Supplementary Information for:

Discovery of podofilox as a novel cGAMP-STING signaling enhancer with antitumor activity

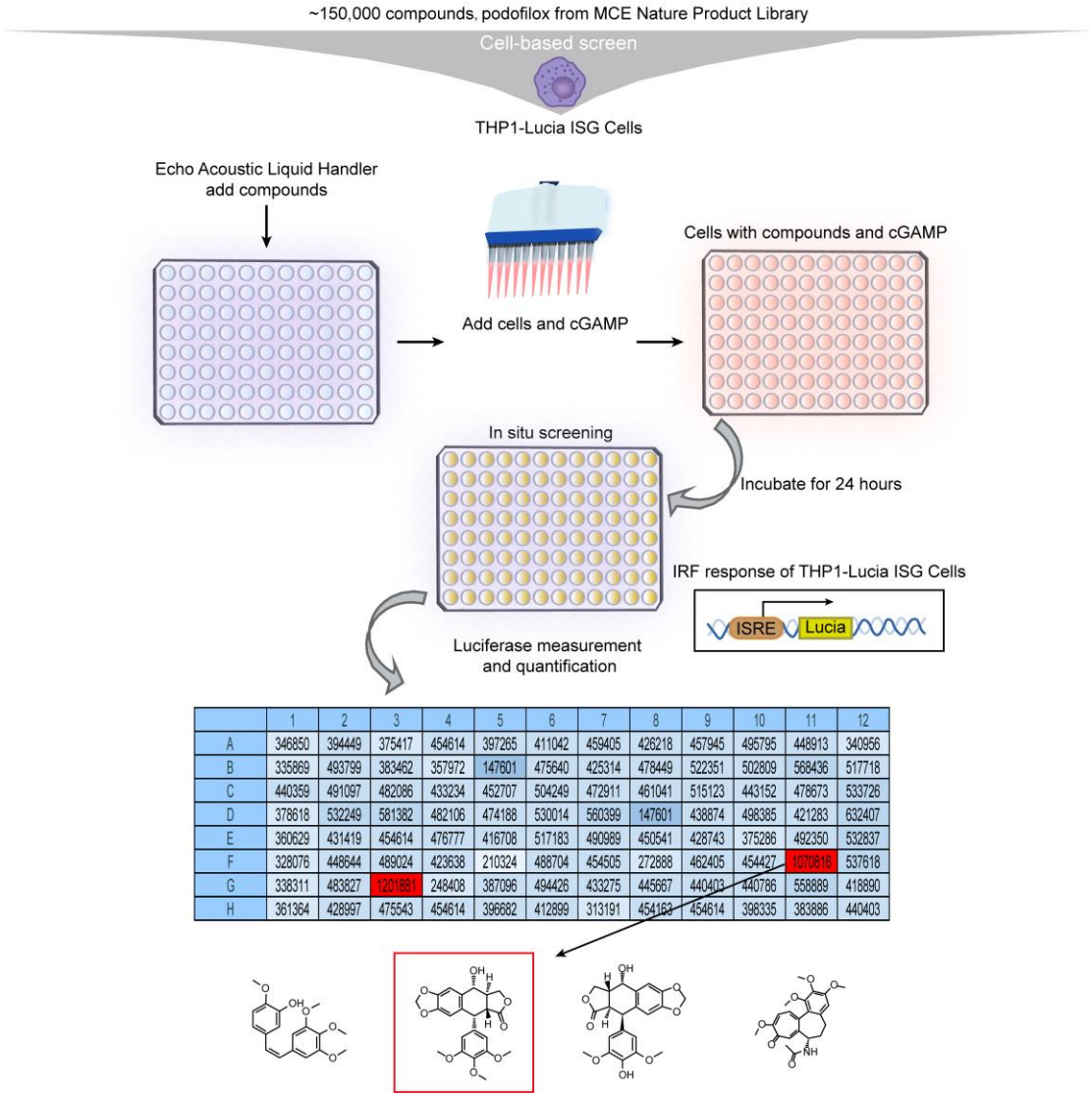
Jing Han[1,2,6], Shuiqing Hu[3,6], Yawei Hu[4,6], Yifang Xu[1], Yanfei Hou[1], Yinlong Yang[1], Huili Su[1], Zhengyin Zhang[1], Peng Liu[4*], Xuxu Sun[5*], and Conggang Zhang[1,2,7*]

Correspondence to: cgzhang@tsinghua.edu.cn

This PDF file includes:

