Original Research Article

Targeting allosteric site of AKT by 5,7-dimethoxy-1,4-phenanthrenequinone suppresses neutrophilic inflammation

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Supplementary Materials and Methods

Superoxide anion-scavenging assay

The superoxide anion-scavenging effect of CLLV-1 was examined in a cell-free xanthine/xanthine oxidase system. The assay buffer contained 50 mM Tris (pH 7.4), 0.3 mM WST-1, and 0.02 U/mL xanthine oxidase. WST-1 was reduced by the superoxide anion after adding 0.1 mM xanthine to the assay buffer at 30 °C. The absorbance was measured at 450 nm.

NADPH oxidase activity

Neutrophils were mixed with 1 mM phenylmethylsulphonyl fluoride for 30 min at 4 °C and sonicated in relaxation buffer (10 mM piperazine-N,N′-bis(2-ethanesulfonic acid) PIPES, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 1 mM ATP; pH 7.3) to produce cytosolic and plasma membrane fractions. NADPH oxidase was activated by the addition of 100 μM SDS before incubation with DMSO or CLLV-1 for 2 min as described.

Elastase activity

Human neutrophils (6×10^5 cells/mL) were activated by the addition of fMLF

(0.1 μM) in the presence of CB (2.5 μg/mL) for 15 min at 37 °C. Elastase was obtained from the supernatant of the cells after they were centrifuged at 1000 *g* for 5 min at 4 °C. Then, the supernatant was equilibrated at 37 °C for 2 min and incubated with or without CLLV-1 for 5 min. After incubation, elastase substrate (100 μM) was added to the reaction solutions. The changes in absorbance were continuously monitored for 10 min at 405 nm to determine the elastase activity.

LDH release

The CytoTox 96 non-radioactive cytotoxicity assay (Promega, Maddison, WI, USA) was used to determine the LDH level. Human neutrophils were treated with CLLV-1 (1 or 3 μM) for 15 or 60 min. The supernatant was obtained for the LDH assay. LDH release was expressed as a percentage of the amount of enzyme liberated following incubation of human neutrophils with 0.1% Triton X-100 for 30 min at 37 °C.

Receptor-binding assay

Receptor binding was assayed by FACScan analysis of the binding of fNLFNYK, a fluorescent analog of fMLF. Cells were incubated with CLLV-1 for 5 min at 4 °C and labeled with fNLFNYK. After 30 min, the cells were pelleted, resuspended in

ice-cold HBSS, and immediately analyzed using a flow cytometer (FACSCalibur™; BD Bioscience).

cAMP concentration

The concentration of cAMP was determined with an enzyme immunoassay kit (GE Healthcare, Little Chalfont, UK). Neutrophils were treated with DMSO or CLLV-1 at 37 °C for 5 min and then activated by 0.1 μM fMLF for another 1 min. The reaction was terminated by adding 0.5% dodecyltrimethylammonium bromide. After samples were centrifuged at 3000 *g* for 5 min at 4 °C, the supernatants were analyzed for cAMP according to the manufacturer's instructions.

Supplementary figure legends

Fig. S1. CLLV-1 has no effect on cell viability, superoxide anion scavenging, NADPH oxidase activity, elastase activity, and FPR1 binding. (a) Neutrophils were incubated with DMSO or CLLV-1 (1 or 3 μM) for 15 and 60 min. Cytotoxicity was evaluated by LDH release as a percentage of total LDH release. (b) The superoxide anion scavenging effect of CLLV-1 (0.1–3 μM) was assayed in a cell-free xanthine/xanthine oxidase system. Reduction of WST-1 was measured spectrophotometrically at 450 nm. SOD (0.5 or 20 U/mL) was used as the positive control. (c) The subcellular NADPH oxidase activity was investigated in an SDS-reconstituted system. A reaction mixture of neutrophil cytosolic fraction and membrane fraction at 30 °C was activated by the addition of 100 μM SDS before incubation with DMSO, CLLV-1 (0.3 or 3 μM), or DPI (10 μM) for 2 min. The reaction was initiated by adding 0.16 mM NADPH, and the changes in absorbance at 550 nm caused by the addition of 0.16 mM NADPH were measured. (d) Human neutrophils were pretreated with fMLF/CB for 15 min. The supernatant was obtained and incubated with DMSO or CLLV-1 (0.1–3 μM) before the addition of substrate (100 μM). Elastase activity was measured spectrophotometrically at 405 nm. (e) Neutrophils were preincubated with DMSO, CLLV-1 (0.1–3 μM), or fMLF (10 μM) for 5 min before labeling with the fluorescence-labeled peptide fNLFNYK (4 nM) for another 30 min. The fluorescence

was determined by flow cytometry. All data are expressed as mean values ± SEM (n = 3); ***$p < 0.001$ compared with the control (Student's *t*-test).

Fig. S2. The representative histograms show an inhibitory effect of CLLV-1 on human neutrophils adhering to ECs. Hoechst 33342-labeled neutrophils were incubated with DMSO or CLLV-1 (0.1–3 μM) for 5 min before activation with fMLF (0.1 μM)/CB (1 μg/mL). Activated neutrophils were then co-cultured with LPS-pre-activated ECs for 30 min. Neutrophils adhering to ECs were detected using microscopy.

Fig. S3. CLLV-1 represses superoxide anion generation or elastase release in NaF-, WKYMVm-, IL-8-, or LTB_4-activated human neutrophils. Human neutrophils were preincubated with DMSO or CLLV-1 (0.03–3 μM) and then activated with or without (a,c) NaF (20 mM)/CB (2 μg/mL), (b,d) WKYMVm (1 nM)/CB (1 μg/mL), (e) IL-8 (50 ng/mL)/CB (2 μg/mL), or (f) LTB_4 (0.1 μM)/CB (0.5 μg/mL). Superoxide anion generation (a-b) and elastase release (c-f) were assayed using cytochrome *c* reduction and elastase substrate by spectrophotometry at 550 nm and 405 nm, respectively. All data are expressed as mean values ± SEM (n = 3); **$p < 0.01$ and ***$p < 0.001$ compared with the DMSO + fMLF group (Student's *t*-test).

