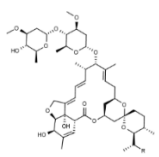
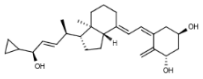
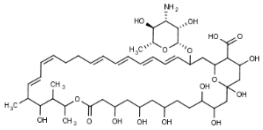
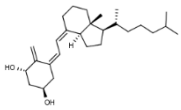
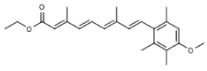
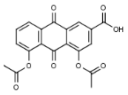
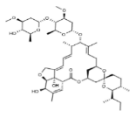
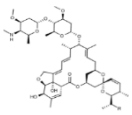
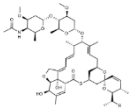
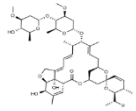
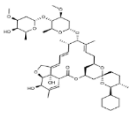
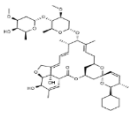
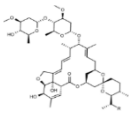
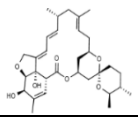
Supplementary Tables

Supplementary Table 1. Prestwick library compounds that passed the FRET-FP screening

Name	Structure	Formula	Molecular Weight
Ivermectin (mixture of 22,23-Dihydroavermectin B_{1a} and B_{1b})		B_{1a}: $C_{48}H_{74}O_{14}$ B_{1b}: $C_{47}H_{72}O_{14}$	875.12 861.08
Calcipotriene		$C_{27}H_{40}O_3$	412.62
Nystatine		$C_{47}H_{75}NO_{17}$	926.12
Alfacalcidol		$C_{27}H_{44}O_2$	400.65
Etretinate		$C_{23}H_{30}O_3$	354.49
Diacerein		$C_{19}H_{12}O_8$	368.30

Supplementary Table 2. IVM analogues showed similar effects on FRET and FP assays

Name	Structure	FRET (IC_{50} µM)	FP(IC_{50} µM)
344-Ivermectin b1a		10.07	2.7
345-Emamectin		6.6	8.3
346-Eprinomectin		92.2	2.8
348-Abamectin		41.7	2.4
349-Selamectin		15.5	1.8
350-Doramectin		23.6	1.6
Ivermectin		9.2	2.4
356-milbemycin A3		30.9	3.7

Supplementary Table 3. Diffraction data collection and structural refinement statistics*

Data Collection	
Space group	H3
Unit cell dimensions (Å)	a=b=324.70 c=198.76 α=β=90° γ=120°
Number of total reflections	200548 (11828)
Number of unique reflections	87279 (6865)
Mean I/σ	2.7 (0.8)
Redundancy	2.5 (1.7)
Merging R factor (%)	27.1 (93.6)
CC (1/2)	0.904 (0.36)
Maximum resolution, (Å)	3.58
Refinement	
Resolution range, (Å)	93.73 – 3.58
Completeness, (%)	99.77
Number of protein atoms	32452
B factors ($Å^2$)	65.2
R factor, (%)	23.75
R free, (%)	24.16
Rms deviations	
Bond lengths, (Å)	0.013
Bond angles, (°)	1.596

* Values in parentheses refer to the respective highest resolution shell (3.77-3.58 Å). A total of 5 % of collected data were used for the calculation of R free.

Supplementary figures legends

Figure S1. A. The Prestwick library of 1,280 compounds was screened by FRET and FP assays. Six compounds passed the combined screening and qualified for further confirmatory assay (actin pyrene assay, trypsin degradation and protein aggregation). Only ivermectin (IVM) passed all phases of the screening and was selected for further studies. B. BLI dose-response curves (3.13-100 μM) for enzalutamide showing that the drug does not interact with HSP27. C. Pyrene labelled actin can polymerize in the absence of HSP27 (pink line) which was decreased after addition of HSP27 (green line). In presence of IVM, HSP27-mediated inhibition of actin polymerization was prevented, as shown by the shift of the red line towards the actin alone signal (orange line). D. Immunoblotting analysis of HSP27 degradation by trypsin. Pure HSP27 was left in the presence of trypsin for the indicated time with or without IVM (130μM). HSP27 alone and with IVM was used as controls. E. Average root mean square deviation (RMSD) of HSP27 24mer backbone atom coordinates from initial X-ray structure over 3 independent MD runs (bold lines) and the deviation of each RMSD from their averages (vertical bars). Blue and red lines represent HSP27 24mer without and with IVM, respectively. F. Contact frequency between HSP27 dimer and IVM molecule averaged from 12 dimers within the 24mer derived from 3 independent MD simulations. The short blue bars indicate the residues of HSP27 in contact with IVM predicted by the docking. The gold and cyan horizontal lines indicate the residues from monomers A and B, respectively, and the black dots indicate the phosphorylatable serines (15, 78 and 82). Contact was defined when the distance between the heavy atoms of IVM molecule and HSP27 residue was lower than 4.5Å.

