 macrophages following IGF-1 treatment

Figure S14A-B. Measurement of mitochondrial ROS and cell viability in MDA-MB-453 cells co-cultured with MEFs.
Figure S15A-B. Measurement of mitochondrial ROS and cell viability in MDA-MB-453 cells post IGF-1R knockdown, co-cultured with MEFs.
Figure S16A-C. Assessment of mitochondrial ROS production, cell viability and transcript level of NRF2 and its downstream target genes in HCC1954 post-lapatinib treatment
Figure S17A-B: Assessment of mitochondrial ROS production in MCF10A and HepG2 cells following lapatinib and tucatinib treatment
Figure S18A-C: Original western blot images
Table S1: EnrichR analysis of lapatinib RD vs. pre-treatment gene expression data from GSE130788
Table S2: ShinyGO analysis of lapatinib RD vs. pre-treatment gene expression data from GSE130788
Table S3: List of qRT-PCR primers
Table S4: List of antibodies and drugs/inhibitors and their sources
Table S5-S9: p-values
Table S10: Gene correlation table IGF-1 vs M2
Table S11: Gene correlation table PIK3CA vs M2
Table S12: Gene correlation table NRF2/NFE2L2 vs M2
Supplemental Method

Figure S1A:

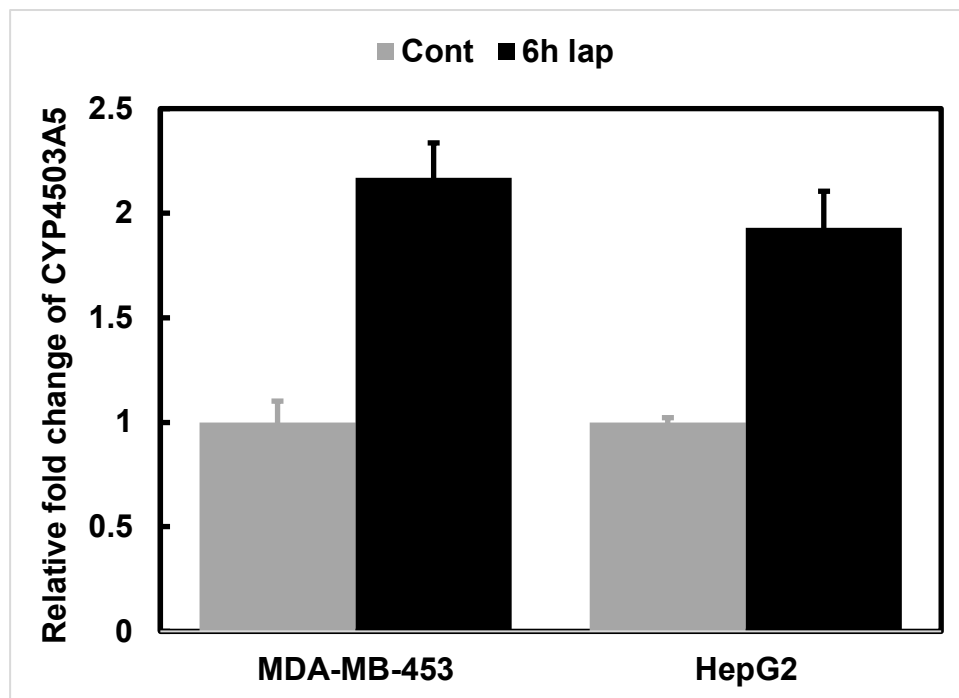


Figure S1B:

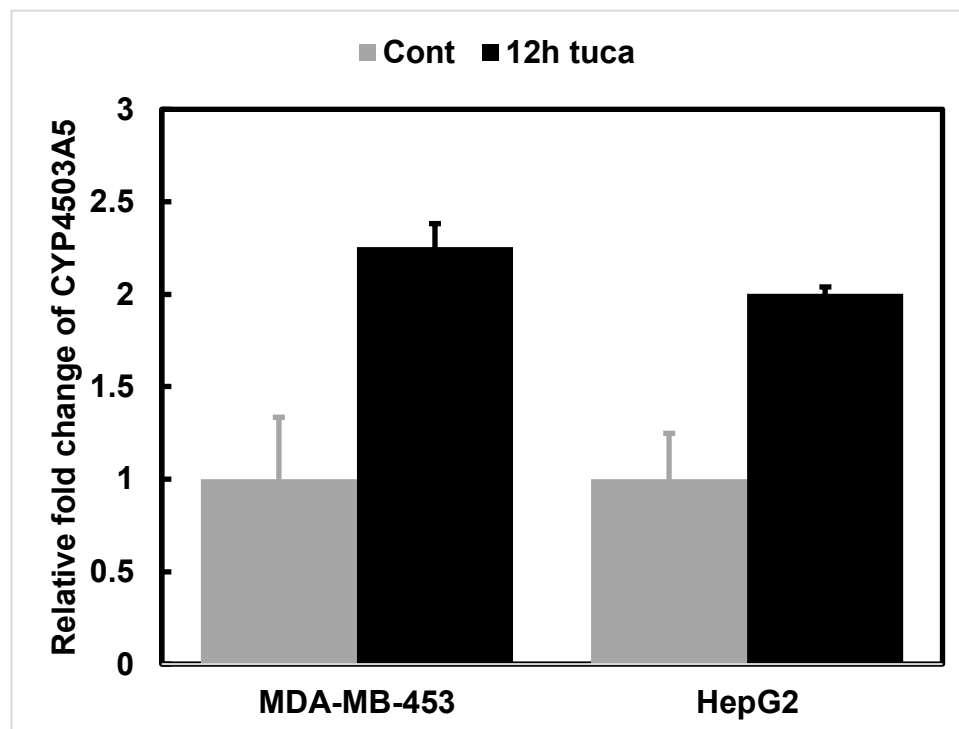


Figure S1: Measurement of transcript levels of CYP4503A5 in MDA-MB-453 and HepG2 cells after lapatinib and tucatinib treatment. MDA-MB-453 and HepG2 cells were treated with lapatinib for 6h (**A**) and tucatinib for 12h (**B**). Transcript levels of CYP4503A5 were measured by RT-qPCR using specific primers and normalized to actin. The p-values are: MDA-MB-453 control vs 6h lap=0.0003693; HepG2 control vs 6h lap=0.004699; MDA-MB-453 control vs 12h tuca= 0.009471; HepG2 control vs 12h tuca=0.03568.

Figure S2A:

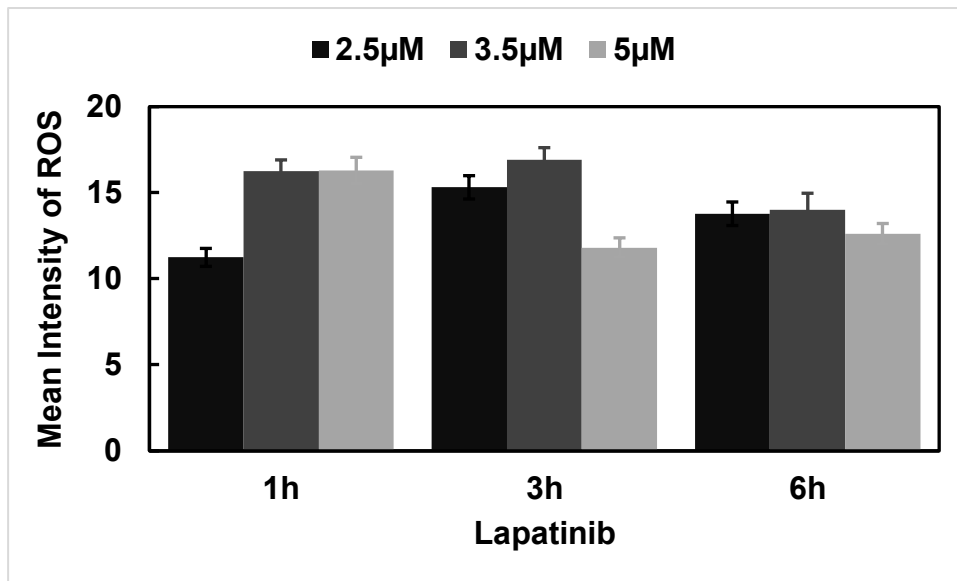


Figure S2B:

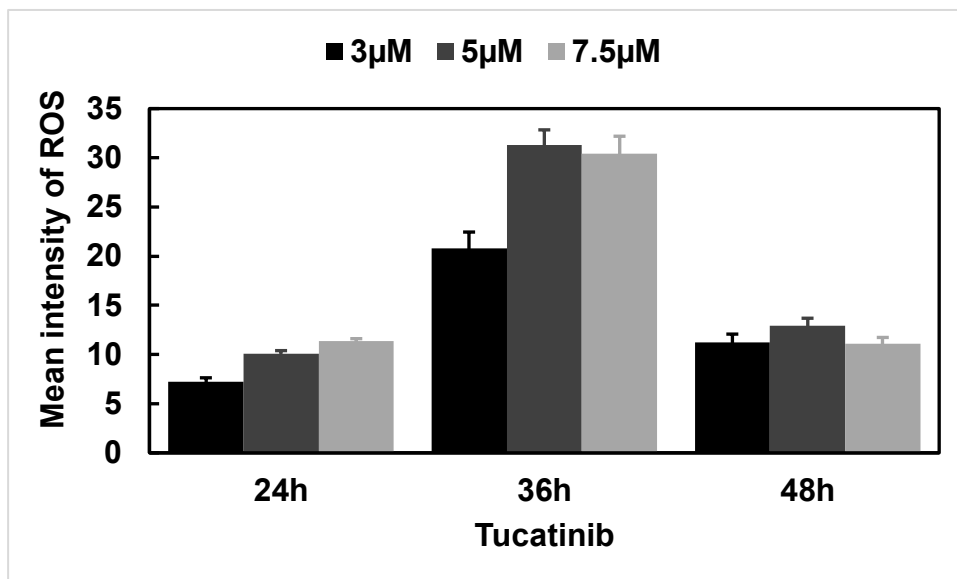


Figure S2: Titration of lapatinib and tucatinib doses for ROS detection. MDA-MB-453 cells were treated with varying concentrations of lapatinib (**A**) and tucatinib (**B**) for different time points before live cell imaging with MitoSox™ Red fluorescence dye to measure mitochondrial ROS production, as described in the Materials and Methods section.

Figure S3A:

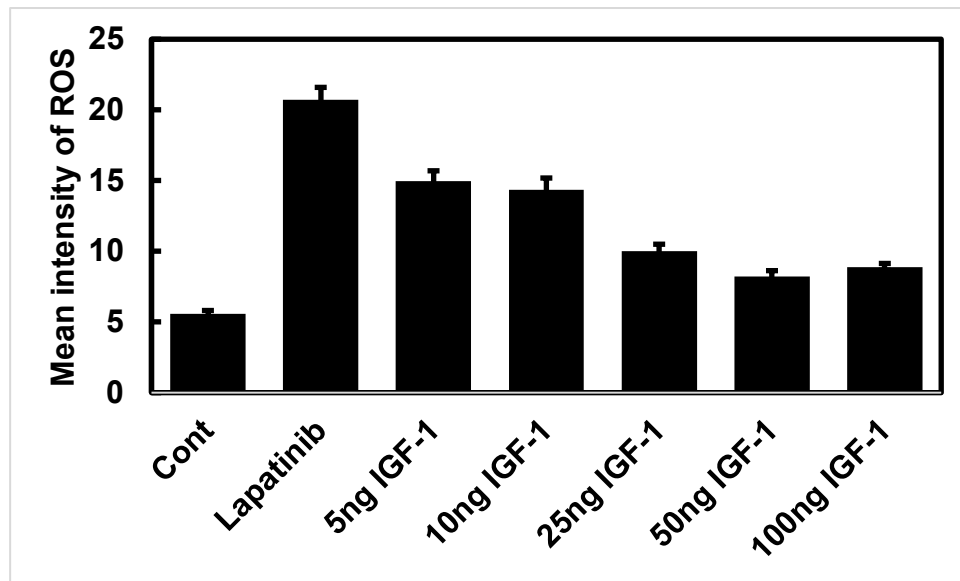


Figure: S3B:

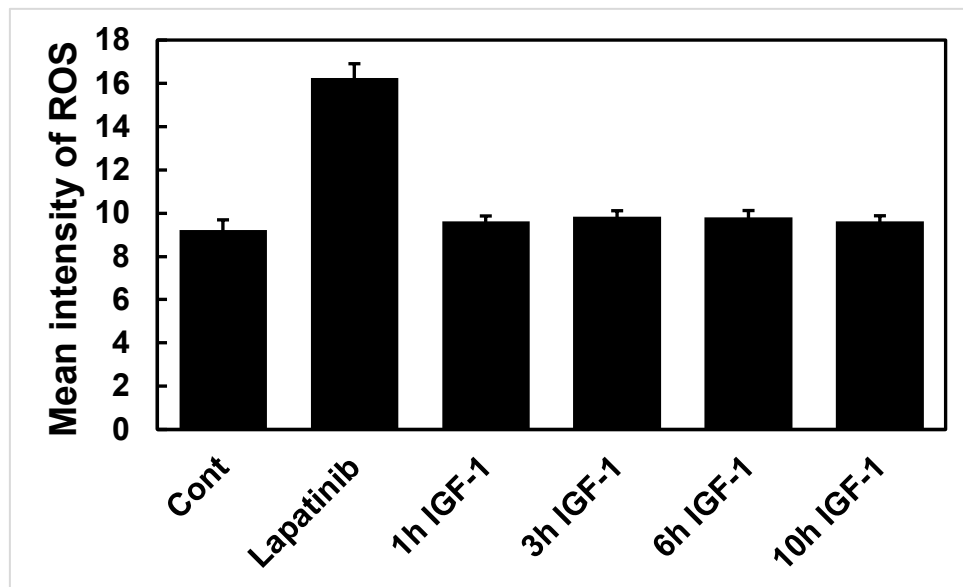


Figure S3: Titration of IGF-1 dose and time for neutralization of lapatinib-induced ROS.

MDA-MB-453 cells were pre-treated with different concentrations of recombinant IGF-1 (A) for different time points (B), followed by treatment with 3.5 μ M lapatinib for 1h. Mitochondrial ROS were measured using the MitoSoxTM red dye, as described previously.