Fig. S4. CLLV-1 inhibits the phosphorylation of AKT in NaF-, WKYMVm-, IL-8-, or LTB_4-activated human neutrophils. Human neutrophils were preincubated with DMSO or CLLV-1 (0.03–3 μM) and then activated with or without (a) NaF (20 mM)/CB (2 μg/mL), (b) WKYMVm (1 nM)/CB (1 μg/mL), (c) IL-8 (50 ng/mL)/CB (2 μg/mL), or (d) LTB_4 (0.1 μM)/CB (0.5 μg/mL). Phosphorylation of AKT was analyzed by immunoblotting, using antibodies against phosphorylated (S473 and T308) and total AKT. All data are expressed as mean values ± SEM (n = 3); $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with the DMSO + fMLF group (Student's *t*-test).

Fig. S5. MK-2206 suppresses superoxide anion generation and elastase release in fMLF-activated human neutrophils and AKT activity *in vitro*. Human neutrophils were preincubated with DMSO or MK-2206 (0.3-10 μM) and then activated with or without fMLF (0.1 μM)/CB (1 μg/mL). (a) Superoxide anion generation was detected using cytochrome *c* reduction by spectrophotometry at 550 nm. (b) Elastase release was measured at 405 nm. (c) The active AKT proteins were immunoprecipitated with phospho-AKT antibodies and treated with DMSO, CLLV-1 (1 or 3 μM), or MK-2206 (0.3–3 μM) for 15 min at 30 °C, followed by treatment with GSK-3 fusion protein (AKT substrate) for another 30 min. The phospho-GSK-3 fusion protein was

examined by immunoblotting. All data are expressed as mean values ± SEM (n = 3); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with the DMSO + fMLF group (A or B) or GSK protein + AKT group (C) (Student's *t*-test).

Fig. S6. The cellular morphology of differentiated HL-60 (dHL-60) cells. The cellular morphologies of HL-60 cells or DMSO-differentiated dHL-60 cells were observed by microscopy for 5 days (D5).

Fig. S7. The representative histograms of flow cytometric analysis show an inhibitory effect of CLLV-1 on ROS formation and F-actin assembly, but not on PIP3 expression in fMLF-activated dHL-60 cells. (a) DHR123 was used to detect the intracellular ROS. (b) Alexa Fluor 594 Phalloidin was used to examine the F-actin assembly. (c) Anti-PIP3 antibodies and FITC-labeled anti-mouse IgG antibodies were used to measure PIP3 expression.

Fig. S8. CLLV-1 does not affect the AKT upstream kinases in fMLF-activated human neutrophils. Phosphorylation of AKT upstream kinases, PDK1, mTORC2, and PI3K was determined by immunoblotting using antibodies against the phosphorylated form and normalized to GAPDH.

Fig. S9. The anti-inflammatory effects of CLLV-1 are independent of cAMP levels and PKA in fMLF-activated human neutrophils. (a-b) Human neutrophils were preincubated for 5 min with H_2O or H89 (3 μM) before the addition of DMSO, CLLV-1 (0.3 μM), or PGE1 (3 μM) and then activated by fMLF (0.1 μM)/CB (1 μg/mL). (a) Superoxide anion generation was detected using cytochrome *c* reduction by spectrophotometry at 550 nm. (b) Elastase release was measured using elastase substrate by spectrophotometry at 405 nm. (c) Human neutrophils were incubated with DMSO or CLLV-1 (0.1 or 1 μM) for 5 min before stimulation with or without fMLF (0.1 μM)/CB (1 μg/mL) for another 1 min. The cAMP levels were measured by enzyme immunoassay kits. All data are expressed as mean values ± SEM (n = 3); ***$p < 0.001$ compared with the H_2O group (Student's *t*-test).

Fig. S10. The chemical interaction of CLLV-1 and synthetic AKT peptides. (a) ^{1}H NMR spectra of CLLV-1, synthetic AKT peptides (304-308 or 314-318), and mixtures of CLLV-1 and AKT peptides. (b) The synthetic AKT peptides $AKT_{304-308}$ or $AKT_{314-318}$ were incubated in the presence or absence of CLLV-1. The molecular masses of the synthetic AKT peptides and their CLLV-1 adducts were detected using MALDI-TOF MS. M, molecular mass of AKT peptides.