Figure S2. A. Immunoblot analysis of phospho and total HSP27 expression after heat shock (42°C for 1 hr) in bladder T24 and breast MCF7 cancer cell lines treated for 24 hours with increasing doses of IVM. B. (Upper panel) PC-3 prostate cancer cells were treated with IVM or IVM analogues (5 μM) for 24 hours. DMSO was used as control. The expression of phosphorylated S78 and S82 as well as total HSP27 was analyzed by western blot. (Lower panel), PC-3 cells were treated as in the upper panel and the expression of S15 was analyzed by western blot. C. HCC-827 lung cancer cells were treated for 24 hours with increasing concentrations of IVM (0 to 5 μM), and phosphorylated levels of p38-MAPK and of HSP27 were analyzed by western blot. D. LNCaP cell

migration was evaluated by scratch assay 42 hours after 5 μM IVM. Data shown as mean ± SEM. *p<0.05,**p<0.01 by Student's t-test. E. E-cadherin mRNA

(CDH1) and protein levels were evaluated 24 hours after IVM treatment by qPCR (upper panel) and western blotting (lower panel), respectively. Data shown as mean ± SEM. *p<0.05, **p<0.01 by unpaired two-tailed Student's ttest with Welch correction, n=3 independent experiments. F. LNCaP cells were treated with increasing doses of IVM (0-10 μM) for 24 hours. Expression of ER-stress related proteins (ATF4, PERK) and apoptosis markers (cleaved caspase 3, PARP) were evaluated by western blotting.