Figure: S4A:

```
import pandas as pd

# Define your dataset
data = {
    'mtROS': [a,b,c,d,e,f],
    'IGF-1': [a,b,c,d,e,f],
    'IGF-1R': [a,b,c,d,e,f]
}

index = ['A', 'B', 'C', 'D', 'E', 'F']

# Create a DataFrame
df = pd.DataFrame(data, index=index)

# Calculate Pearson correlation coefficient
correlation_matrix = df.corr()

print("Correlation Matrix:")
print(correlation_matrix)
```

Figure: S4B:

Drugs	Parameters	Time in hours					
		0h	1h	3h	6h	10h	24h
Lapatinib	mtROS	9.207380952	16.23886	16.90138	14.02095	14.20071	10.48705
	IGF-1	1	1.196263	10.94188	12.22144	14.98342	1.515955
	IGF-1R	1	1.223816	11.62053	12.01015	4.092162	0.10145

Drugs	Parameters	Time in hours					
		0h	12h	24h	36h	40h	48h
Tucatinib	mtROS	4.57152381	8.70005	7.76081	31.2852	18.5827	12.9331
	IGF-1	1	0.87436	1.93289	2.54554	2.74464	0.81258
	IGF-1R	1	1.00357	1.61303	2.52014	1.64588	0.03786

Figure S4: Correlation analysis of mitochondrial ROS, IGF-1 and IGF-1R transcript

levels after lapatinib and tucatinib treatment. A. Python code used for correlation analysis:

“a,b,c,d,e,f” in the data section of the code reflects the values of each group at different time points and “A, B, C, D, E, F” in the index section of the code reflects the different time points.

B. Values of different groups after lapatinib and tucatinib treatment that were used for correlation analysis.

Figure S5A:

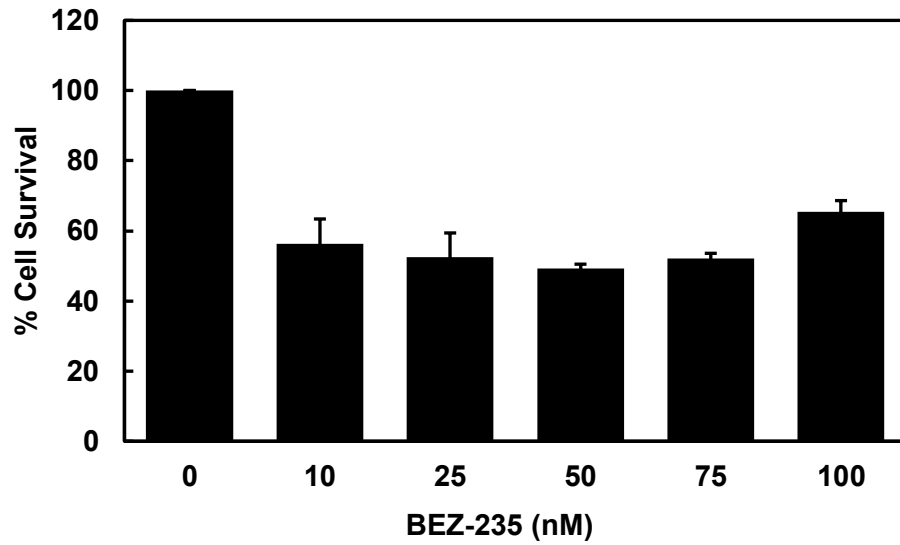


Figure: S5B:

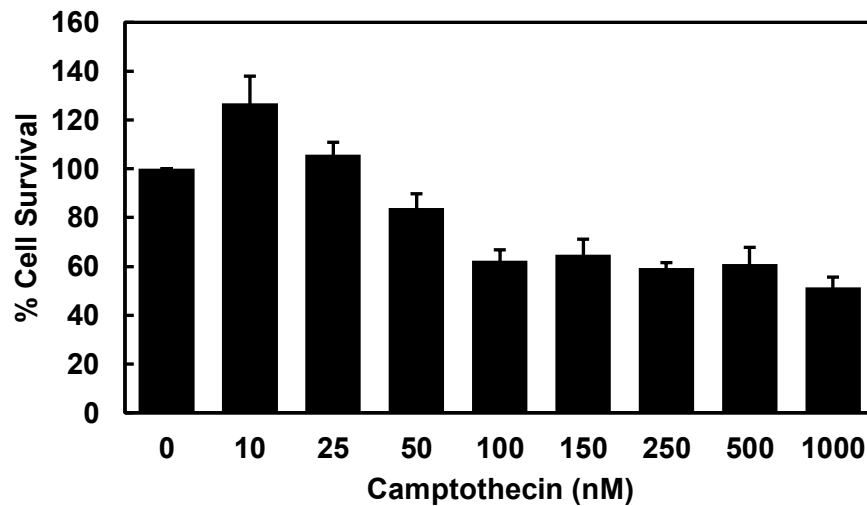


Figure S5: Dose optimization of BEZ-235 and Camptothecin in MDA-MB-453 cells. MDA-MB-453 cells were exposed to different concentrations of BEZ-235 (**A**) or Camptothecin (**B**) for 48h followed by MTT assay to measure the cell survival. Each bar graph shows the average of three replicates, with the calculated standard error.

Figure S6A:

```
import pandas as pd

# Define your dataset
data = {
    'mtROS': [a,b,c,d,e,f],
    'IGF-1': [a,b,c,d,e,f],
    'IGF-1R': [a,b,c,d,e,f]
    'NRF2': [a,b,c,d,e,f]
}

index = ['A', 'B', 'C', 'D', 'E', 'F']

# Create a DataFrame
df = pd.DataFrame(data, index=index)

# Calculate Pearson correlation coefficient
correlation_matrix = df.corr()

print("Correlation Matrix:")
print(correlation_matrix)
```

Figure: S6B:

Drugs	Parameters	Time in hours					
		0h	1h	3h	6h	10h	24h
Lapatinib	mtROS	9.207380952	16.23886	16.90138	14.02095	14.20071	10.48705
	IGF-1	1	1.196263	10.94188	12.22144	14.98342	1.515955
	IGF-1R	1	1.223816	11.62053	12.01015	4.092162	0.10145
	NRF2	6.833	9.4912	15.3098	14.9728	14.5298	9.9524

Drugs	Parameters	Time in hours					
		0h	12h	24h	36h	40h	48h
Tucatinib	mtROS	4.57152381	8.70005	7.76081	31.2852	18.5827	12.9331
	IGF-1	1	0.87436	1.93289	2.54554	2.74464	0.81258
	IGF-1R	1	1.00357	1.61303	2.52014	1.64588	0.03786
	NRF2	9.483	10.1892	19.7858	25.8346	27.3436	3.856

Figure S6: Correlation analysis of mitochondrial ROS, IGF-1 and IGF-1R transcript levels after lapatinib and tucatinib treatment. A. Python code used for correlation analysis: “a,b,c,d,e,f” in the data section of the code reflects the values of each group at different time points and “A, B, C, D, E, F” in the index section of the code reflects the different time points. **B.** Values of different groups after lapatinib and tucatinib treatment that were used for correlation analysis.

Figure S7A:

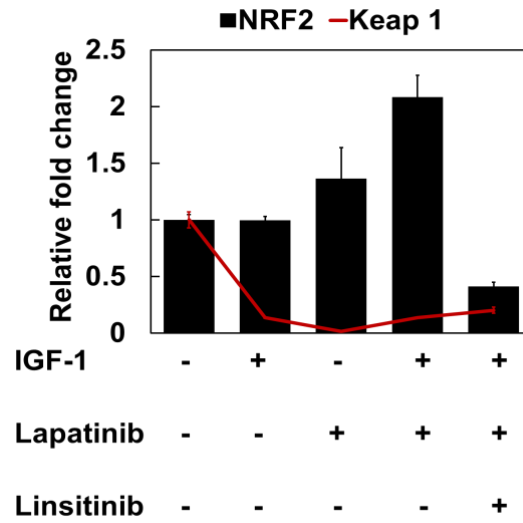


Figure S7B:

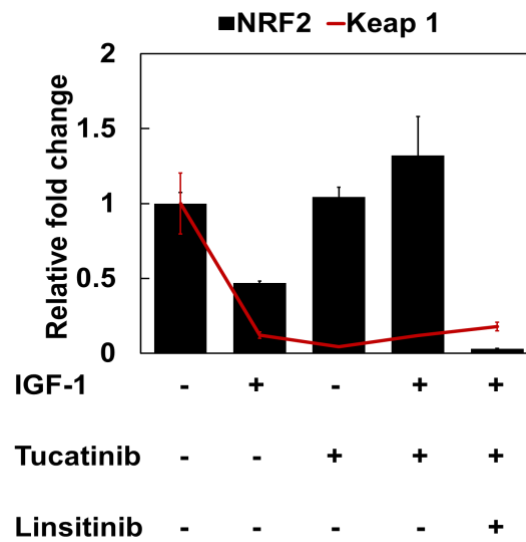


Figure S7. Measurement of Keap1 and NRF2 transcript levels. MDA-MB-453 cells were pretreated with IGF-1 with or without linsitinib for 6h, followed by treatment with lapatinib for 1h (A) and tucatinib for 36h (B). Transcript levels of Keap1 and NRF2 were measured using RT-qPCR with specific primers and normalized against actin. The p-values for Keap1 are: control vs lap=0.002235; control vs lap+IGF-1=0.04154; control vs lap+IGF-1+lin=0.3082; control vs tuca=0.002235; control vs tuca+IGF-1=0.04154; control vs tuca+IGF-1+lin=0.3082.

Figure S8:

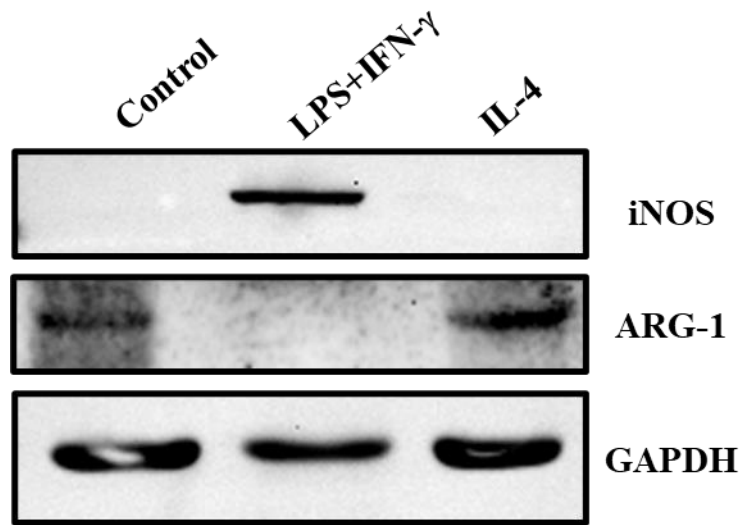
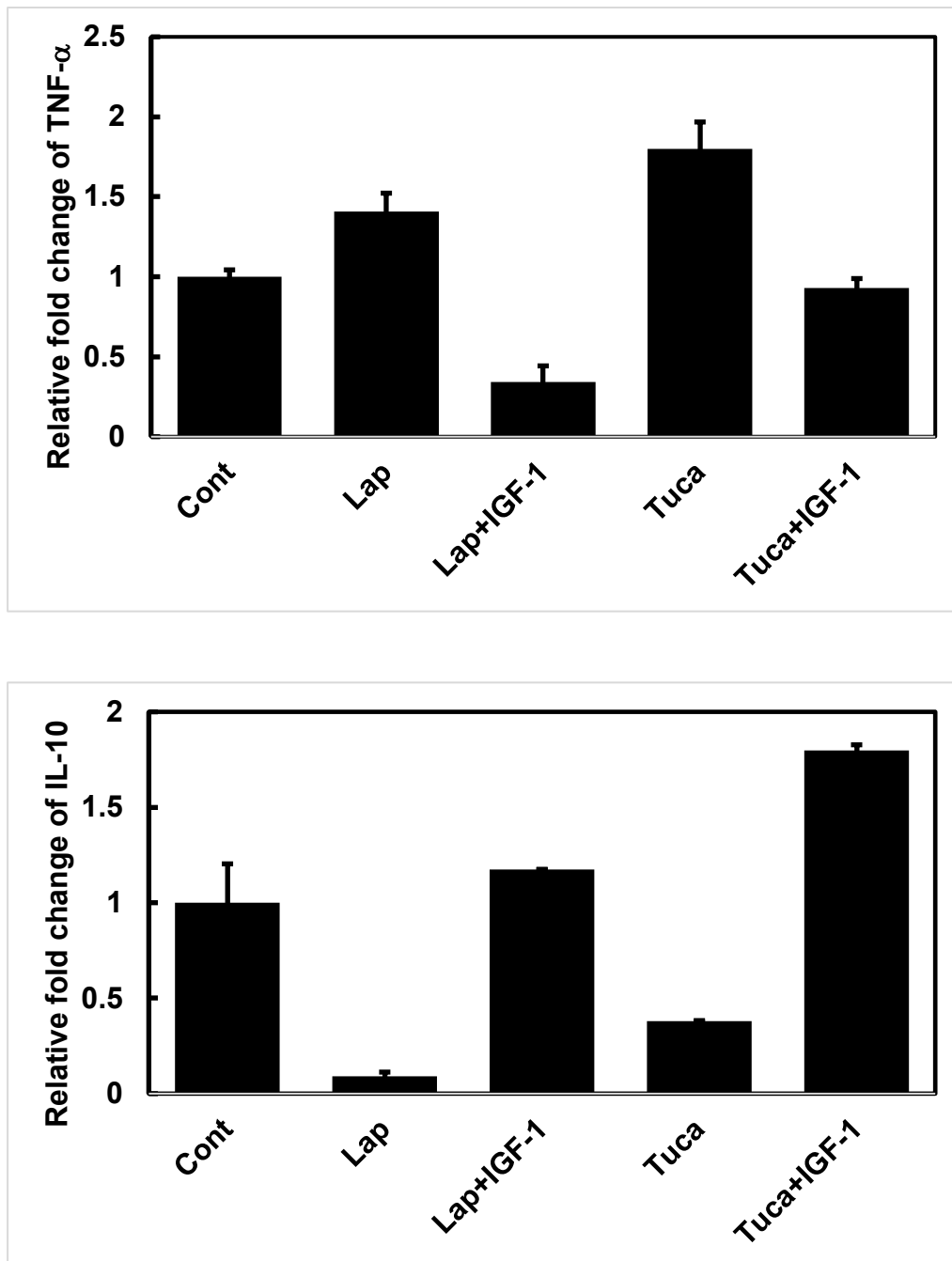


Figure S8: Evaluation of M1 and M2 markers in RAW264.7 macrophages treated with canonical stimuli. RAW264.7 cells were exposed to Lipopolysaccharide (LPS) and Interferon- γ (IFN- γ) to promote M1 macrophage polarization, and to Interleukin-4 (IL-4) to induce M2 macrophage polarization for 24h. Whole-cell lysates were collected, and proteins were extracted to measure iNOS and ARG-1 levels. Appearance of ARG-1 band in unstimulated control cells can be attributed to the presence of serum in the RAW264.7 culture media. Note that, it is increased after IL-4 stimulation.