Fig. S1

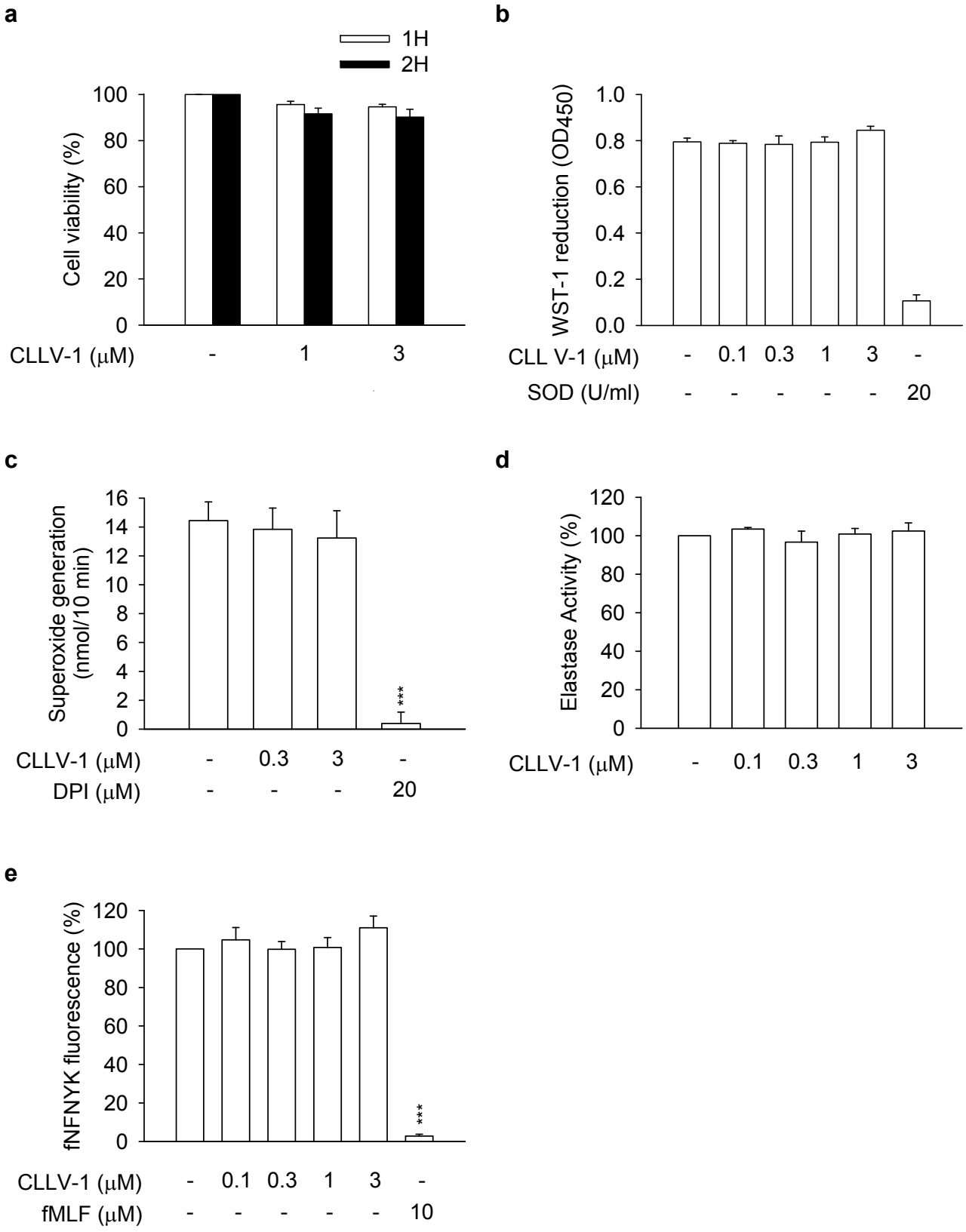

a
1H
2H
Cell viability (%)
100
80
60
40
20
0
CLLV-1 (μM) - 1 3
b
WST-1 reduction (OD450)
1.0
0.8
0.6
0.4
0.2
0.0
CLL V-1 (μM) - 0.1 0.3 1 3 -
SOD (U/ml) - - - - - 20
c
Superoxide generation (nmol/10 min)
16
14
12
10
8
6
4
2
0
***
CLLV-1 (μM) - 0.3 3 -
DPI (μM) - - - 20
d
Elastase Activity (%)
120
100
80
60
40
20
0
CLLV-1 (μM) - 0.1 0.3 1 3
e
fNFNYK fluorescence (%)
120
100
80
60
40
20
0
***
CLLV-1 (μM) - 0.1 0.3 1 3 -
fMLF (μM) - - - - - 10