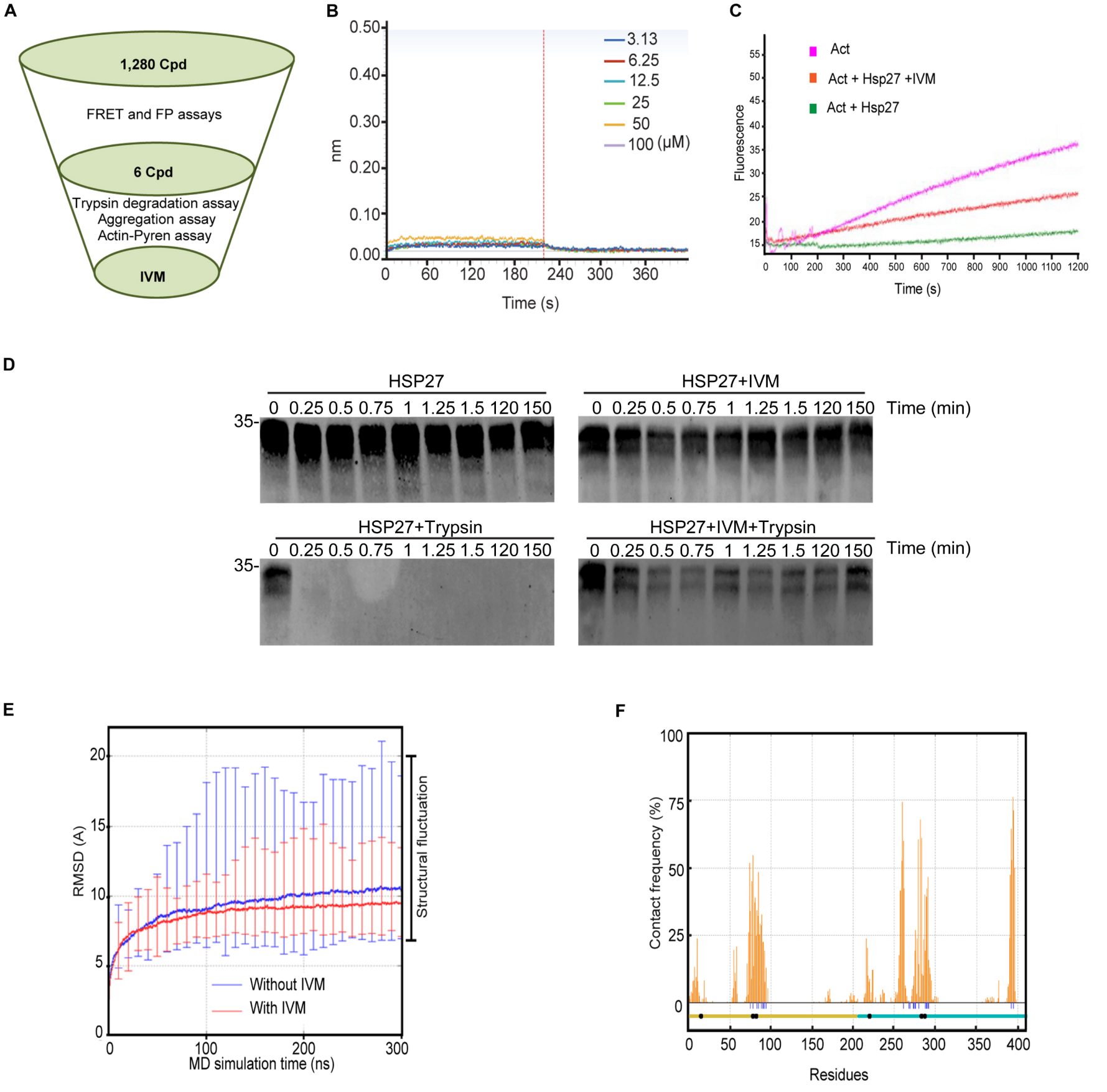
Figure S3. A. mRNA levels of EGFR were evaluated in HCC-827 cells after IVM (5 μM for 24 hours) treatment or HSP27 silencing (20nM); DMSO and si SCR were used as controls, respectively. B. Proximity ligation assay between HSP27and pEGFR in HCC-827 cells after treatment with increasing doses of IVM. Confocal microscopy was used to detect the interaction (red dots). DNA was counterstained with DAPI (blue). Scale bar,20 μm. C. A549 and HCC-827 erlotinib resistant (HCC-827 ER) lung cancer cells, BT474 breast and HCT-116 colorectal cells were treated with increasing doses of HER receptor inhibitors (erlotinib, trastuzumab, and cetuximab respectively) with or without IVM. Surviving cells were quantified by crystal violet. Data shown as mean ± SEM.*p<0.05,**p<0.01,****p<0.0001 by one way ANOVA with Bonferroni's correction. D. (Left panel). BL6 mice were treated with IVM by gavage daily, for 5 days at the dose of 10 mg/kg. The serum concentration of IVM were assessed 0.5,1,2, 4, 8, 12, 24 and 144 hours after the first treatment by mass spectrometry. Bars: SD, (N=5 mice per group). (Middle panel). Balb-c mice xenograft with PC3 prostate cancer cells were treated with IVM 10 mg/Kg orally, 3 times / week, for 6 weeks. IVM concentration was assessed in the serum and in the tumor tissue using Mass Spectrometry (MS). The tumor/serum ratio was 0.68 demonstrating that IVM has a high solubility and distribution in tumor tissue, after oral administration. Bars: SD, (N=3 mice). (Right panel). Balb-c mice xenograft with HCC-827 non small cell lung cancer cells were randomized to receive vehicle alone (CTRL), erlotinib (ERL) 15 mg/kg, ivermectin (IVM) 10 mg/kg, or the combination of both (IVM+ERL) administered orally, 3 times/week. The graph shows the mice weight change during the treatment. Bars: SEM, (N=8 mice per group) E. IHC scoring of pEGFR, pHSP27, Ki-67, SHPTP1 and TUNEL staining in tumor tissue from HCC-827 xenografts treated with control, erlotinib, IVM or the combination of the two drugs, as specified

in figure 3G. Data shown as mean ± SEM,* p<0.05, **p<0.01 by one way ANOVA with Tukey correction n= 8 mice per group. F. (Left panel). Representative images of total and pEGFR levels evaluated in vivo by immunohistochemistry in A549 xenografts treated with SCR-B as control, OGX-427, erlotinib or the combination, as previously reported.(11) (Righpanel). IHC scoring of EGF and pEGFR staining. Data shown as mean ± SEM,**p<0.01,****p<0.0001 by one way ANOVA with Tukey correction n= 8 mice per group.