Figure S9:



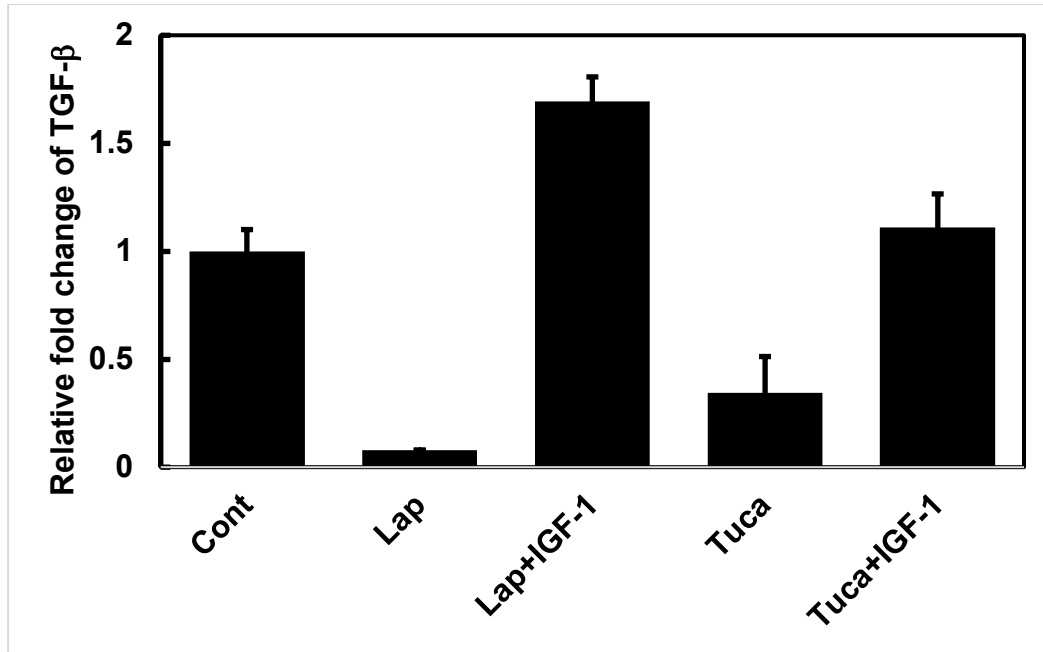


Figure S9. Assessment of M1 and M2 markers in RAW264.7 cells treated with conditioned media from MDA-MB-453 cells. After pretreatment with IGF-1, MDA-MB-453 cells were treated with lapatinib and tucatinib for 24h, followed by incubation with serum-free media for 48h for conditioned media (CM) collection. Naïve RAW264.7 macrophages were exposed to the CM for 24h. Transcript levels of M1 (TNF- α) and M2 (IL-10, TGF- β)-specific cytokines were measured by qRT-PCR with specific primers and normalized against actin. The p-values for TNF- α are: lap vs lap+IGF-1=0.0009459, tuca vs tuca+IGF-1=0.003512; p-values for IL-10 are: lap vs lap+IGF-1=0.05986, tuca vs tuca+IGF-1=0.01594, p-values for TGF- β are: lap vs lap+IGF-1=8.16E-05, tuca vs tuca+IGF-1=0.01114.

Figure S10:

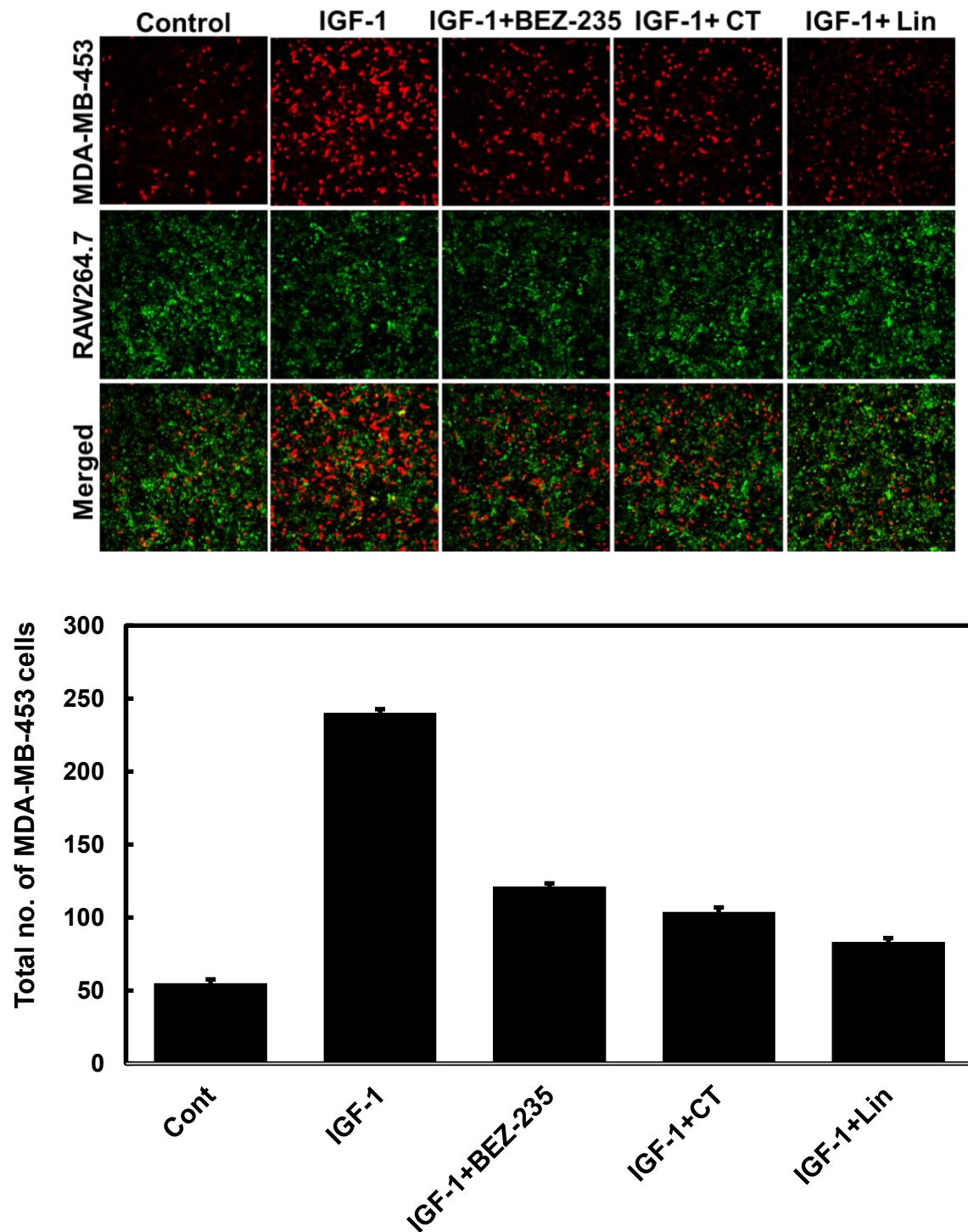


Figure S10: Evaluation of RAW264.7 macrophage-mediated immune clearance of cancer cells exposed to IGF-1 with or without blocking IGF-1R/PI3K/NRF2 axis. MDA-MB-453 cells were divided into five groups: one untreated and four treated with IGF-1 alone or in

combination with IGF-1R/ PI3K/ NRF2 inhibitors for 6h, labeled with CellTracker Orange. RAW264.7 cells were activated with LPS and IFN- γ for 24h and tracked with CellTracker Green. Labeled cancer cells and macrophages were co-cultured in a 1:1 ratio for 48h. The average number of MDA-MB-453 cells was counted to assess immune clearance (top panel=microscopic images and bottom panel=quantitation).

Figure S11:

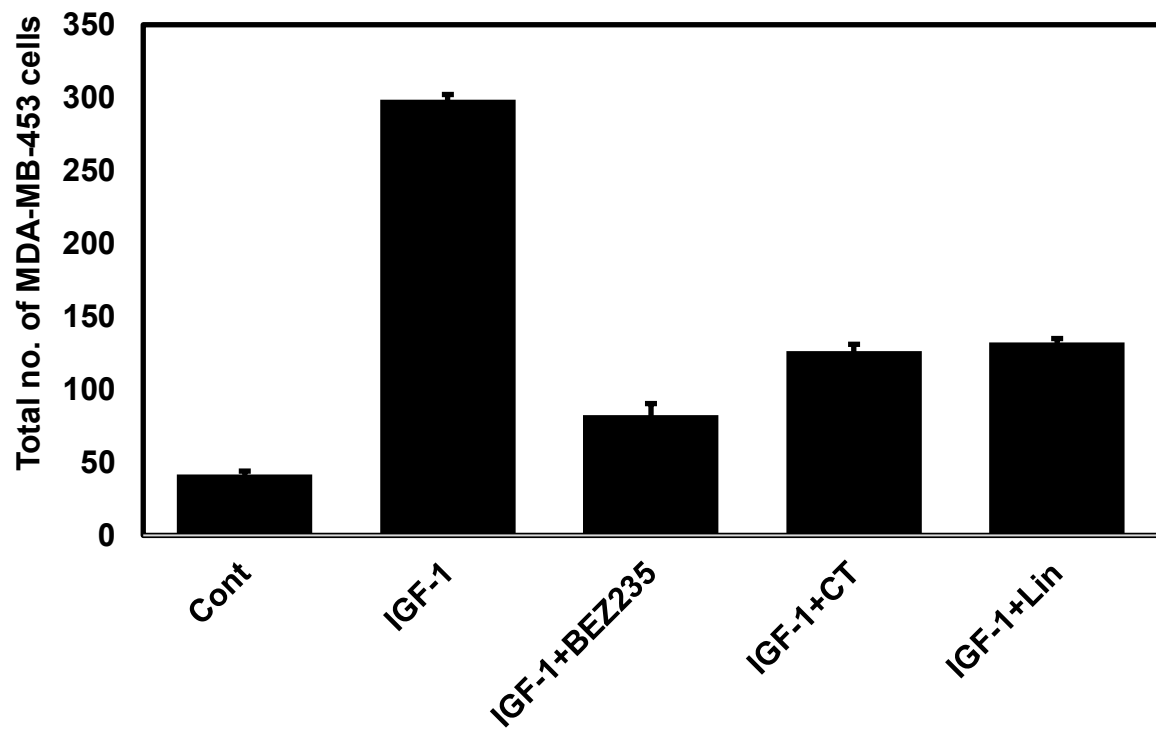
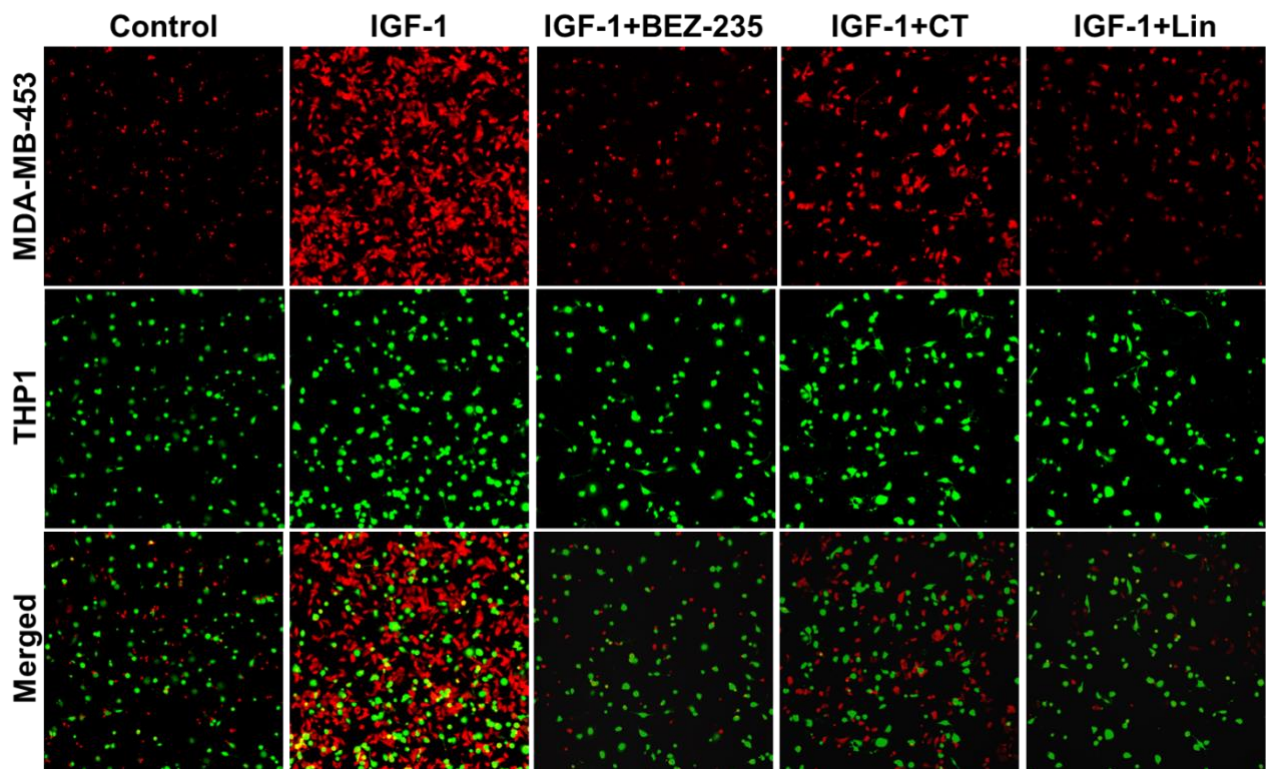


Figure S11: Evaluation of M1-activated THP1-mediated immune clearance of cancer cells exposed to IGF-1 with or without blocking IGF-1R/PI3K/NRF2 axis. MDA-MB-453 cells were divided into five groups: one untreated and four treated with IGF-1 alone or in combination with IGF-1R/ PI3K/ NRF2 inhibitors for 6h, labeled with CellTracker Orange. THP1 monocytes were first differentiated to M0 macrophages with 150nM PMA for 24h, cultured for 5 days, and further activated to M1 phenotype with LPS and IFN- γ for 24h and tracked with CellTracker Green. Labeled cancer cells and macrophages were co-cultured in a 1:1 ratio for 48h. The average number of cancer cells was counted to assess immune clearance.