Fig. S2

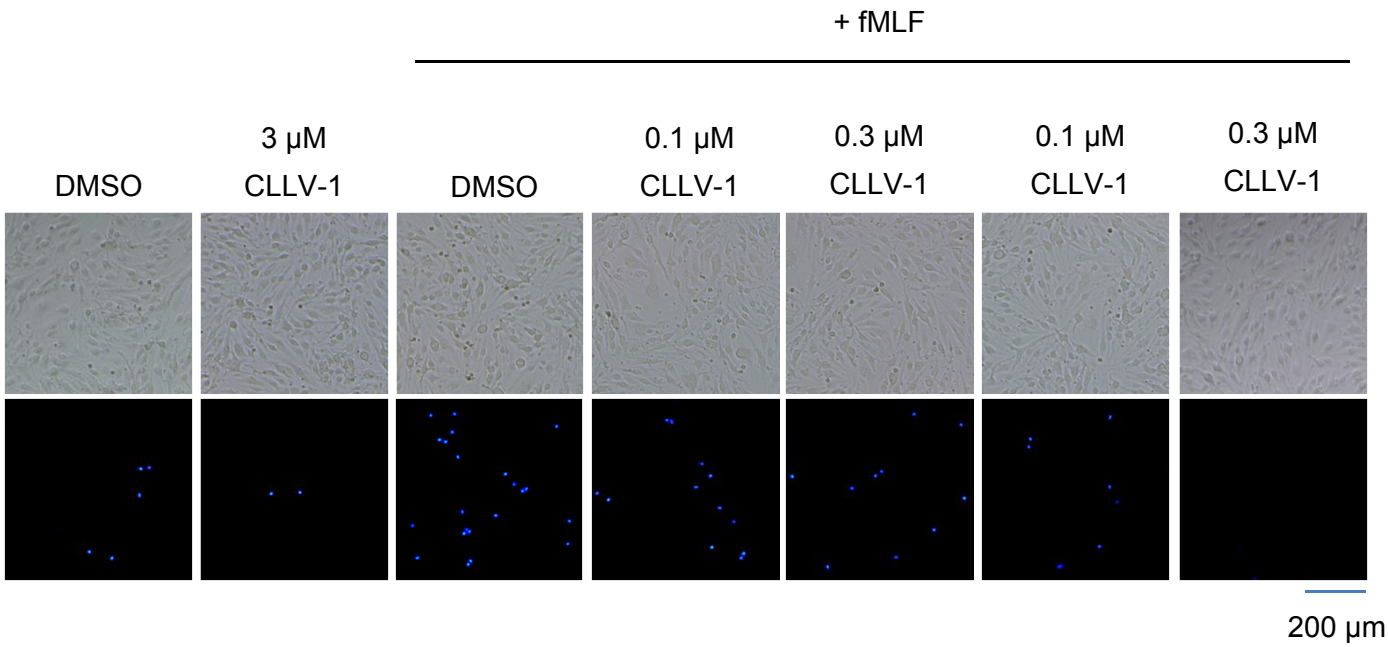

+ fMLF
DMSO
3 μM CLLV-1
DMSO
0.1 μM CLLV-1
0.3 μM CLLV-1
0.1 μM CLLV-1
0.3 μM CLLV-1
200 μm

Fig. S3

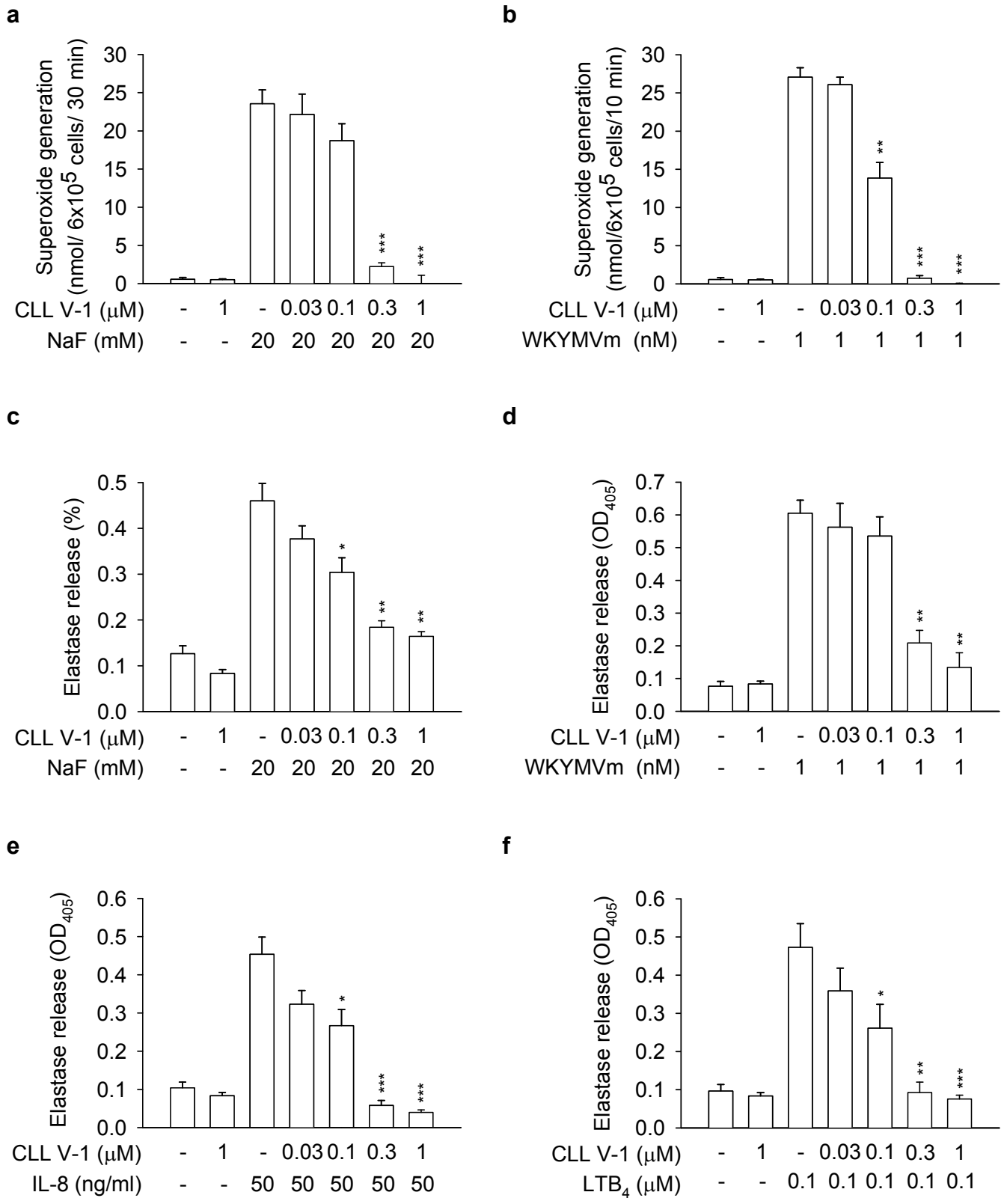

a
Superoxide generation (nmol/ 6x10^5 cells/ 30 min)
30
25
20
15
10
5
0
***
***
CLL V-1 (μM) - 1 - 0.03 0.1 0.3 1
NaF (mM) - - 20 20 20 20 20
b
Superoxide generation (nmol/6x10^5 cells/10 min)
30
25
20
15
10
5
0
**
***
***
CLL V-1 (μM) - 1 - 0.03 0.1 0.3 1
WKYMVm (nM) - - 1 1 1 1 1
c
Elastase release (%)
0.5
0.4
0.3
0.2
0.1
0.0
*
**
**
CLL V-1 (μM) - 1 - 0.03 0.1 0.3 1
NaF (mM) - - 20 20 20 20 20
d
Elastase release (OD405)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
**
CLL V-1 (μM) - 1 - 0.03 0.1 0.3 1
WKYMVm (nM) - - 1 1 1 1 1
e
Elastase release (OD405)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
***
***
CLL V-1 (μM) - 1 - 0.03 0.1 0.3 1
IL-8 (ng/ml) - - 50 50 50 50 50
f
Elastase release (OD405)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
**
***
CLL V-1 (μM) - 1 - 0.03 0.1 0.3 1
LTB4 (μM) - - 0.1 0.1 0.1 0.1 0.1