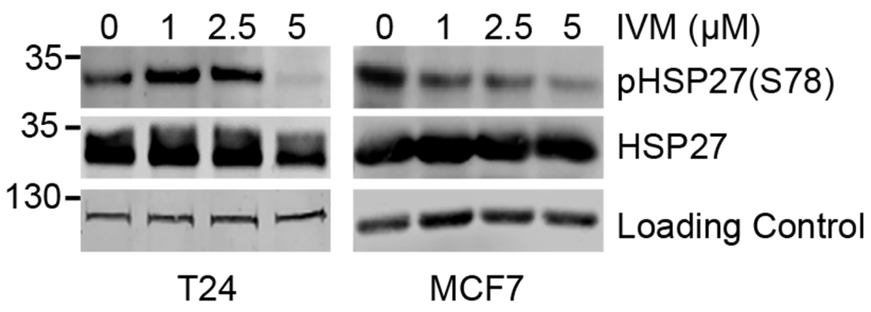
Figure S4. A. mRNA levels of AR and AR-V7 were evaluated in LNCaP and 22RV1 cells after IVM treatment (5 µM for 24 hours); DMSO was used as control. B. IHC scoring of pHSP27, TUNEL and Ki-67 staining in tumor tissue from the PC3-DR xenografts treated with control (CREL), micellar paclitaxel (PACLI), IVM or the combination of the two drugs (PACLI+IVM), as specified in figure 5D. Data shown as mean ± SEM,* p<0.05, **p<0.01 , *** p<0.001, ****p<0.0001 by one way ANOVA with Tukey correction n= 8 mice per group.

Supplementary movies 1 and 2

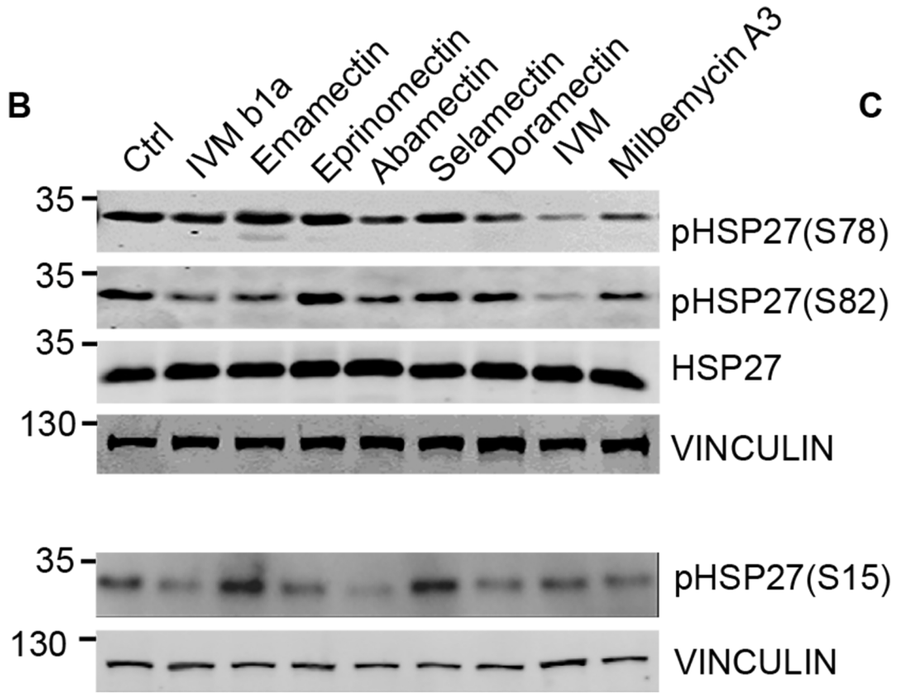
The Molecular Dynamic simulation of 24- mer assembly of HSP27 protein in the absence (file 1, first 454ns of simulations) and in the presence of bound IVM molecules (file 2, first 232 ns of simulations).