Figure S12:

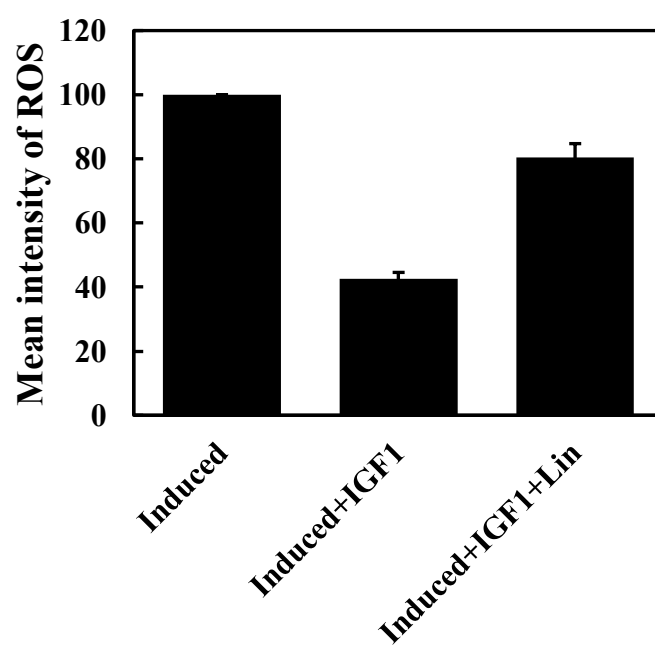
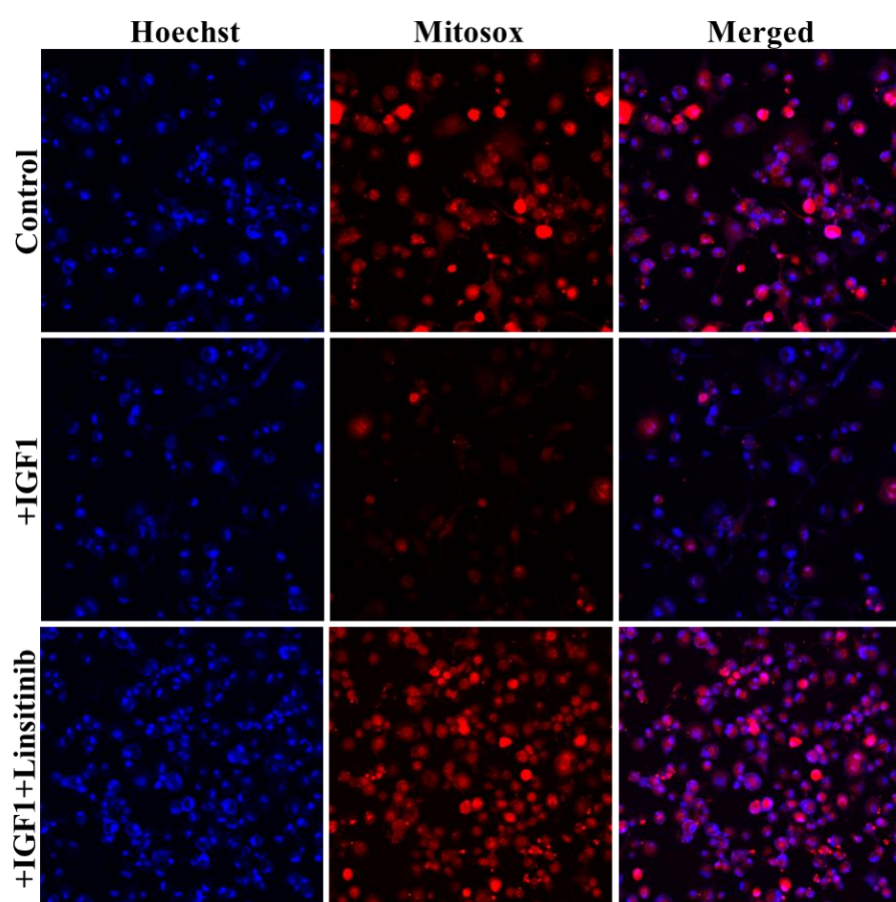


Figure S12: Assessment of ROS production by M1-activated THP1 macrophages following IGF-1 treatment. THP1 monocytes were first differentiated to M0 macrophages with 150nM PMA for 24h, cultured for 5 days, and further divided into three groups: one untreated and two pretreated with IGF-1 alone or with linsitinib, followed by activation with LPS and IFN- γ . Mitochondrial ROS was measured using the dye MitoSoxTM Red. Top panel represents microscopic images and bottom panel represents quantification.

Figure S13:

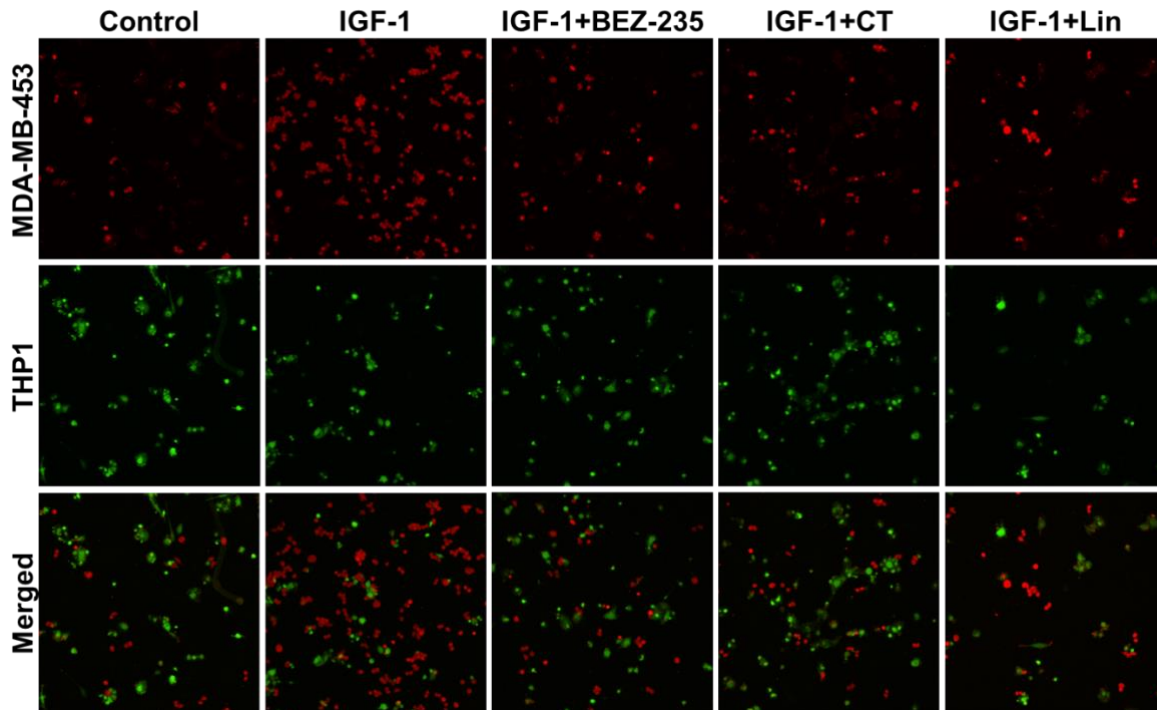


Figure S13: Assessment of cancer cell clearance co-cultured with IGF-1-exposed macrophages with or without blocking IGF-1R/PI3K/NRF2 axis. THP1 macrophages (induced from monocytes by PMA exposure) were divided into five groups: one untreated and four treated with IGF-1 alone or in combination with IGF-1R/ PI3K/ NRF2 inhibitors for 6h. Macrophages were then activated with LPS and IFN- γ for 24h and tracked with CellTracker Green. Drug naïve MDA-MB-453 cells were labeled with CellTracker Orange and co-cultured with activated THP1 macrophages in a 1:1 ratio for 48h. The average number of MDA-MB-453 cells was counted to assess immune clearance.

Figure S14A:

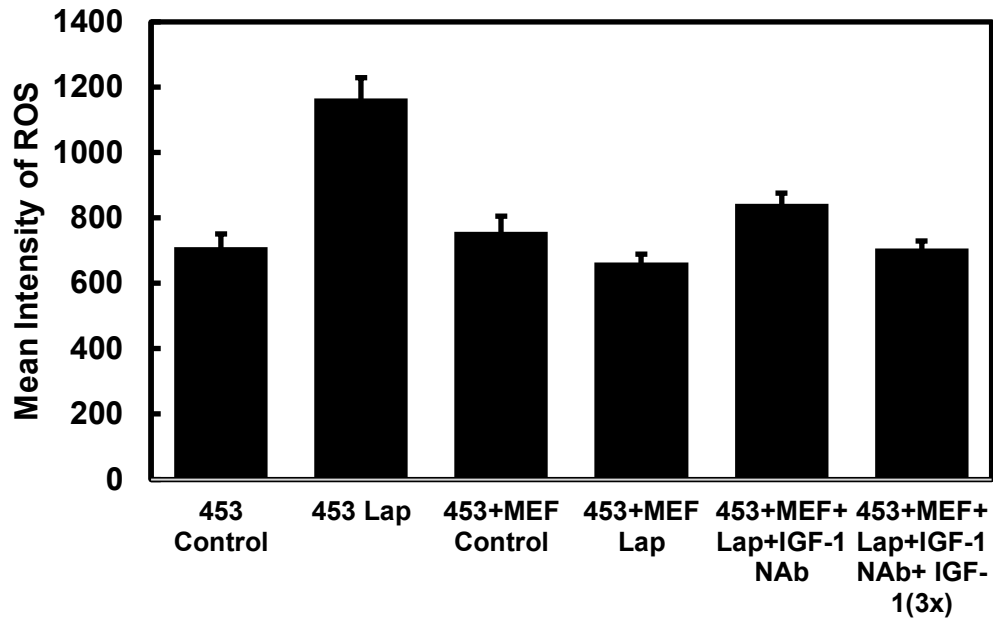


Figure S14B:

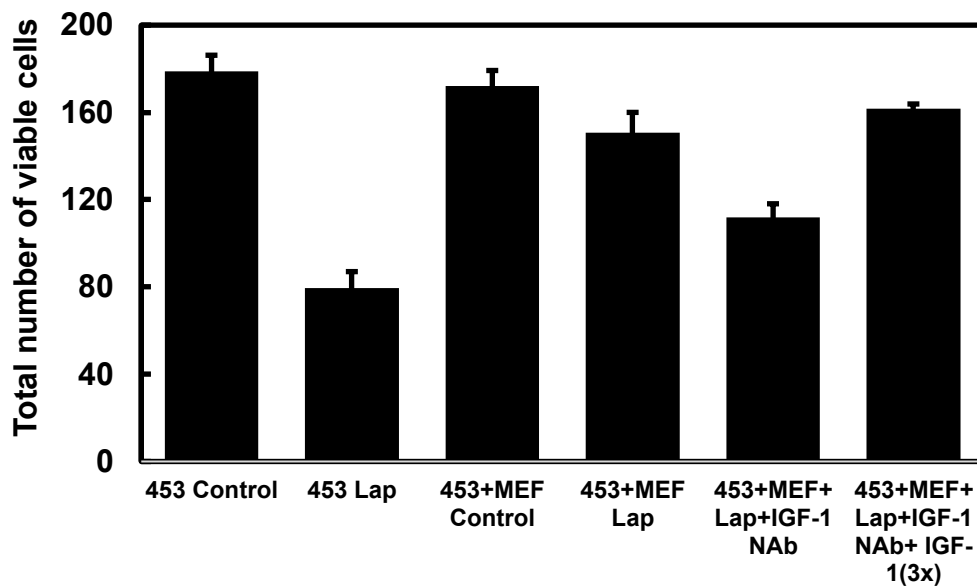


Figure S14: Measurement of mitochondrial ROS (A) and cell viability (B) in MDA-MB-453 cells co-cultured with mouse embryonic fibroblasts (MEFs). MDA-MB-453 cells were co-cultured with MEFs at a 1:1 ratio, then pretreated with IGF-1 neutralizing antibody (NAb)

alone or in combination with IGF-1 (3x) for 6h. This was followed by lapatinib treatment for 1h to measure mitochondrial ROS (**A**) and for 48h to measure cell viability (**B**). The p-values for S14A are: MDA-MB-453 lap vs MDA-MB-453+MEF lap = 4.08E-13, MDA-MB-453+MEF lap vs MDA-MB-453+MEF lap+IGF-1 NAb = 0.02882, MDA-MB-453+MEF lap vs MDA-MB-453+MEF lap+IGF-1 NAb+IGF-1(3x) = 2.12E-11. The p-values for S14B are: MDA-MB-453 lap vs MDA-MB-453+MEF lap = 0.0001286, MDA-MB-453+MEF lap vs MDA-MB-453+MEF lap+IGF-1 NAb = 0.02058, MDA-MB-453+MEF lap vs MDA-MB-453+MEF lap+IGF-1 NAb+IGF-1(3x) = 3.07E-05.