Fig. S4

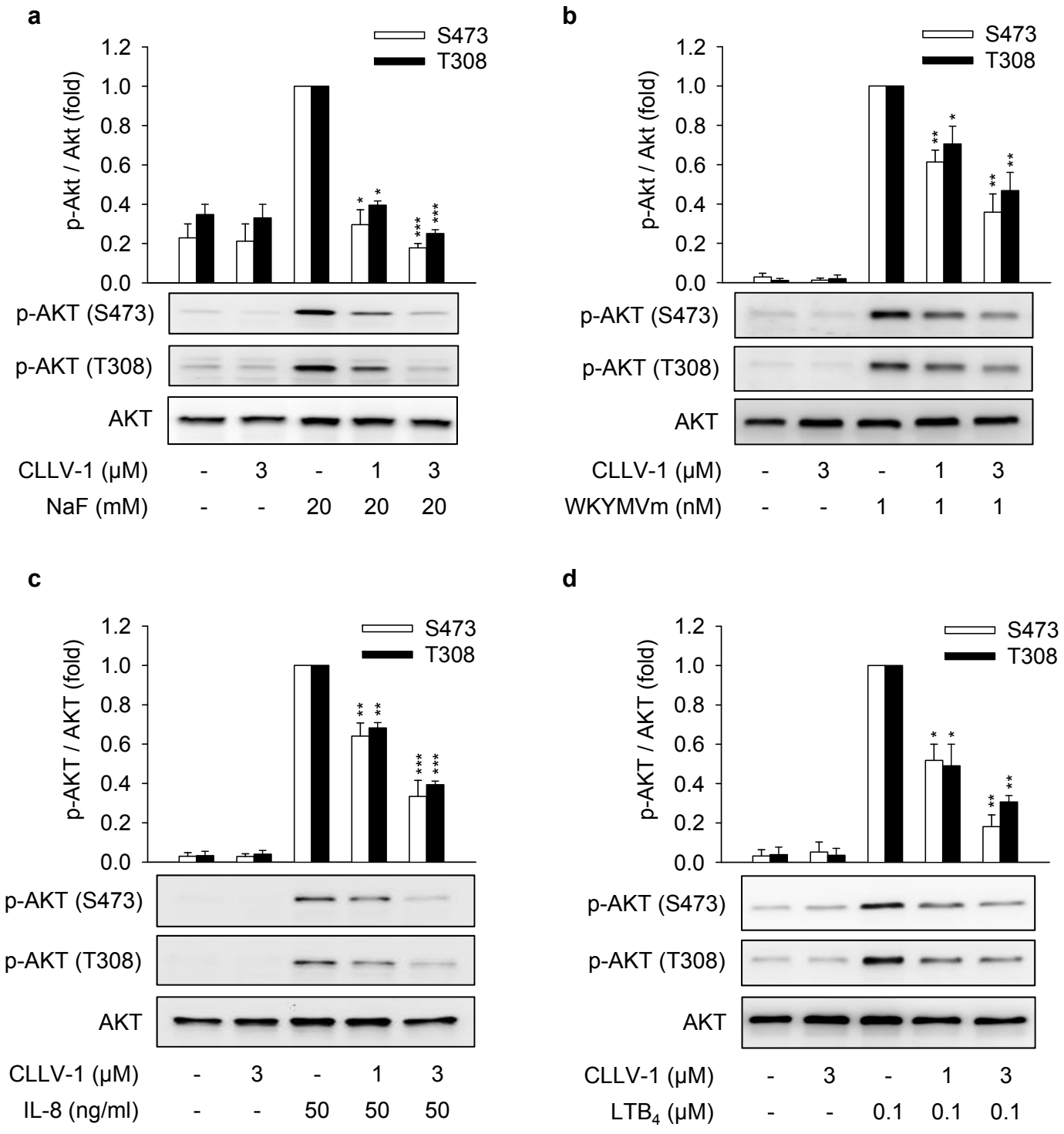

a
S473
T308
p-Akt / Akt (fold)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
p-AKT (S473)
p-AKT (T308)
AKT
CLLV-1 (μM) - 3 - 1 3
NaF (mM) - - 20 20 20
b
S473
T308
p-Akt / Akt (fold)
p-AKT (S473)
p-AKT (T308)
AKT
CLLV-1 (μM) - 3 - 1 3
WKYMVm (nM) - - 1 1 1
c
S473
T308
p-AKT / AKT (fold)
p-AKT (S473)
p-AKT (T308)
AKT
CLLV-1 (μM) - 3 - 1 3
IL-8 (ng/ml) - - 50 50 50
d
S473
T308
p-AKT / AKT (fold)
p-AKT (S473)
p-AKT (T308)
AKT
CLLV-1 (μM) - 3 - 1 3
LTB4 (μM) - - 0.1 0.1 0.1

Fig. S5

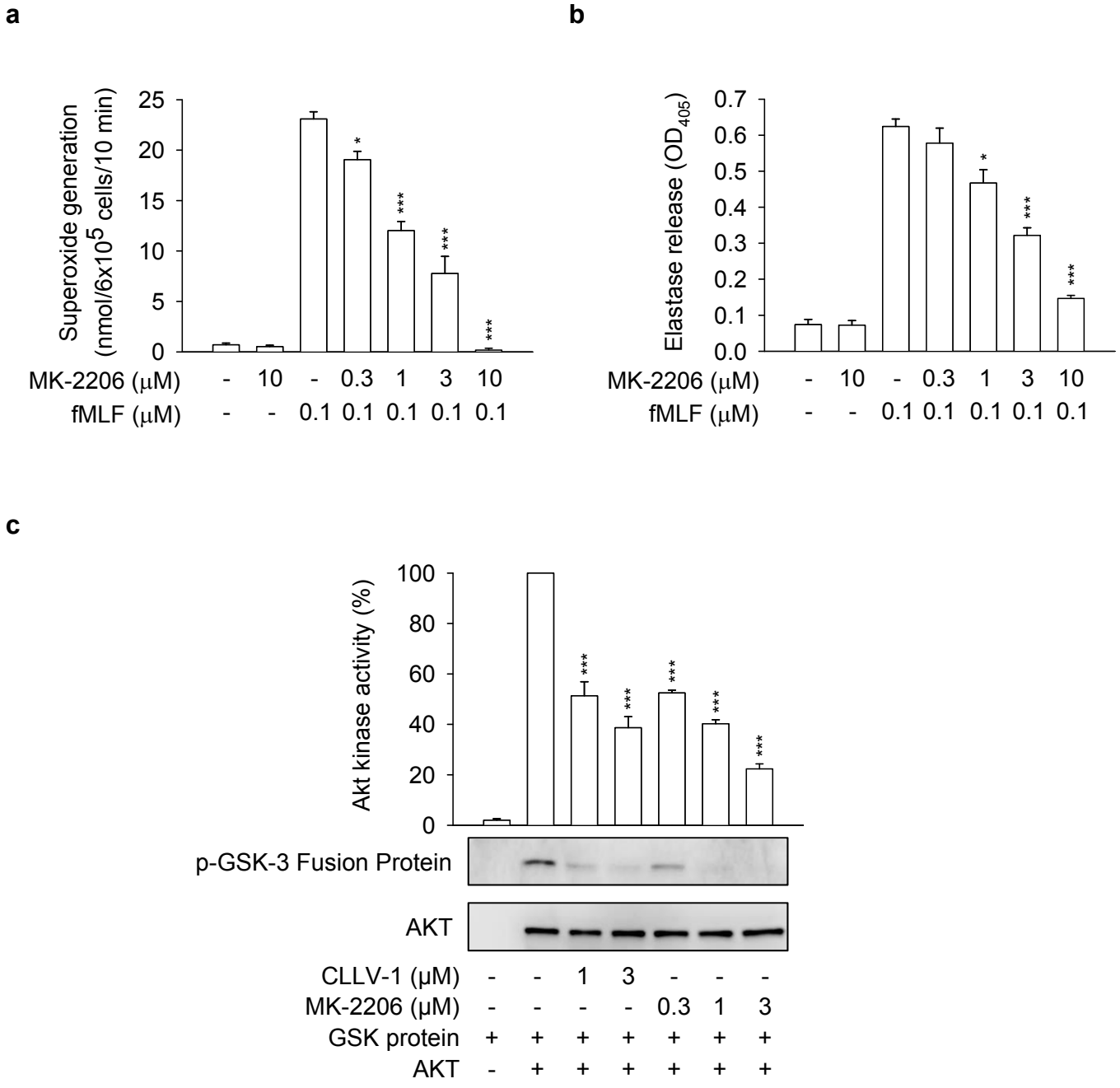

a
Superoxide generation (nmol/6x10^5 cells/10 min)
25
20
15
10
5
0
*
***
***
***
MK-2206 (μM) - 10 - 0.3 1 3 10
fMLF (μM) - - 0.1 0.1 0.1 0.1 0.1
b
Elastase release (OD405)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
***
***
MK-2206 (μM) - 10 - 0.3 1 3 10
fMLF (μM) - - 0.1 0.1 0.1 0.1 0.1
c
Akt kinase activity (%)
100
80
60
40
20
0
***
***
***
***
***
p-GSK-3 Fusion Protein
AKT
CLLV-1 (μM) - - 1 3 - - -
MK-2206 (μM) - - - - 0.3 1 3
GSK protein + + + + + + +
AKT - + + + + + +