A
1,280 Cpd
FRET and FP assays
6 Cpd
Trypsin degradation assay
Aggregation assay
Actin-Pyren assay
IVM
B
nm
Time (s)
3.13
6.25
12.5
25
50
100 (μM)
C
Act
Act + Hsp27 +IVM
Act + Hsp27
Fluorescence
Time (s)
D
HSP27
HSP27+IVM
HSP27+Trypsin
HSP27+IVM+Trypsin
0 0.25 0.5 0.75 1 1.25 1.5 120 150
Time (min)
35-
E
RMSD (A)
MD simulation time (ns)
Structural fluctuation
Without IVM
With IVM
F
Contact frequency (%)
Residues

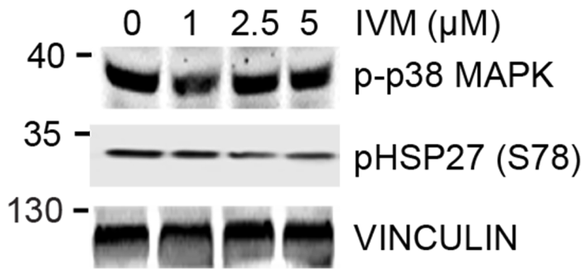
A


0 1 2.5 5 0 1 2.5 5 IVM (μM)
35
pHSP27(S78)
35
HSP27
130
Loading Control
T24
MCF7

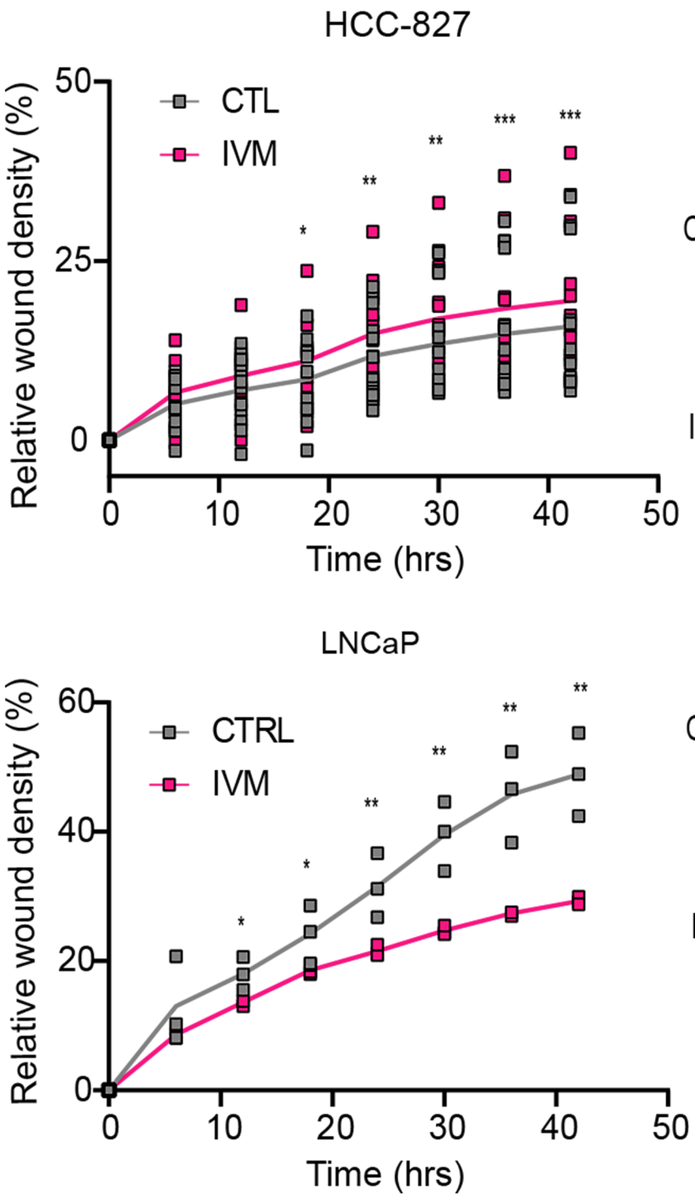
B


Ctrl
IVM b1a
Emamectin
Eprinomectin
Abamectin
Selamectin
Doramectin
IVM
Milbemycin A3
35
pHSP27(S78)
35
pHSP27(S82)
35
HSP27
130
VINCULIN
35
pHSP27(S15)
130
VINCULIN