Figure S15A:

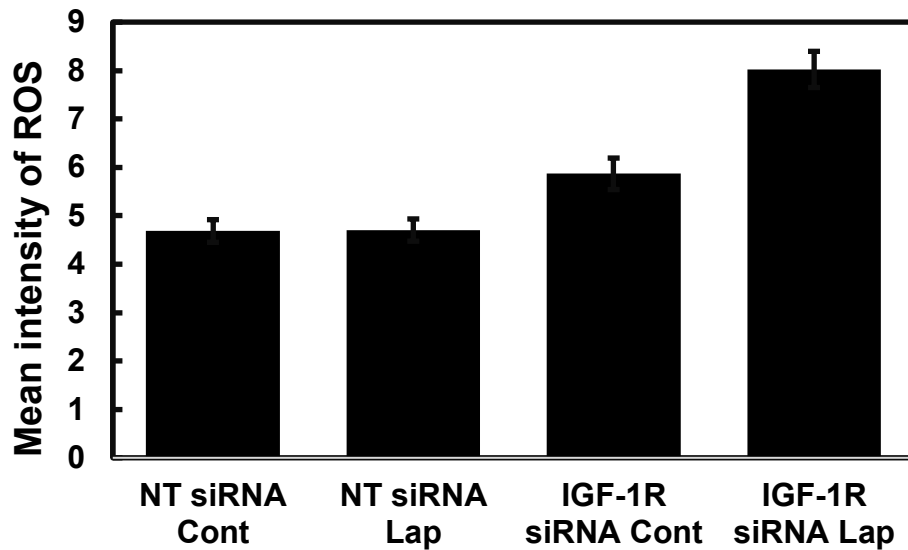


Figure S15B:

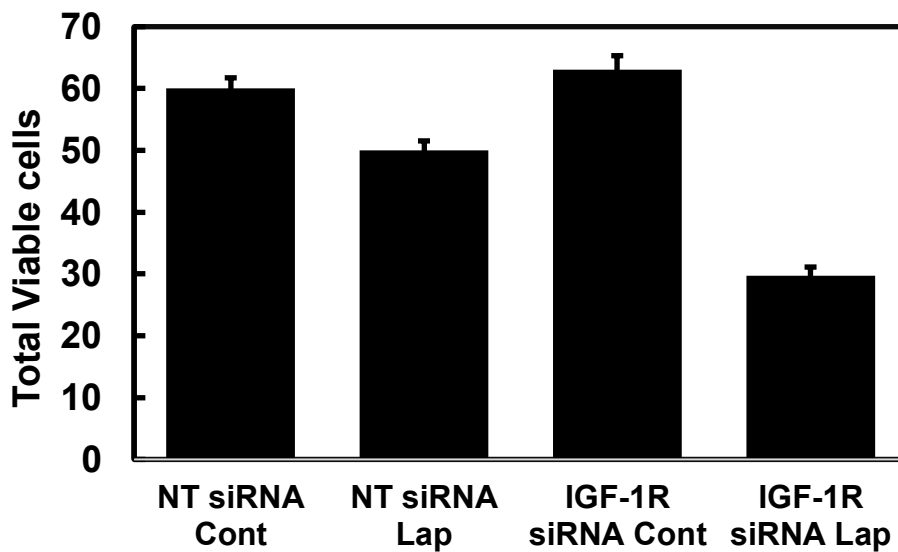


Figure S15: Measurement of mitochondrial ROS (A) and cell viability (B) in MDA-MB-453 cells post IGF-1R knockdown, co-cultured with mouse embryonic fibroblasts (MEFs). Following IGF-1R knockdown in MDA-MB-453 cells, these cells were co-cultured with MEFs at a 1:1 ratio. This was followed by lapatinib treatment for 1h to measure mitochondrial ROS (A) and for 24h to measure cell viability (B). The p-values for S15A are:

NT siRNA cont vs NT siRNA lap = 0.9996, IGF-1R siRNA cont vs IGF-1R siRNA lap = 1.24E-05, NT siRNA lap vs IGF-1R siRNA lap = 7.63E-11. The p-values for S15B are: NT siRNA cont vs NT siRNA lap = 0.01766, IGF-1R siRNA cont vs IGF-1R siRNA lap = 4.94E-06, NT siRNA lap vs IGF-1R siRNA lap = 0.0001934.

Figure S16A:

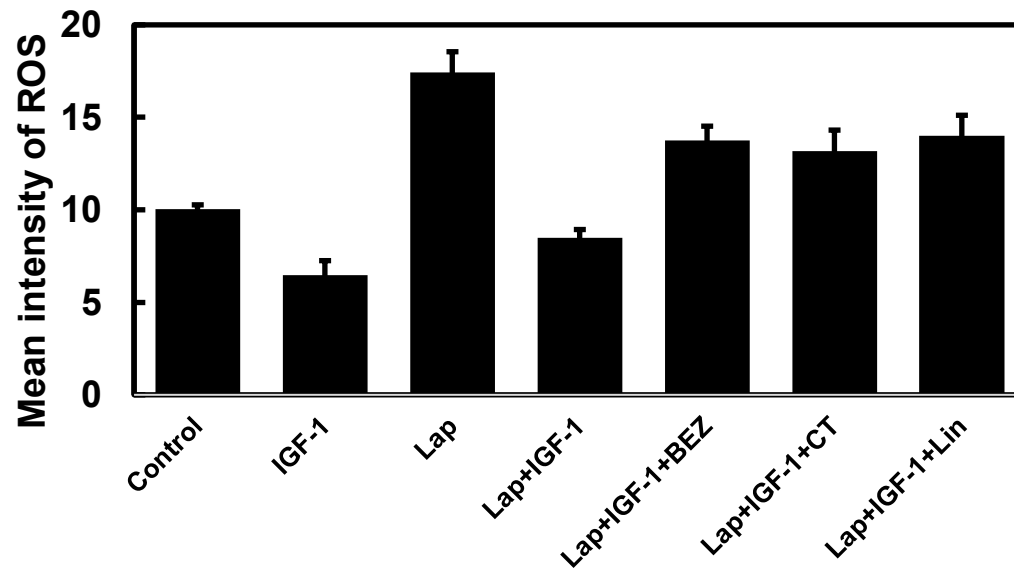


Figure S16B:

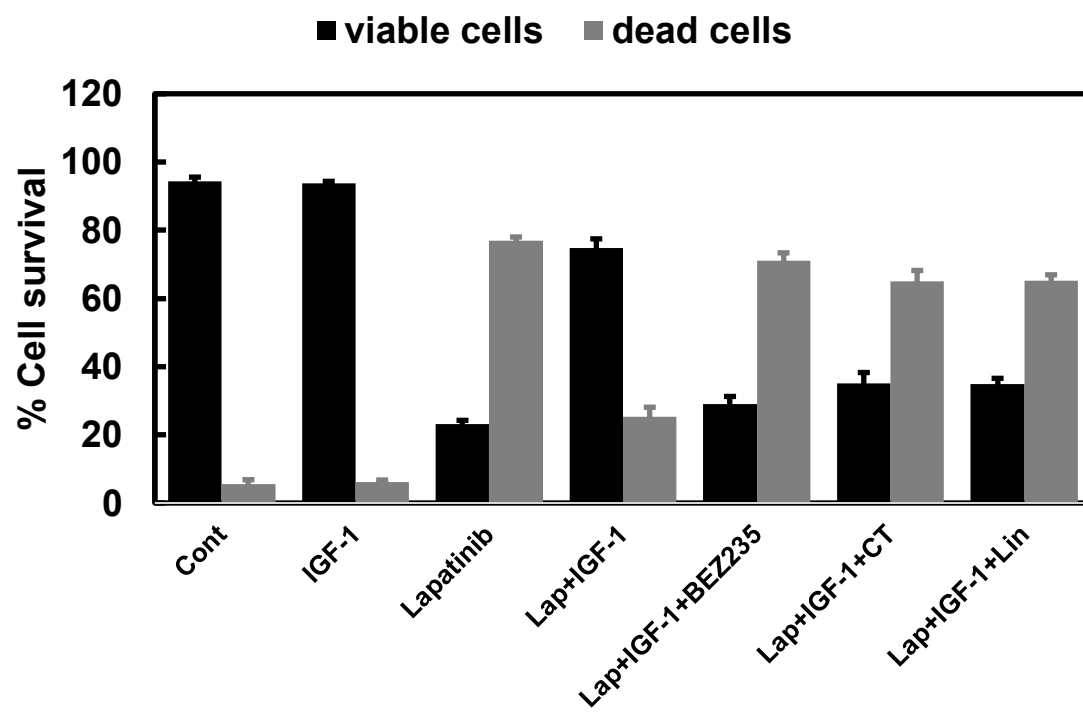


Figure S16C:

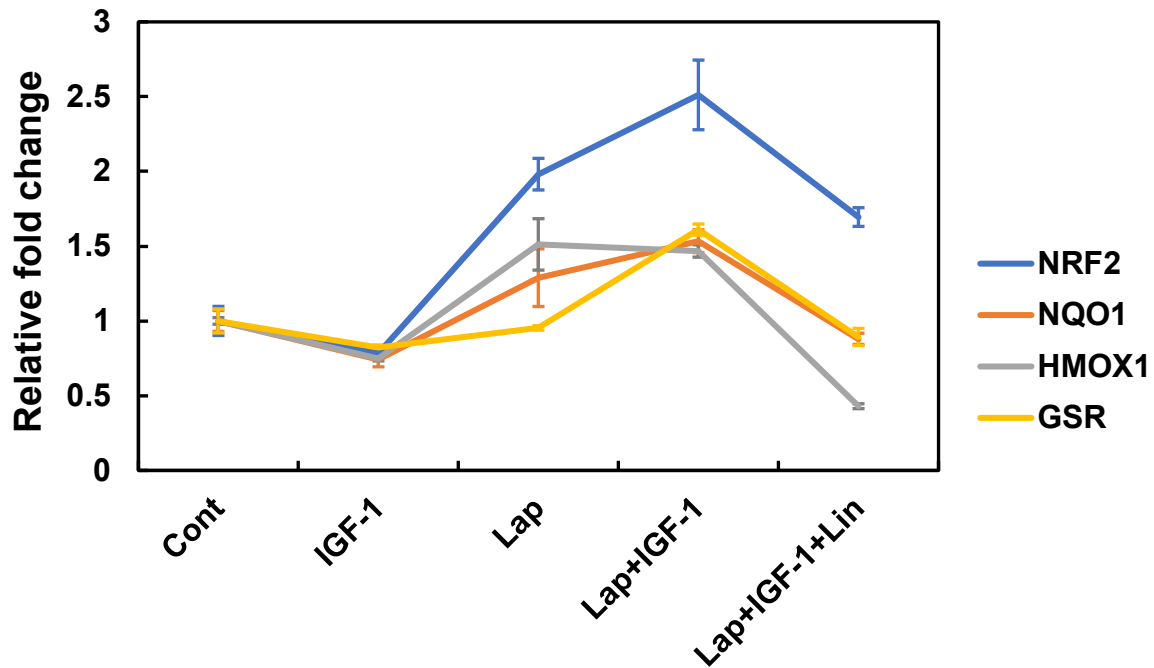


Figure S16: Assessment of mitochondrial ROS production (A), cell viability (B) and transcript levels of NRF2 and its downstream target genes (C) in HCC1954 post-lapatinib treatment. HCC1954 cells were cultured in RPMI plus 10% FBS at 37°C and 5% CO₂ in a humidified incubator, pretreated with 50ng/ml IGF-1 alone or in combination with 100nM BEZ-235, 250nM CT or 40μM linsitinib for 6h before exposing them to 3.5μM lapatinib for 3h for measuring mitochondrial ROS (A), and 48h for measuring cell viability (B). For measuring the transcript levels of NRF2, NQO1, HMOX1 and GSR, HCC1954 cells were pretreated with IGF-1 with or without linsitinib for 6h, followed by lapatinib treatment for 3h. mRNA levels were measured using RT-qPCR with specific primers and normalized against actin (C).

Figure S17A:

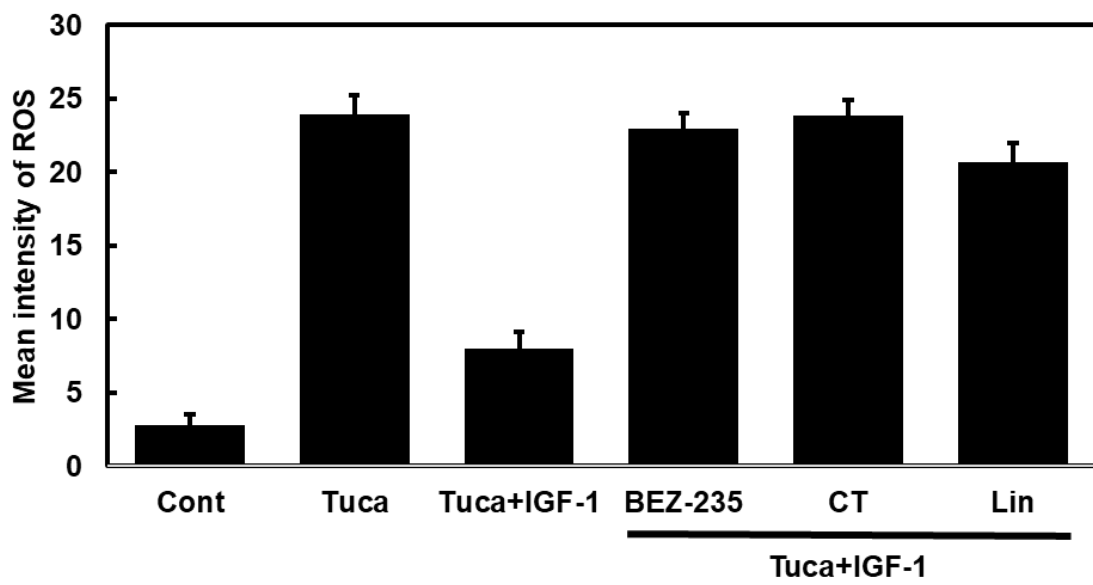
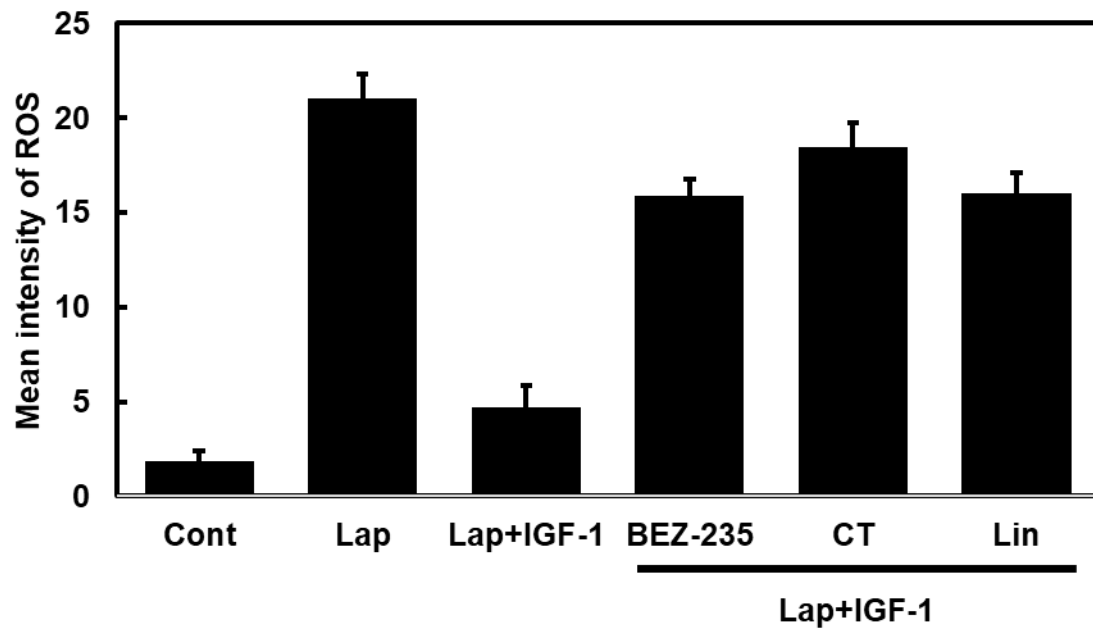