Fig. S6

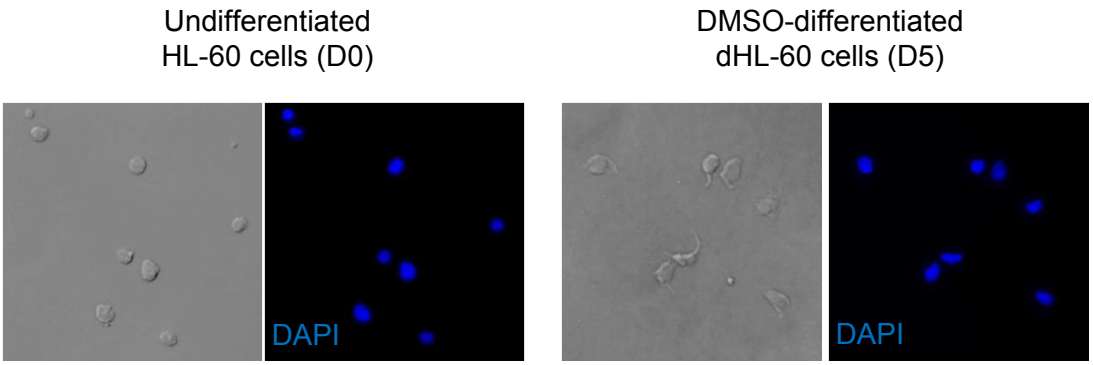

Undifferentiated
HL-60 cells (D0)
DMSO-differentiated
dHL-60 cells (D5)
DAPI
DAPI

Fig. S7

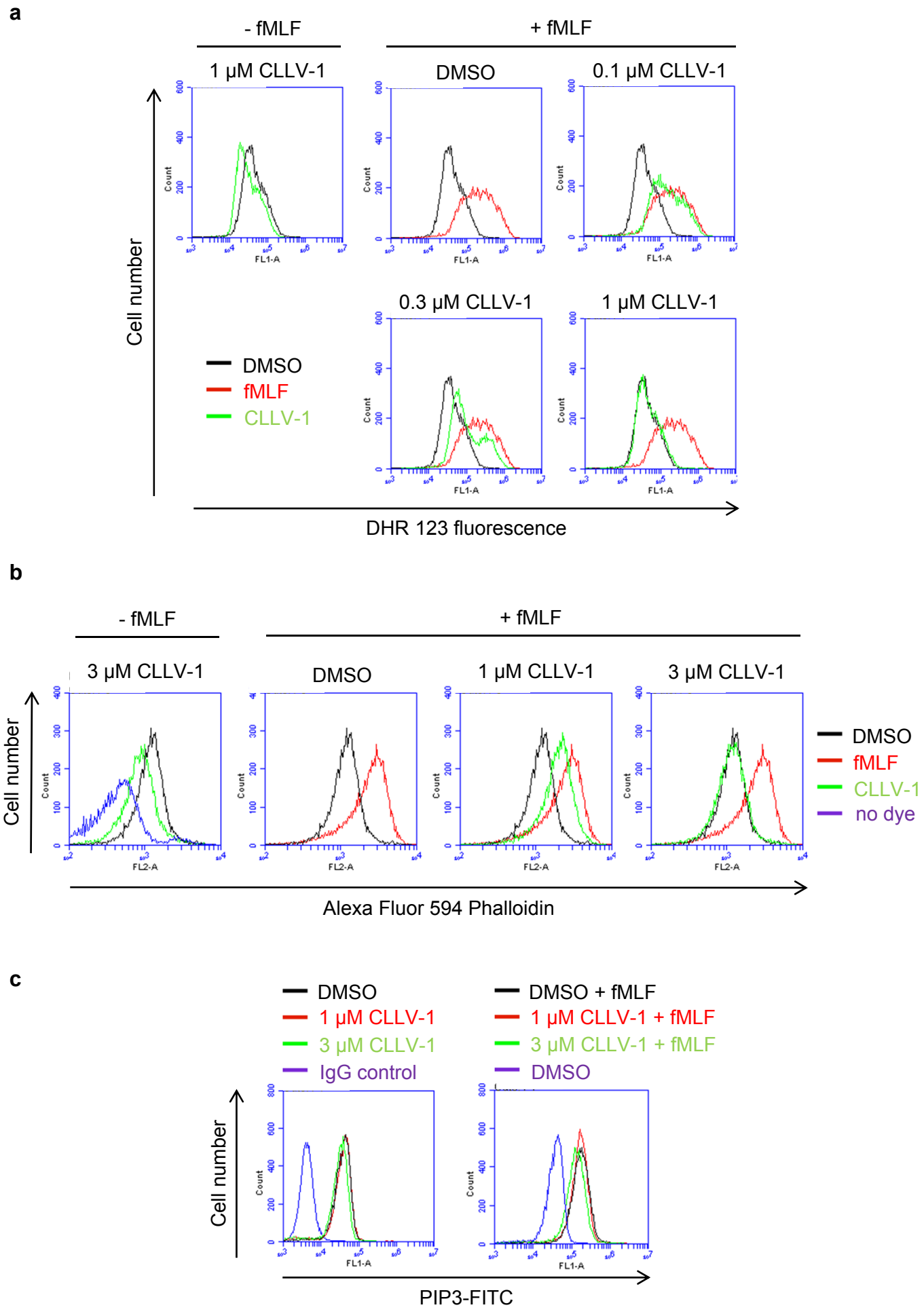

a
- fMLF
+ fMLF
1 μM CLLV-1
DMSO
0.1 μM CLLV-1
0.3 μM CLLV-1
1 μM CLLV-1
Cell number
DMSO
fMLF
CLLV-1
Count
FL1-A
DHR 123 fluorescence
b
- fMLF
+ fMLF
3 μM CLLV-1
DMSO
1 μM CLLV-1
3 μM CLLV-1
Cell number
DMSO
fMLF
CLLV-1
no dye
Count
FL2-A
Alexa Fluor 594 Phalloidin
c
DMSO
1 μM CLLV-1
3 μM CLLV-1
IgG control
DMSO + fMLF
1 μM CLLV-1 + fMLF
3 μM CLLV-1 + fMLF
DMSO
Cell number
Count
FL1-A
PIP3-FITC