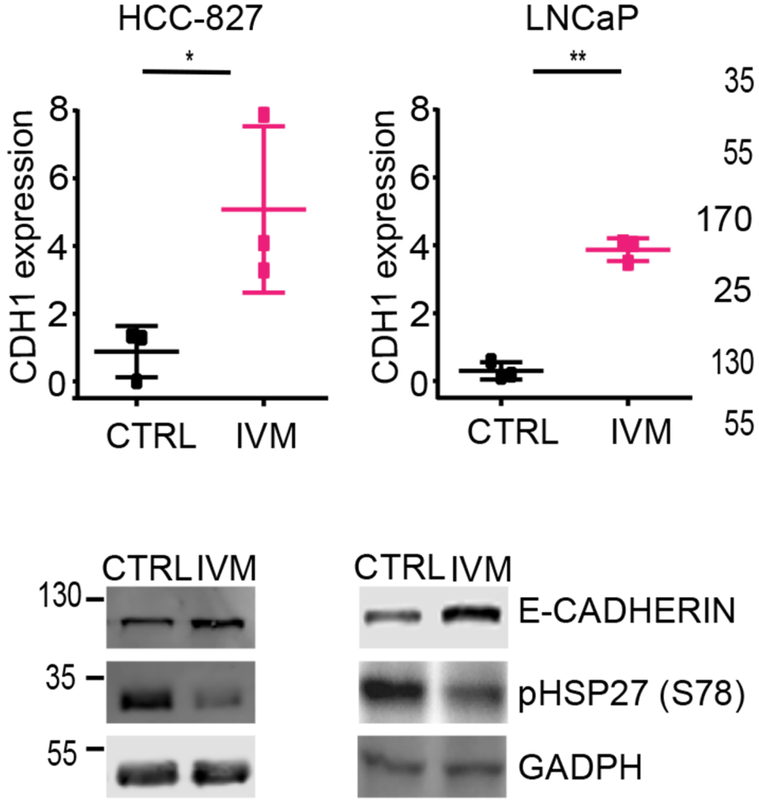
C


0 1 2.5 5 IVM (μM)
40
p-p38 MAPK
35
pHSP27 (S78)
130
VINCULIN

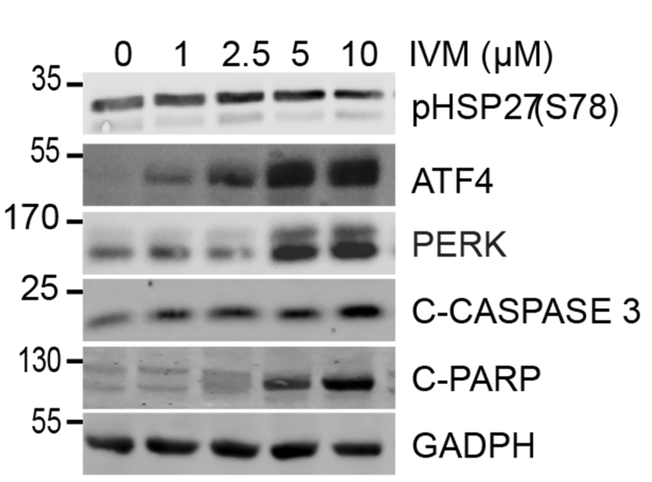
D