Supplementary Figures S1 to S9 with Legends

Supplementary Figure S1

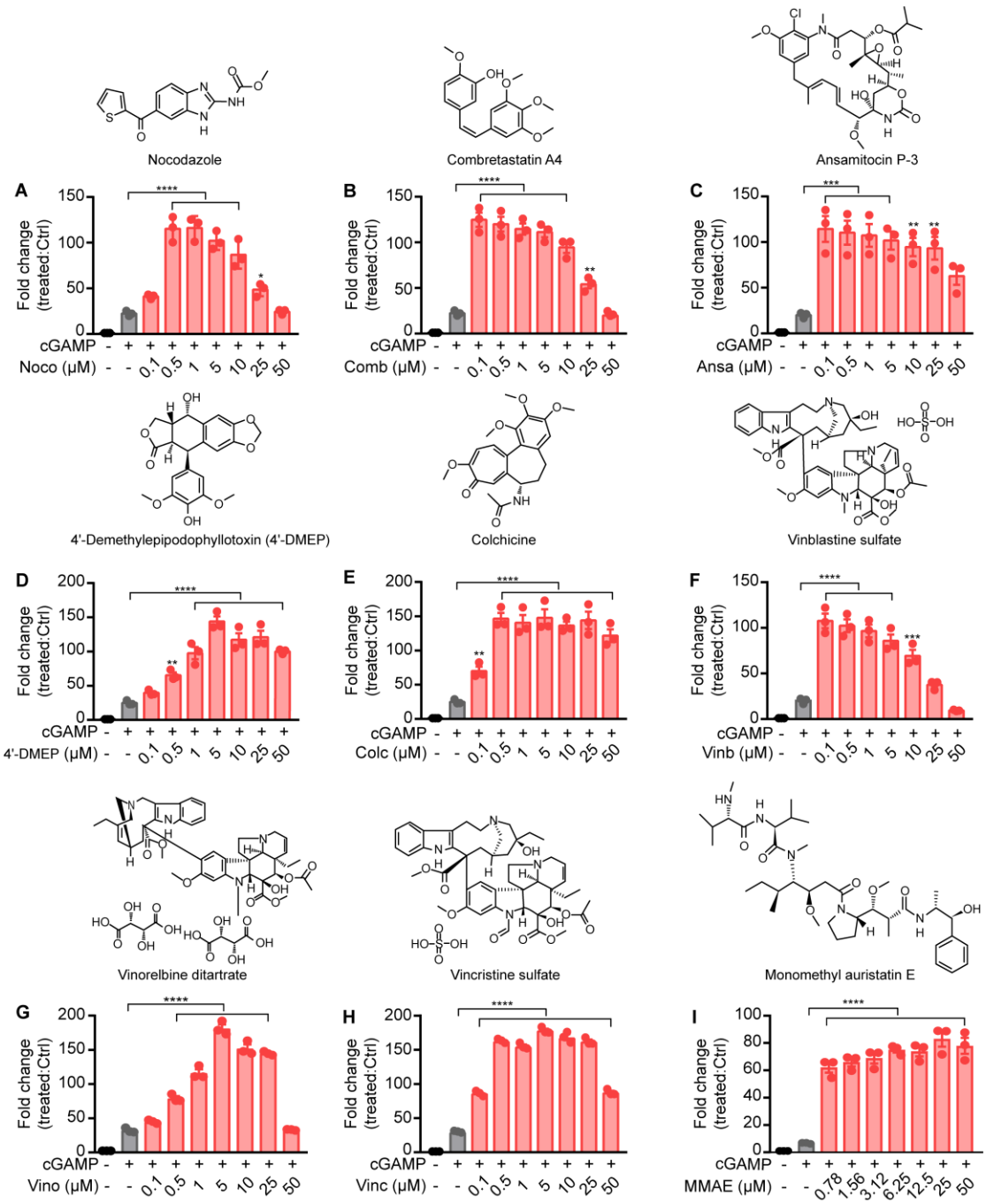


	1	2	3	4	5	6	7	8	9	10	11	12
A	346850	394449	375417	454614	397265	411042	459405	426218	457945	495795	448913	340956
B	335869	493799	383462	357972	147601	475640	425314	478449	522351	502809	568436	517718
C	440359	491097	482086	433234	452707	504249	472911	461041	515123	443152	478673	533726
D	378618	532249	581382	482106	474188	530014	560399	147601	438874	498385	421283	632407
E	360629	431419	454614	476777	416708	517183	490989	450541	428743	375286	492350	532837
F	328076	448644	489024	423638	210324	488704	454505	272888	462405	454427	1070616	537618
G	338311	483827	1201881	248408	387096	494426	433275	445667	440403	440786	558889	418890
H	361364	428997	475543	454614	396682	412899	313191	454153	454614	398335	383886	440403

Supplementary Fig. S1. A high-throughput chemical screening system to identify small molecules that regulate the cGAS-STING pathway.