Fig. S8

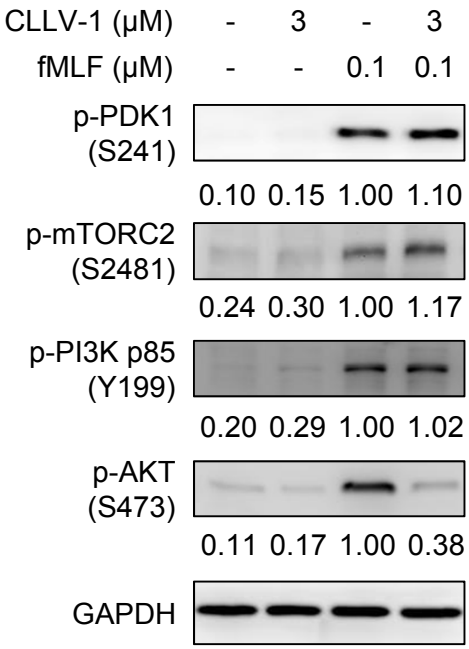
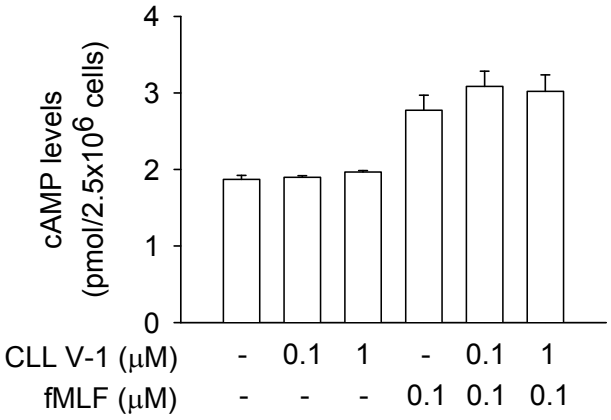
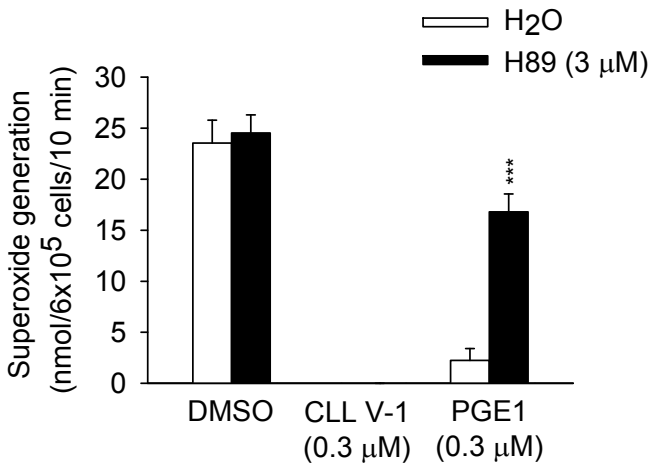

CLLV-1 (μM) - 3 - 3
fMLF (μM) - - 0.1 0.1
p-PDK1 (S241)
0.10 0.15 1.00 1.10
p-mTORC2 (S2481)
0.24 0.30 1.00 1.17
p-PI3K p85 (Y199)
0.20 0.29 1.00 1.02
p-AKT (S473)
0.11 0.17 1.00 0.38
GAPDH

Fig. S9

a


cAMP levels
(pmol/2.5x10^6 cells)
4
3
2
1
0
CLL V-1 (μM) - 0.1 1 - 0.1 1
fMLF (μM) - - - 0.1 0.1 0.1

b


H2O
H89 (3 μM)
Superoxide generation
(nmol/6x10^5 cells/10 min)
30
25
20
15
10
5
0
***
DMSO
CLL V-1
(0.3 μM)
PGE1
(0.3 μM)