Figure S17B:

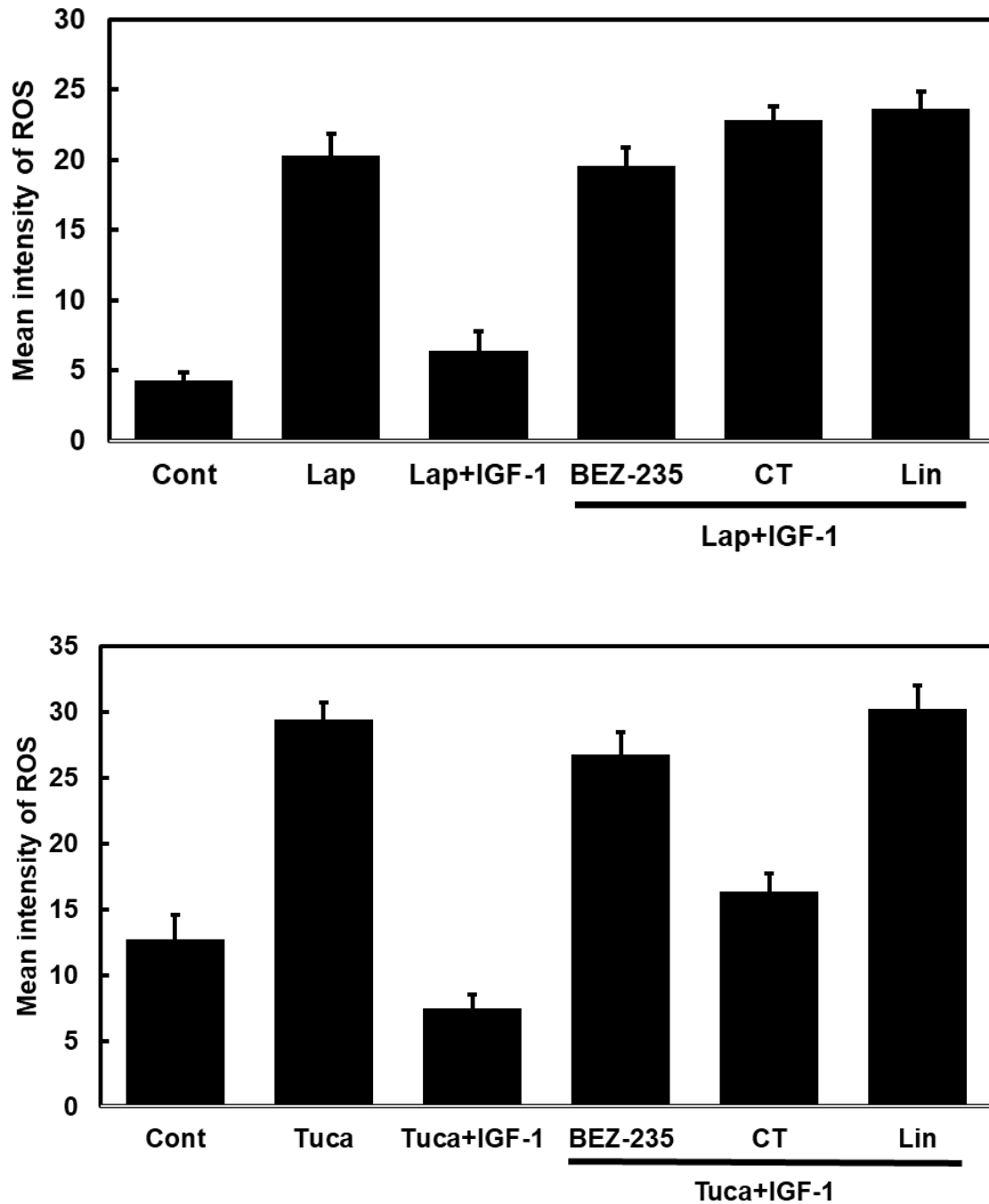
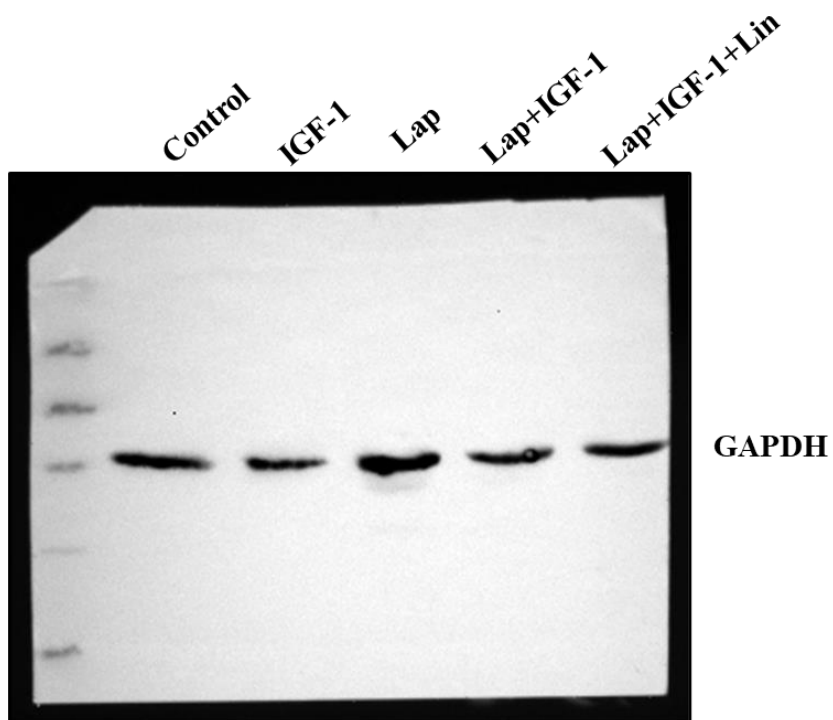
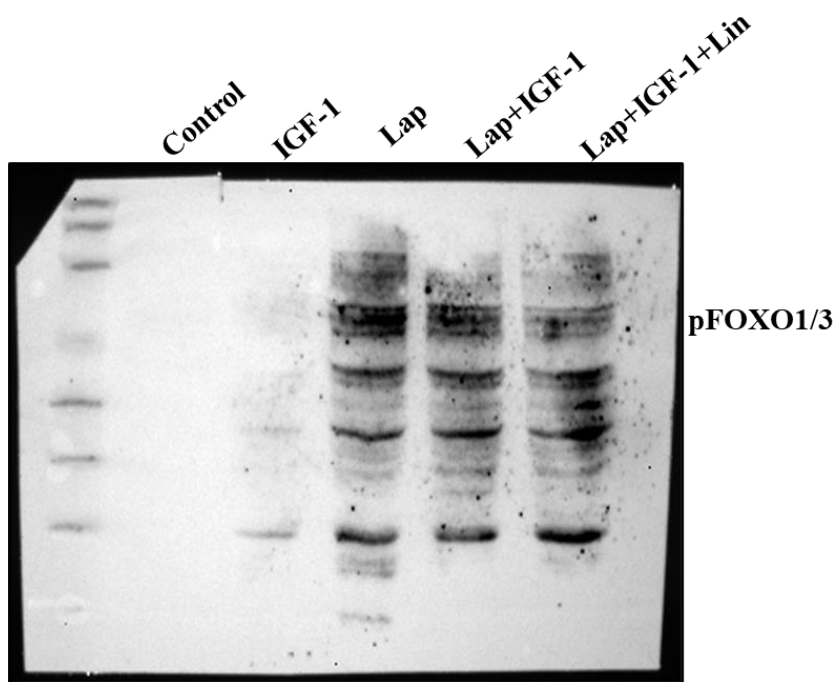


Figure S17: Assessment of mitochondrial ROS production in MCF10A (A) and HepG2 (B) cells following lapatinib and tucatinib treatment. MCF10A cells were cultured in complete growth media containing DMEM/F12 plus 2.5% horse serum, epidermal growth factor, hydrocortisone, and cholera toxin, at 37°C and 5% CO₂ in a humidified incubator.

HepG2 cells were cultured in MEM plus 10% FBS at 37°C and 5% CO₂ in a humidified incubator. Both cell lines were pretreated with 50ng/ml IGF-1 alone or in combination with 100nM BEZ-235, 250nM CT or 40μM linsitinib for 6h before exposing them to 3.5μM lapatinib for 3h and 5μM tucatinib for 6h. Mitochondrial ROS were measured using MitoSox™ Red dye as previously described.

Figure S18A:



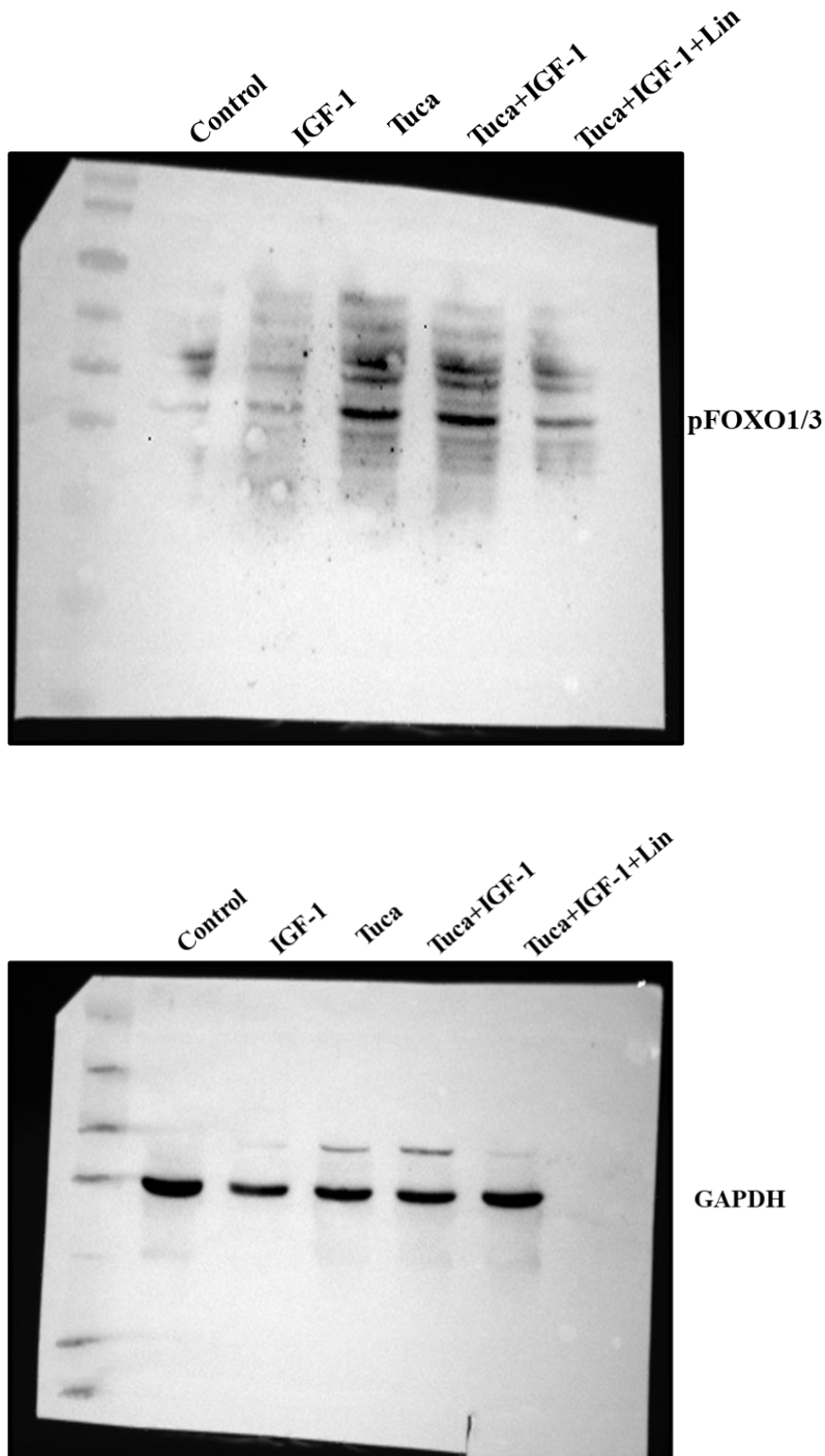


Figure S18A: Original western blot images of Figure 5S-T. The first two images show the whole western blot from Figure 5S, and the next two show the western blots from Figure 5T, with the top panel showing pFOXO1/3 and the bottom panel showing GAPDH, respectively.