Screening workflow. ISG-THP1 cells were treated with 2'3'-cGAMP along with compounds and fold change of luminescence was normalized to DMSO-treated cells. The following experiment verified candidates selected from the primary screening.

Supplementary Figure S2

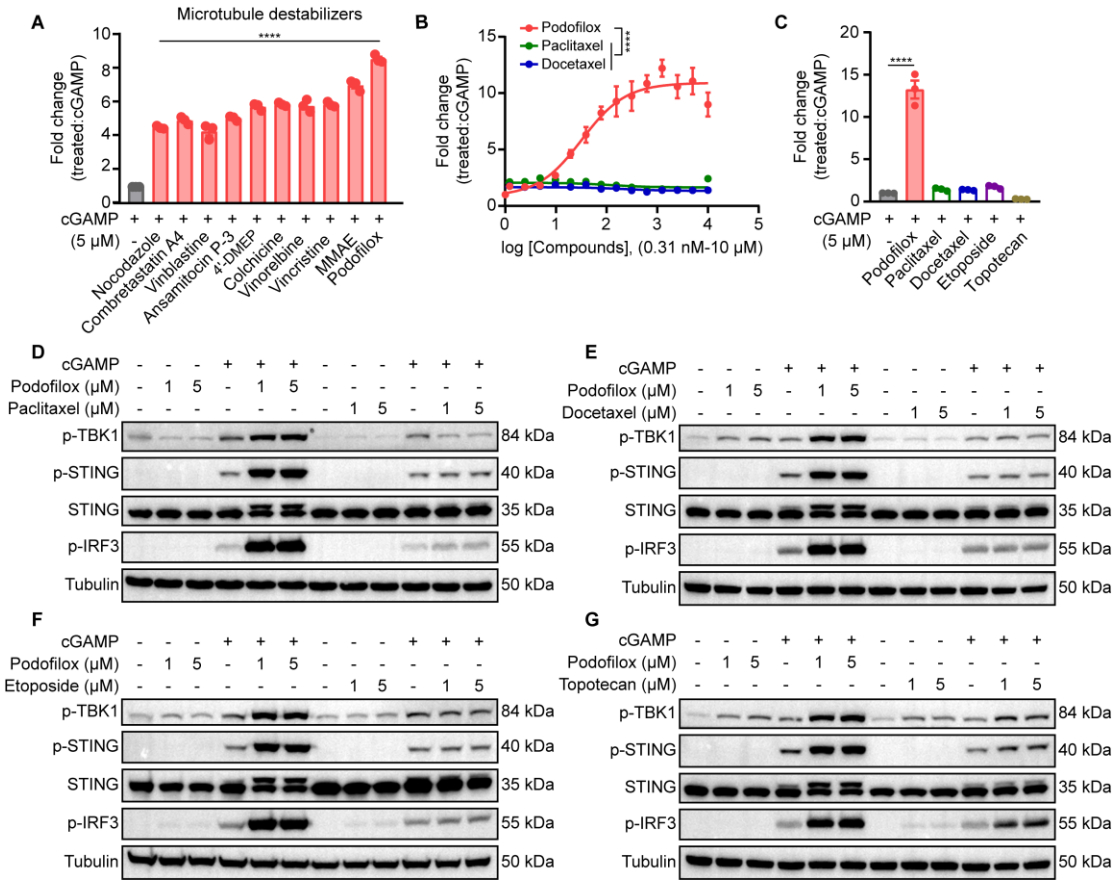


Supplementary Fig. S2. Microtubule destabilizers enhanced ISRE-luciferase activity induced by cGAMP.

A-I, ISG-THP1 cells were treated with cGAMP (0.5 μM) alone, or a combination of cGAMP and nocodazole, combretastatin A4, ansamitocin P-3, 4'-Demethylepipodophyllotoxin (4'-DMEP), colchicine, vinblastine sulfate, vinorelbine detartrate, vincristine sulfate, or

monomethyl auristatin E with increasing concentrations for 24 h. Normalized values of ISRE luciferase activity induced by cGAMP along with microtubule destabilizers are shown in comparison to the activity triggered by DMSO. Data are representative of three independent experiments; each bar represents mean ± SEM. One-way ANOVA was applied for more than two datasets, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supplementary Figure S3