c

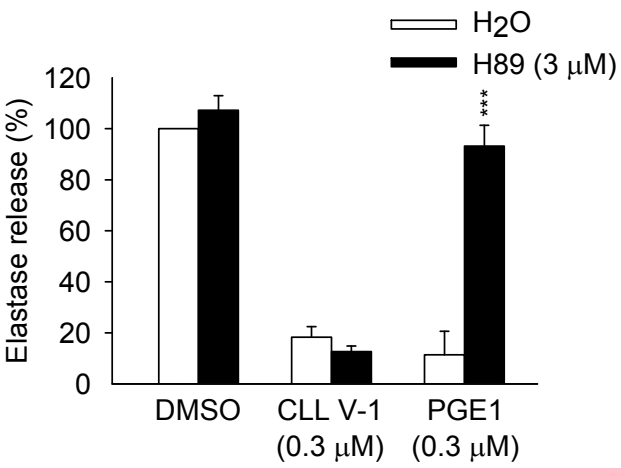
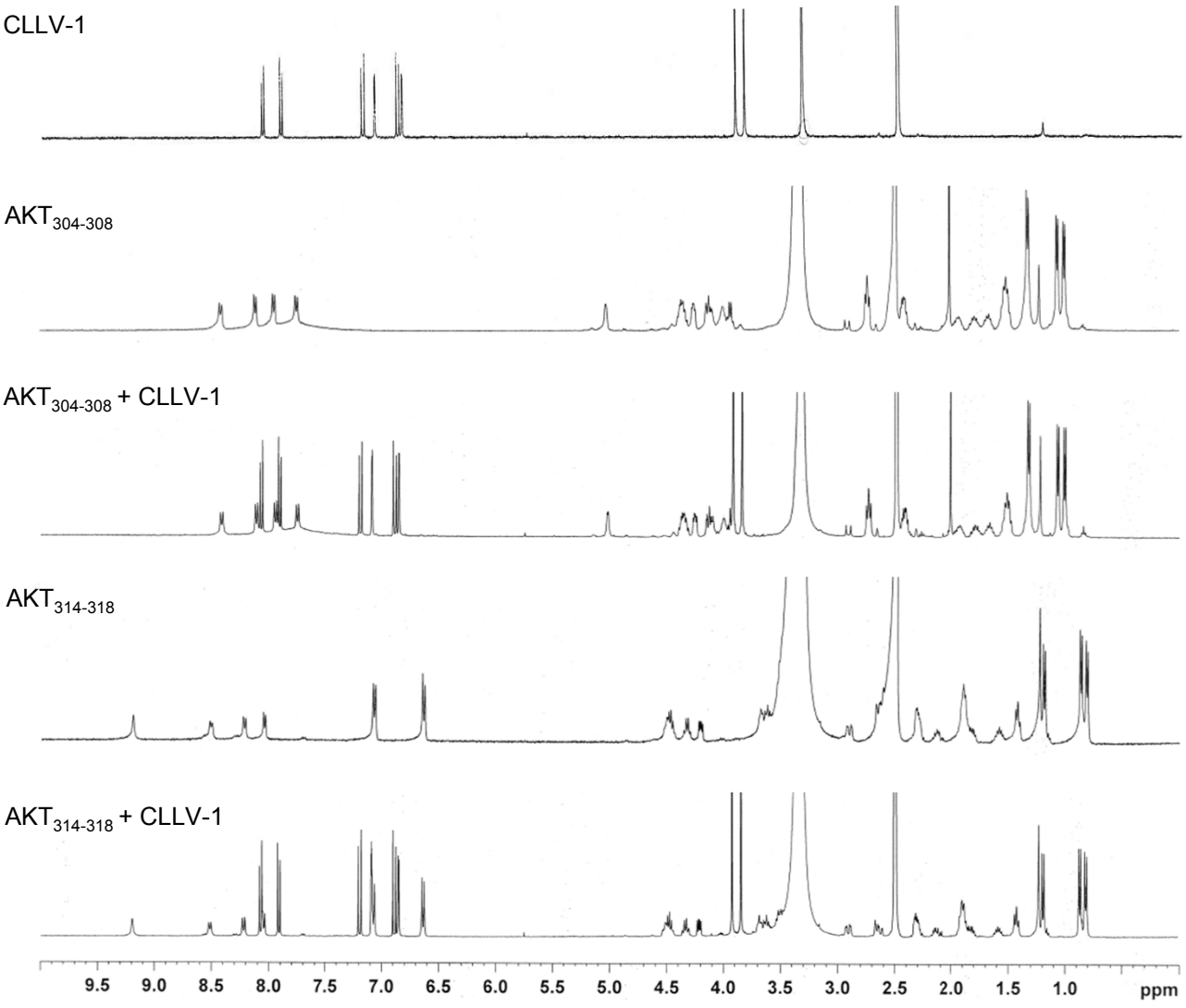
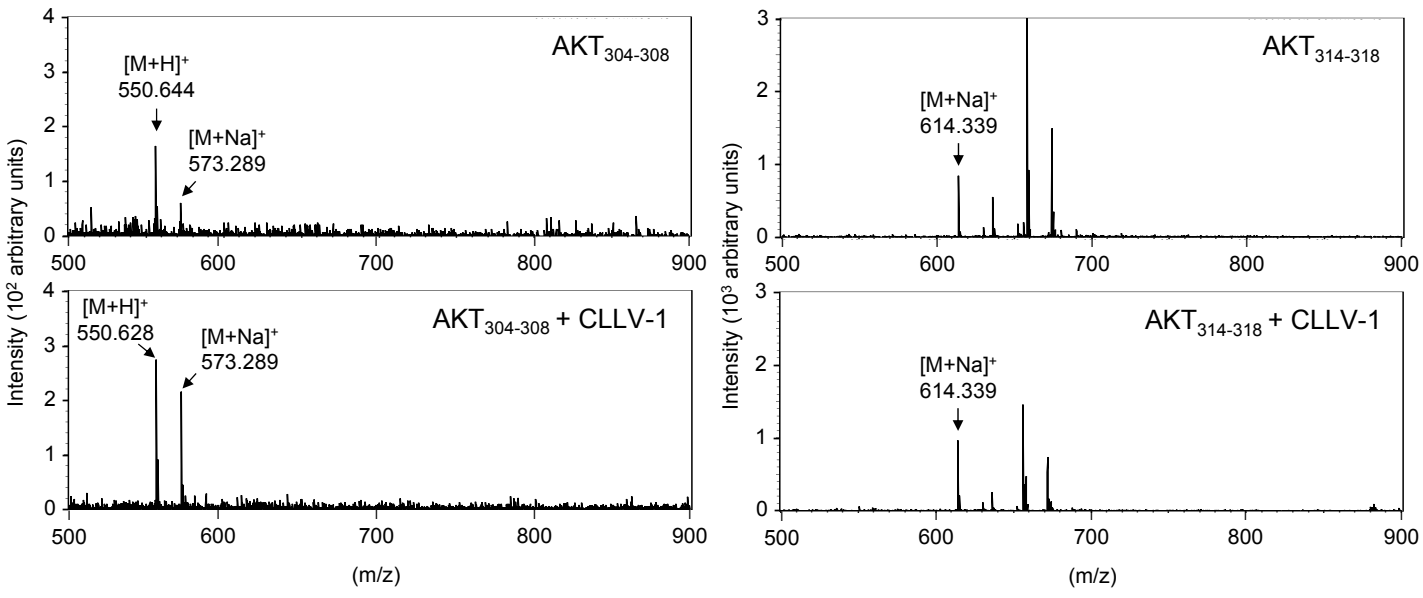

H2O
H89 (3 μM)
Elastase release (%)
120
100
80
60
40
20
0
***
DMSO
CLL V-1
(0.3 μM)
PGE1
(0.3 μM)

Fig. S10

a


CLLV-1
AKT304-308
AKT304-308 + CLLV-1
AKT314-318
AKT314-318 + CLLV-1
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
ppm

b


AKT304-308
[M+H]+
550.644
[M+Na]+
573.289
AKT304-308 + CLLV-1
[M+H]+
550.628
[M+Na]+
573.289
AKT314-318
[M+Na]+
614.339
AKT314-318 + CLLV-1
[M+Na]+
614.339
Intensity (10² arbitrary units)
Intensity (10³ arbitrary units)
(m/z)
(m/z)