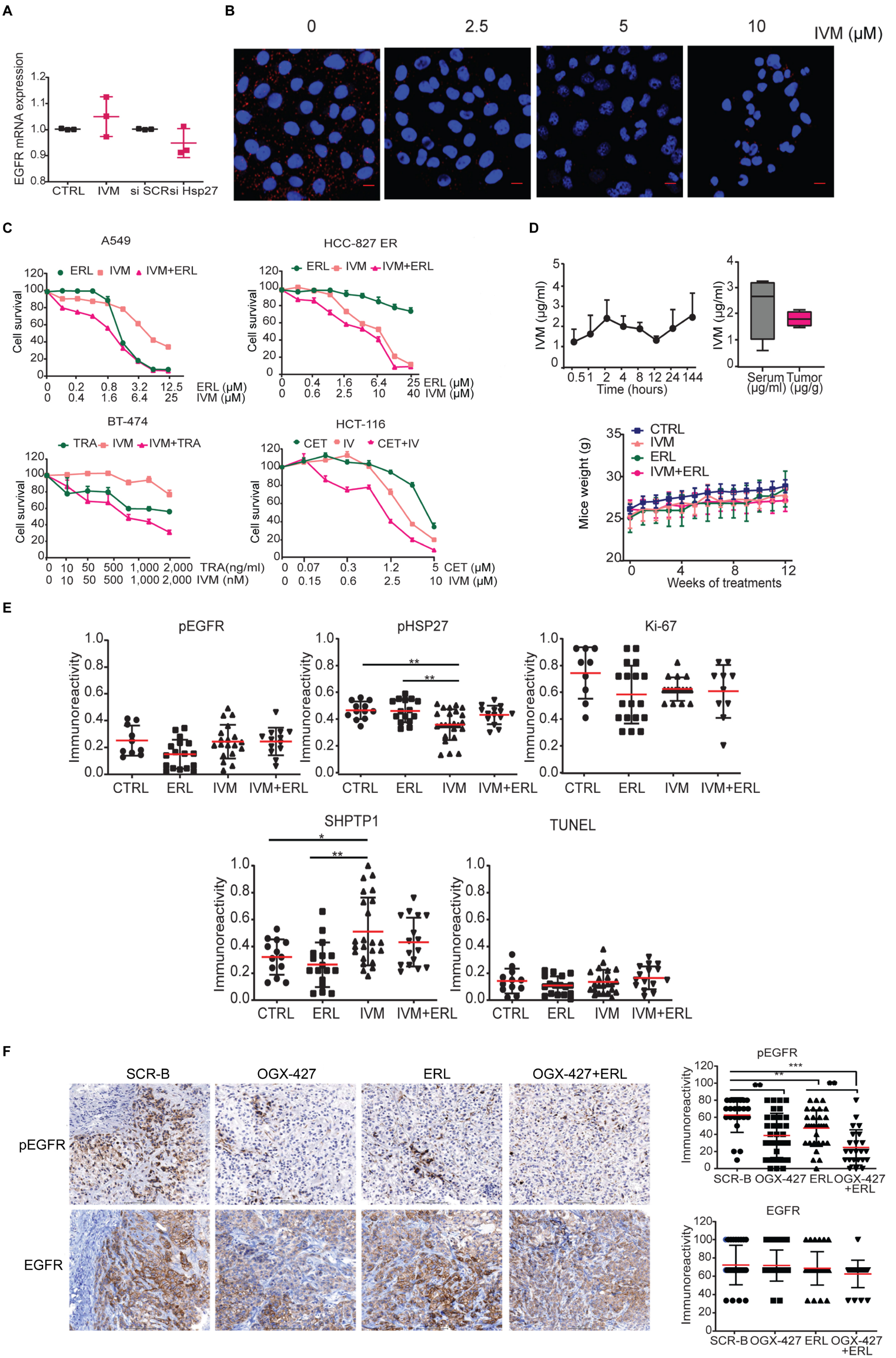

HCC-827
CTL
IVM
Relative wound density (%)
Time (hrs)
T0
42h
CTRL
IVM
LNCaP
CTRL
IVM
Relative wound density (%)
Time (hrs)
T0
42h
CTRL
IVM