Figure S18B:

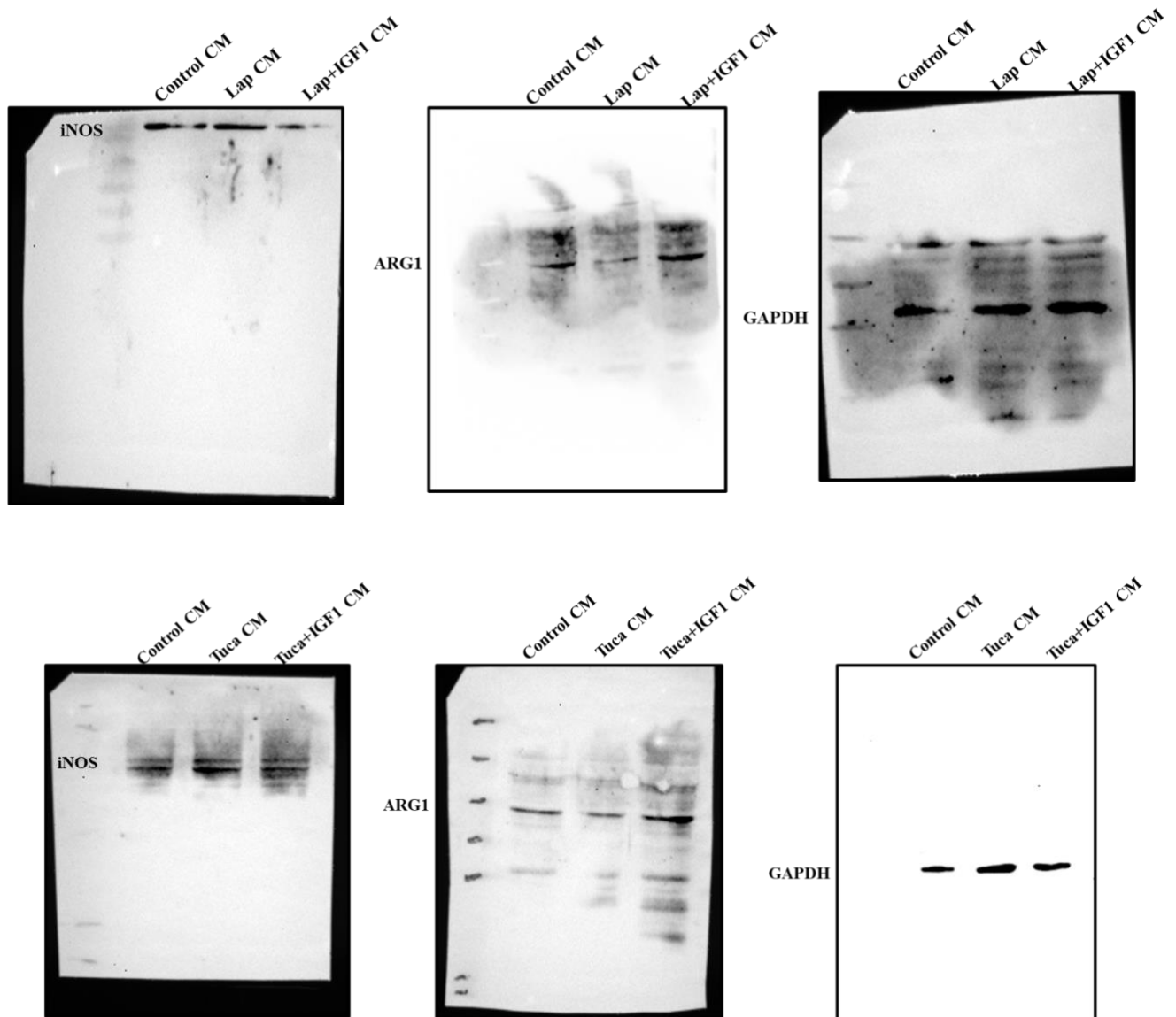


Figure S18B: Original western blot images of Figure 6D-E. The upper panel images show the whole western blot images from Figure 6D, and the lower panel images show the whole western blot images from Figure 6E. The images show the levels of iNOS (right-hand side), ARG1 (middle), and GAPDH (left-hand side).

Figure S18C:

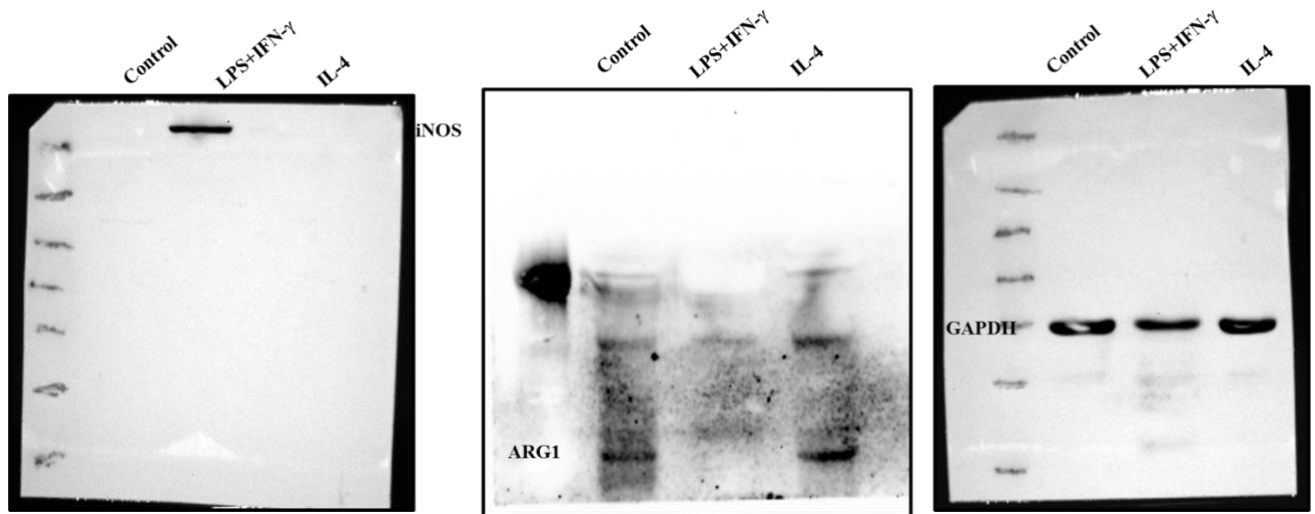


Figure S18C: Original western blot images of Supplemental Figure S8. The images show the whole western blot images from Figure S8. The images show the levels of iNOS (right-hand side), ARG1 (middle), and GAPDH (left-hand side).

Table S3: List of RT-qPCR primers

Gene	Sequence of primers 5'-3'
IGF-1 F	CTCTTCAGTTCGTGTGTGGAGAC
IGF-1 R	CAGCCTCCTTAGATCACAGCTC
IGF-1R F	CCTGCACAACCTCCATCTTCGTG
IGF-1R R	CGGTGATGTTGTAGGTGTCTGC
Actin F	GCCTCGCCTTTGCCGA
Actin R	GGAATCCTTCTGACCCATGC
GSR F1	CGGTGCCAGCTTAGGAATAA
GSR R1	GCCATCTCCACAGCAATGTA
HMOX1 F	CCAGGCAGAGAATGCTGAGTTC
HMOX1 R	AAGACTGGGCTCTCCTTGTTGC
NQO1 F	CCTGCCATTCTGAAAGGCTGGT
NQO1 R	GTGGTGATGGAAAGCACTGCCT
NRF2 F	CACATCCAGTCAGAAACCAGTGG
NRF2 R	GGAATGTCTGCGCCAAAAGCTG
Keap1 F	CAACTTCGCTGAGCAGATTGGC
Keap1 R	TGATGAGGGTCACCAGTTGGCA
CYP4503A5 F	ACGGTCATTGCTGTCTCCAACC
CYP4503A5 R	GTGACAGGCTTGCCTTTCTCTG
TGF-β1 F	TGATACGCCTGAGTGGCTGTCT
TGF-β1 R	CACAAGAGCAGTGAGCGCTGAA
IL10 F	CGGGAAGACAATAACTGCACCC
IL10 R	CGGTTAGCAGTATGTTGTCCAGC
TNF-α F	GGTGCCTATGTCTCAGCCTCTT
TNF-α R	GCCATAGAAGCTGATGAGAGGGAG

Table S4: List of antibodies and drugs/inhibitors used and their sources

Antibodies	Sources
pFoxO1/3	Cell Signaling Technology (Danvers, MA, USA) Cat. No. 9464
Arginase 1, iNOS	Affinity Biosciences. Arginase 1 Cat. No. AF0199, iNOS Cat. No. DF6657
GAPDH	Elabscience Cat. No. E-AB-40337
Recombinant IGF-1	R&D Systems (Minnesota, USA) Cat. No. 291-G1
Drugs/Inhibitors	Sources
Lapatinib	Selleckchem, Texas, USA Cat. No. S2111
Camptothecin	APExBIO Technology, Texas, USA Cat. No. A2877
BEZ-235; Linsitinib; Tucatinib	MedChemExpress (New Jersey, USA) BEZ-235 Cat. No. HY-50673; Linsitinib Cat. No. HY-10191; Tucatinib Cat. No. HY-16069
B-27 supplement	Gibco, Cat. No. 17504-044
Human recombinant Epidermal Growth Factor (EGF)	HiMedia Laboratories, Cat. No. TC228

Table S5: The p-values for different comparisons displayed in Figure 2

Figure 2		p-values
A	cont vs lap	2.13E-13
	lap vs lap+NAC	1.70E-05
B	cont vs tuca	5.96E-12
	lap vs tuca+NAC	0.0001749
C	cont vs lap	1.99E-11
	lap vs lap+NAC	2.27E-14
D	cont vs tuca	1.99E-11
	lap vs tuca+NAC	5.40E-06
E	cont vs lap	4.38E-07
	cont vs lap+NAC	0.1383
F	cont vs tuca	3.01E-11
	cont vs tuca+NAC	0.06814
G	cont vs lap	1.39E-06
	cont vs lap+NAC	0.005206
H	cont vs tuca	2.04E-06
	cont vs tuca+NAC	0.06016
I	cont vs lap	2.27E-14
	lap vs lap+NAC	2.27E-14
J	cont vs tuca	1.08E-06
	tuca vs tuca+NAC	5.40E-06

Table S6: The p-values for different comparisons displayed in Figure 3

Figure 3		p-values
B	NTlap vs NTlap+IGF-1	3.13E-08
	siIGF-1R lap vs siIGF-1R lap+IGF-1	0.003248
C	NTtuca vs NTtuca+IGF-1	1.76E-06
	siIGF-1R tuca vs siIGF-1R tuca+IGF-1	0.7247
D	NTlap vs NTlap+IGF-1	0.000235
	siIGF-1R lap vs siIGF-1R lap+IGF-1	0.7125
E	NTtuca vs NTtuca+IGF-1	1.42E-06
	siIGF-1R tuca vs siIGF-1R tuca+IGF-1	0.679
F	lap vs lap+IGF-1	2.38E-10
	Lap+IGF-1 vs lap+IGF-1+lin	3.10E-05
G	tuca vs tuca+IGF-1	2.36E-06
	tuca+IGF-1 vs tuca+IGF-1+lin	0.0003176
H	lap vs lap+IGF-1	1.70E-06
	lap+IGF-1 vs lap+IGF-1+lin	2.27E-14
I	tuca vs tuca+IGF-1	4.46E-12
	tuca+IGF-1 vs tuca+IGF-1+lin	1.85E-10
J	0h vs 1h lap mtROS	1.97E-11
	0h vs 3h lap mtROS	3.61E-13
	0h vs 6h lap mtROS	4.51E-06
	0h vs 10h lap mtROS	1.80E-06
	0h vs 24h lap mtROS	0.7012
	0h vs 1h lap IGF-1	1
	0h vs 3h lap IGF-1	0.002326
	0h vs 6h lap IGF-1	0.000822
	0h vs 10h lap IGF-1	0.000105
	0h vs 24h lap IGF-1	0.9997
	0h vs 1h lap IGF-1R	1
	0h vs 3h lap IGF-1R	2.26E-05
	0h vs 6h lap IGF-1R	1.56E-05
	0h vs 10h lap IGF-1R	0.204
	0h vs 24h lap IGF-1R	0.9755
K	0h vs 12h tuca mtROS	0.01252
	0h vs 24h tuca mtROS	0.03801
	0h vs 36h tuca mtROS	1.21E-17
	0h vs 40h tuca mtROS	1.38E-10
	0h vs 48h tuca mtROS	1.73E-06
	0h vs 12h tuca IGF-1	1
	0h vs 24h tuca IGF-1	0.03097
	0h vs 36h tuca IGF-1	0.01904

	0h vs 40h tuca IGF-1	0.000239
	0h vs 48h tuca IGF-1	1
	0h vs 12h tuca IGF-1R	0.6825
	0h vs 24h tuca IGF-1R	0.6274
	0h vs 36h tuca IGF-1R	0.01983
	0h vs 40h tuca IGF-1R	0.5789
	0h vs 48h tuca IGF-1R	0.2066
M	cont vs 3.5μM lap	2.44E-04
	cont vs 5μM lap	5.78E-07
	cont vs 5μM tuca	6.44E-05
	cont vs 7.5μM tuca	3.08E-06
	3.5μM lap vs 5μM lap	3.57E-12
	5μM tuca vs 7.5μM tuca	4.83E-12
	3.5μM lap vs 3.5μM lap+IGF-1	1
	5μM lap vs 5μM lap+IGF-1	0.9797
	5μM tuca vs 5μM tuca+IGF-1	1
	7.5μM tuca vs 7.5μM tuca+IGF-1	0.04229
	50nM doxo vs 50nM doxo+IGF-1	0.0001
N	cont vs 3.5μM lap	7.66E-07
	cont vs 5μM lap	0
	cont vs 5μM tuca	1.59E-08
	cont vs 7.5μM tuca	3.82E-11
	3.5μM lap vs 5μM lap	0.00E+00
	5μM tuca vs 7.5μM tuca	0.2224
	3.5μM lap vs 3.5μM lap+IGF-1	1.00E+00
	5μM lap vs 5μM lap+IGF-1	0.8651
	5μM tuca vs 5μM tuca+IGF-1	0.8651
	7.5μM tuca vs 7.5μM tuca+IGF-1	6.86E-10
	50nM doxo vs 50nM doxo+IGF-1	2.44E-10

Table S7: The p-values for different comparisons displayed in Figure 4

Figure 4		p-values
A	lap+IGF-1 vs lap+IGF-1+BEZ-235	1.02E-05
	lap+IGF-1 vs lap+IGF-1+CT	1.40E-07
B	tuca+IGF-1 vs tuca+IGF-1+BEZ-235	0.001341
	tuca+IGF-1 vs tuca+IGF-1+CT	5.81E-06
C	lap+IGF-1 vs lap+IGF-1+BEZ-235	2.27E-14
	lap+IGF-1 vs lap+IGF-1+CT	2.27E-14
D	tuca+IGF-1 vs tuca+IGF-1+BEZ-235	4.54E-10
	tuca+IGF-1 vs tuca+IGF-1+CT	2.52E-11
F	NTlap vs NTlap+IGF-1	3.71E-07
	siNRF2lap vs siNRF2lap+IGF-1	0.9899
G	NTtuca vs NTtuca+IGF-1	3.13E-08
	siNRF2tuca vs siNRF2tuca+IGF-1	0.1074
H	NTlap vs NTlap+IGF-1	1.95E-11
	siNRF2lap vs siNRF2lap+IGF-1	0.02534
I	NTtuca vs NTtuca+IGF-1	2.92E-14
	siNRF2tuca vs siNRF2tuca+IGF-1	0.9617
J	cont vs lap (NRF2)	0.000105
	cont vs lap+IGF-1 (NRF2)	3.78E-08
	cont vs lap+IGF-1+lin (NRF2)	0.9904
	Lap+IGF-1 vs Lap+IGF-1+lin (NRF2)	2.96E-08
	cont vs lap (NQO1)	0.05425
	cont vs lap+IGF-1 (NQO1)	0.01274
	cont vs lap+IGF-1+lin (NQO1)	0.7341
	Lap+IGF-1 vs Lap+IGF-1+lin (NQO1)	0.03145
	cont vs lap (HMOX1)	0.8717
	cont vs lap+IGF-1 (HMOX1)	0.002251
	cont vs lap+IGF-1+lin (HMOX1)	0.9998
	Lap+IGF-1 vs Lap+IGF-1+lin (HMOX1)	0.001798
	cont vs lap (GSR)	0.462
	cont vs lap+IGF-1 (GSR)	2.33E-06
	cont vs lap+IGF-1+lin (GSR)	0.3907
	Lap+IGF-1 vs Lap+IGF-1+lin (GSR)	2.04E-06
K	cont vs tuca (NRF2)	1.05E-04
	cont vs tuca+IGF-1 (NRF2)	3.78E-08
	cont vs tuca+IGF-1+lin (NRF2)	0.9904
	Lap+IGF-1 vs Lap+IGF-1+lin (NRF2)	2.96E-08
	cont vs tuca (NQO1)	0.9376
	cont vs tuca+IGF-1 (NQO1)	2.43E-03
	cont vs tuca+IGF-1+lin (NQO1)	0.01579
	Lap+IGF-1 vs Lap+IGF-1+lin (NQO1)	5.43E-05
	cont vs tuca (HMOX1)	0.008517
	cont vs tuca+IGF-1 (HMOX1)	1.56E-04

	cont vs tuca+IGF-1+lin (HMOX1)	0.7747
	Lap+IGF-1 vs Lap+IGF-1+lin (HMOX1)	3.73E-04
	cont vs tuca (GSR)	0.6466
	cont vs tuca+IGF-1 (GSR)	4.74E-03
	cont vs tuca+IGF-1+lin (GSR)	3.39E-04
	Lap+IGF-1 vs Lap+IGF-1+lin (GSR)	7.81E-06
P	0h vs 1h lap (NRF2)	0.3001
	0h vs 3h lap (NRF2)	6.25E-06
	0h vs 6h lap (NRF2)	1.19E-05
	0h vs 10h lap (NRF2)	2.79E-05
	0h vs 24h lap (NRF2)	0.1594
Q	0h vs 12h tuca (NRF2)	0.9974
	0h vs 24h tuca (NRF2)	1.15E-05
	0h vs 36h tuca (NRF2)	3.02E-09
	0h vs 40h tuca (NRF2)	5.14E-10
	0h vs 48h tuca (NRF2)	0.0163

Table S8: The p-values for different comparisons displayed in Figure 5

Figure 5		p-values
B	cont vs lap	3.13E-08
	lap vs lap+IGF-1	3.13E-08
C	cont vs tuca	1.06E-13
	tuca vs tuca+IGF-1	5.68E-16
G	cont vs lap	0.4218
	lap vs lap+IGF-1	0.000575
H	cont vs tuca	0.5764
	tuca vs tuca+IGF-1	9.52E-06
I	lap+IGF-1 vs lap+IGF-1+BEZ235	1.37E-06
	lap+IGF-1 vs lap+IGF-1+CT	0.00039
	lap+IGF-1 vs lap+IGF-1+Lin	3.13E-08
J	tuca+IGF-1 vs tuca+IGF-1+BEZ235	0.01817
	tuca+IGF-1 vs tuca+IGF-1+CT	1.98E-07
	tuca+IGF-1 vs tuca+IGF-1+Lin	1.34E-06
K	lap+IGF-1 vs lap+IGF-1+BEZ235	2.45E-06
	lap+IGF-1 vs lap+IGF-1+CT	7.46E-06
	lap+IGF-1 vs lap+IGF-1+Lin	2.80E-07
L	tuca+IGF-1 vs tuca+IGF-1+BEZ235	4.68E-05
	tuca+IGF-1 vs tuca+IGF-1+CT	5.82E-05
	tuca+IGF-1 vs tuca+IGF-1+Lin	3.11E-06

Table S9: The p-values for different comparisons displayed in Figure 6

Figure 6		p-values
B	LPS vs IGF-1+LPS	6.71E-08
	IGF-1+LPS vs IGF-1+Lin+LPS	4.71E-08
D	cont vs IGF-1	3.24E-05
	IGF-1 vs IGF-1+BEZ-235	1.29E-05
	IGF-1 vs IGF-1+CT	9.60E-06
	IGF-1 vs IGF-1+Lin	4.13E-05
E	cont vs IGF-1	0.001213
	IGF-1 vs IGF-1+BEZ-235	0.000573
	IGF-1 vs IGF-1+CT	0.000357
	IGF-1 vs IGF-1+Lin	0.000352

Supplemental Methods:

Cell Proliferation and Viability Assays:

Cell proliferation was performed by manually counting with a haemocytometer slide (Japson, Haryana, India) or using Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma-Aldrich) colorimetric assay. Cells were seeded into 96-well plates at a density of 5000 cells/well and allowed to adhere overnight, followed by treatment with varying concentrations of lapatinib and tucatinib for 48h. Post-treatment, 5mg/ml MTT reagent was added and incubated for 2h at 37°C. Formazan crystals were solubilized in DMSO, and optical density was quantified at 595nm in a microplate reader.

Viability was assessed with 1µM calcein AM (detects live cells) and 1µM propidium iodide/PI (detects dead cells), obtained from HiMedia Laboratories after pretreating cells with 50ng/ml recombinant IGF-1 (R&D Systems, Minnesota, USA) for 6h, followed by TKI treatment for 48h (lapatinib: 3.5µM and tucatinib: 5µM). Cells were imaged at 20X magnification using FITC/TRITC filters on a Leica DMEL LED Fluo microscope (Leica Microsystems, Inc., Wetzlar, Germany).

Drug Rechallenge Assay:

MDA-MB-453 persister cells that survived the initial 48h TKI exposure (with or without 50ng/ml IGF-1 pretreatment) were allowed to grow for 15 days. Every third day, cells were refed with fresh media. On day 15, cells were trypsinized and replated at 5000 cells/well in serum-free media to eliminate confounding growth factor signaling. After overnight adherence, cells were rechallenged with lapatinib (3.5µM and 5µM), tucatinib (5µM and 7.5µM), or doxorubicin (50nM) for 48h. We did not add IGF-1 during this secondary challenge. Mitochondrial ROS was measured 1h after treatment using MitoSOX™ Red (Thermo Fisher

Scientific, Massachusetts, USA), and 48h viability was assessed with calcein AM/PI staining. Fluorescence signals were captured with an inverted Leica DMEL LED Fluo microscope at 10X and quantified using ImageJ software (National Institutes of Health, Maryland, USA).

RNAi and Real-time PCR:

IGF-1 and NRF-2 siRNA (GAAGGGAAUUACUCCUUCUdTdT and GUCACUUGUCCUGAUUAUdTdT, respectively) purchased from Eurogentec were transfected in MDA-MB-453 cells at 25nM each, using Lipofectamine RNAiMax (Thermo Fisher Scientific). RNA was isolated 24h later with TRIzol (Sigma-Aldrich), treated with TURBO DNase (Thermo Fisher Scientific), and quantified via NanoDrop (Thermo Fisher Scientific). Real-time PCR employed GoTaq 1-Step RT-qPCR (Promega Corporation, Wisconsin, USA) with CFX96 (Bio-Rad Laboratories, California, USA), using actin as control.

Immunofluorescence Assay:

Cells grown on poly-L-lysine (Sigma-Aldrich)-coated coverslips were fixed with 4% paraformaldehyde in PBS for 20min, permeabilized with 0.2% Triton X-100 for 10min, and blocked with 10% FBS for 1 h. They were then incubated overnight at 4°C with primary antibody (1:1000), washed, and stained with Alexa Fluor 555 secondary antibody (1:500) for 1h. After washing, cells were counterstained with 1µg/ml DAPI (Thermo Fisher Scientific) for 20min, mounted in anti-fade in DMF (Thermo Fisher Scientific) and glycerol, and imaged at 100X using a Nikon Eclipse Ti-2 confocal microscope. ImageJ was used for analysis.

Live Cell Imaging for Detection of Mitochondrial Reactive Oxygen Species (ROS), Mitochondrial Membrane Potential and Lipid Peroxidation:

Mitochondrial ROS following TKI were detected by live-cell imaging at 20X using a TRITC filter with 1 μ M MitoSOX™ Red after 30min incubation, imaged on a Leica DMEL LED Fluo microscope. Nuclei were counterstained with Hoechst 33342. Mitochondrial membrane potential was measured similarly with 20nM TMRM (Thermo Fisher Scientific) after TKI treatment. Hoechst (Thermo Fisher Scientific) was used as a counterstain. Lipid peroxidation was assessed using BODIPY 581/591 C11 (Thermo Fisher Scientific).

Immunoblot Analysis:

Total cellular proteins were extracted with RIPA buffer containing protease and phosphatase inhibitors, and quantified using the Bradford reagent (HiMedia Laboratories). Proteins were denatured in 5X dye at 95°C for 10min, run on 12% SDS-PAGE, and transferred to nitrocellulose membrane (MDI Membrane Technologies, Inc., Pennsylvania, USA). Nonspecific binding was blocked with 5% BSA or 10% milk in TBST for 1h, then incubated overnight at 4°C with primary antibody (1:1000). After three washes, secondary antibody (1:10000) was applied for 1h. After a second wash, chemiluminescence was detected with SuperSignal substrate (Thermo Fisher Scientific), and the signals were imaged with the Bio-Rad imaging system. GAPDH (1:1000) served as a loading control.

Assessment of Organoid Structural Integrity:

We assessed organoid structural integrity using fluorescence microscopy images in Fiji (ImageJ). We thresholded the CellTracker Orange channel and excluded objects smaller than

100 pixels to remove noise. For each view, the cross-sectional area (μm^2) and perimeter (μm) of the largest object were measured with the Analyze Particles function. Circularity was then calculated as $4\pi \times \text{Area} / \text{Perimeter}^2$, with 1 representing a perfect circle and lower values indicating irregularities or disruptions. We derived a comprehensive structural integrity score by averaging the min-max-normalized area and circularity metrics across all conditions, with 1 indicating maximum integrity and 0 indicating highly disrupted morphology.

Single-cell Transcriptomics Profiling:

To delineate the cellular origin of IGF-1 in the breast tumor microenvironment, we analyzed scRNA-seq data using the TISCH2 database. We queried the GSE114727 (inDrop) dataset to quantify and visualize IGF-1 transcripts across microenvironmental compartments. We compared expression levels, measured as $\log(\text{TPM}/10+1)$, among malignant, stromal, and immune cells.

Clinical Trial Transcriptomics Analysis of GSE130788

To evaluate therapy-induced microenvironmental and intracellular transcriptomic changes in human patients, we obtained microarray data from the TRIO-B-07 trial (GSE130788) from GEO. We analyzed differential gene expression in the "Residual Disease/RD" (lapatinib non-responder) group. Paired comparisons between pre-treatment and two-week post-lapatinib biopsies assessed therapy-induced Log2 fold changes (LogFC) and -log10 p-values for key microenvironmental and antioxidant effectors, including IGFBP1, MMP1, MMP9, and NQO1. We used the EnrichR web tool to generate pathway enrichment signatures for post-lapatinib gene sets relative to pre-treatment samples.

Baseline Predictive Transcriptomics of GSE240671

To evaluate baseline predictive biomarkers of therapy resistance, we obtained pre-treatment transcriptomic data from HER2-positive breast cancer patients from the NCBI GEO database (GSE240671). Patients were grouped by their response to neoadjuvant chemotherapy: complete responders (no residual disease) and non-responders (residual disease-minimal, moderate, extensive). Differential gene expression analysis using GEO2R, a web-based tool, identified baseline transcriptomic patterns related to persister cell survival and assessed the predictive role of the stroma-macrophage resistance axis.

Tumor Immune Profiling (TIMER2.0)

The Tumor Immune Estimation Resource 2.0 (TIMER2.0) web server was used to assess correlations between microenvironmental gene expression profiles and specific tumor-infiltrating immune cell populations, particularly tumor-associated macrophages, in the breast invasive carcinoma (BRCA) cohort.