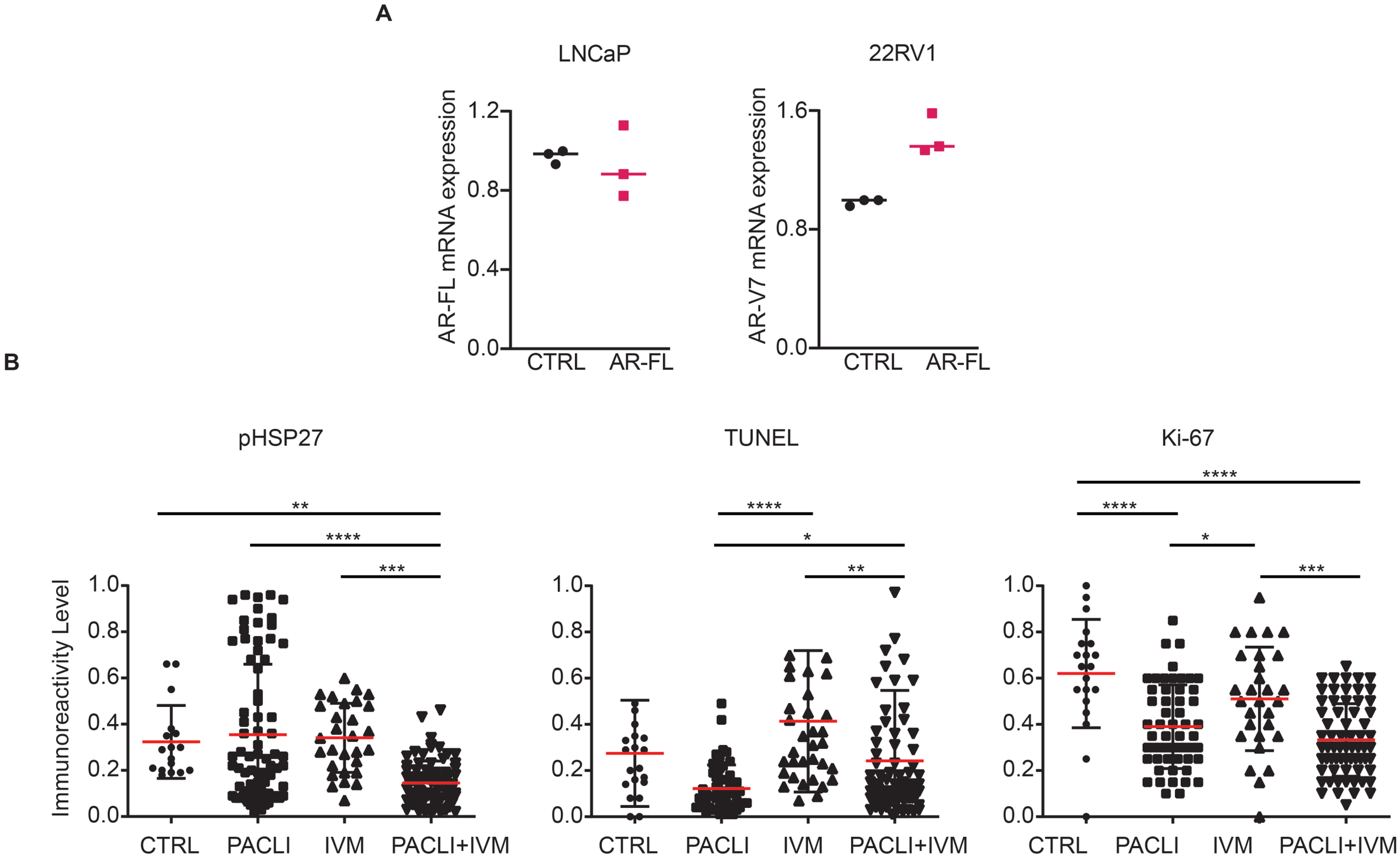
E


HCC-827
CDH1 expression
CTRL
IVM
LNCaP
CDH1 expression
CTRL
IVM
CTRL IVM
130
35
55
CTRL IVM
E-CADHERIN
pHSP27 (S78)
GADPH

F


0 1 2.5 5 10 IVM (μM)
35
pHSP27(S78)
55
ATF4
170
PERK
25
C-CASPASE 3
130
C-PARP
55
GADPH


A
EGFR mRNA expression
1.2
1.1
1.0
0.9
0.8
CTRL
IVM
si SCR
si Hsp27
B
0
2.5
5
10
IVM (μM)
C
A549
ERL
IVM
IVM+ERL
Cell survival
0 0.2 0.8 3.2 12.5 ERL (μM)
0 0.4 1.6 6.4 25 IVM (μM)
HCC-827 ER
0 0.4 1.6 6.4 25 ERL (μM)
0 0.6 2.5 10 40 IVM (μM)
BT-474
TRA
IVM+TRA
0 10 50 500 1,000 2,000 TRA(ng/ml)
0 10 50 500 1,000 2,000 IVM (nM)
HCT-116
CET
IV
CET+IV
0 0.07 0.3 1.2 5 CET (μM)
0 0.15 0.6 2.5 10 IVM (μM)
D
IVM (μg/ml)
0.5 1 2 4 8 12 24 144
Time (hours)
Serum (μg/ml)
Tumor (μg/g)
CTRL
IVM
ERL
IVM+ERL
Mice weight (g)
Weeks of treatments
E
pEGFR
pHSP27
Ki-67
Immunoreactivity
CTRL
ERL
IVM
IVM+ERL
SHPTP1
TUNEL
F
SCR-B
OGX-427
ERL
OGX-427+ERL
pEGFR
EGFR
SCR-B OGX-427 ERL OGX-427 +ERL


A
LNCaP
AR-FL mRNA expression
1.2
0.8
0.4
0.0
CTRL
AR-FL
22RV1
AR-V7 mRNA expression
1.6
0.8
0.0
CTRL
AR-FL
B
pHSP27
TUNEL
Ki-67
Immunoreactivity Level
1.0
0.8
0.6
0.4
0.2
0.0
**
****
***
*
CTRL
PACLI
IVM
PACLI+IVM