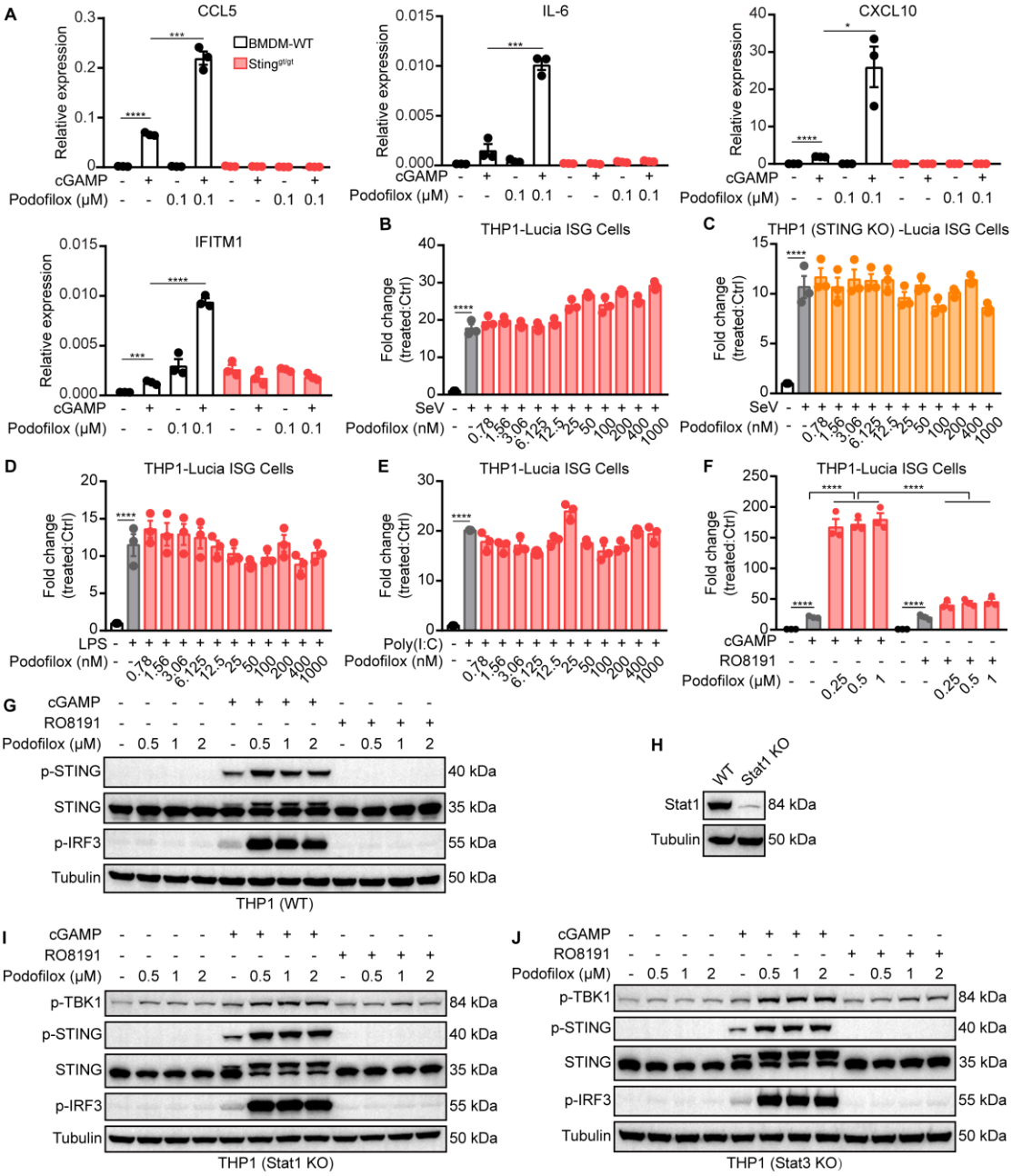


Supplementary Fig. S3. Microtubule destabilizers may specifically enhance the cGAMP-STING pathway.

A-C, ISG-THP1 cells were treated with cGAMP (0.5 μM), or cGAMP along with nocodazole, combretastatin A4, vinblastine sulfate, ansamitocin P-3, 4'-Demethylepipodophyllotoxin (4'-DMEP), colchicine, vinorelbine detartrate, vincristine sulfate, monomethyl auristatin E, podofilox, paclitaxel (stabilizer of tubulin polymerization), docetaxel (microtubule stabilizer), etoposide (DNA topoisomerase II inhibitor, antitumor chemotherapeutic agents), or topotecan (DNA topoisomerase I inhibitor, induced cell cycle arrest) for 24 h, and fold change of luminescence was normalized to cGAMP-treated cells. **D-G,** ISG-THP1 cells were treated with cGAMP (0.5 μM) and/or podofilox, paclitaxel, docetaxel, etoposide, or

topotecan for 4 h. Activation of the STING pathway were analyzed by immunoblotting.

Supplementary Figure S4

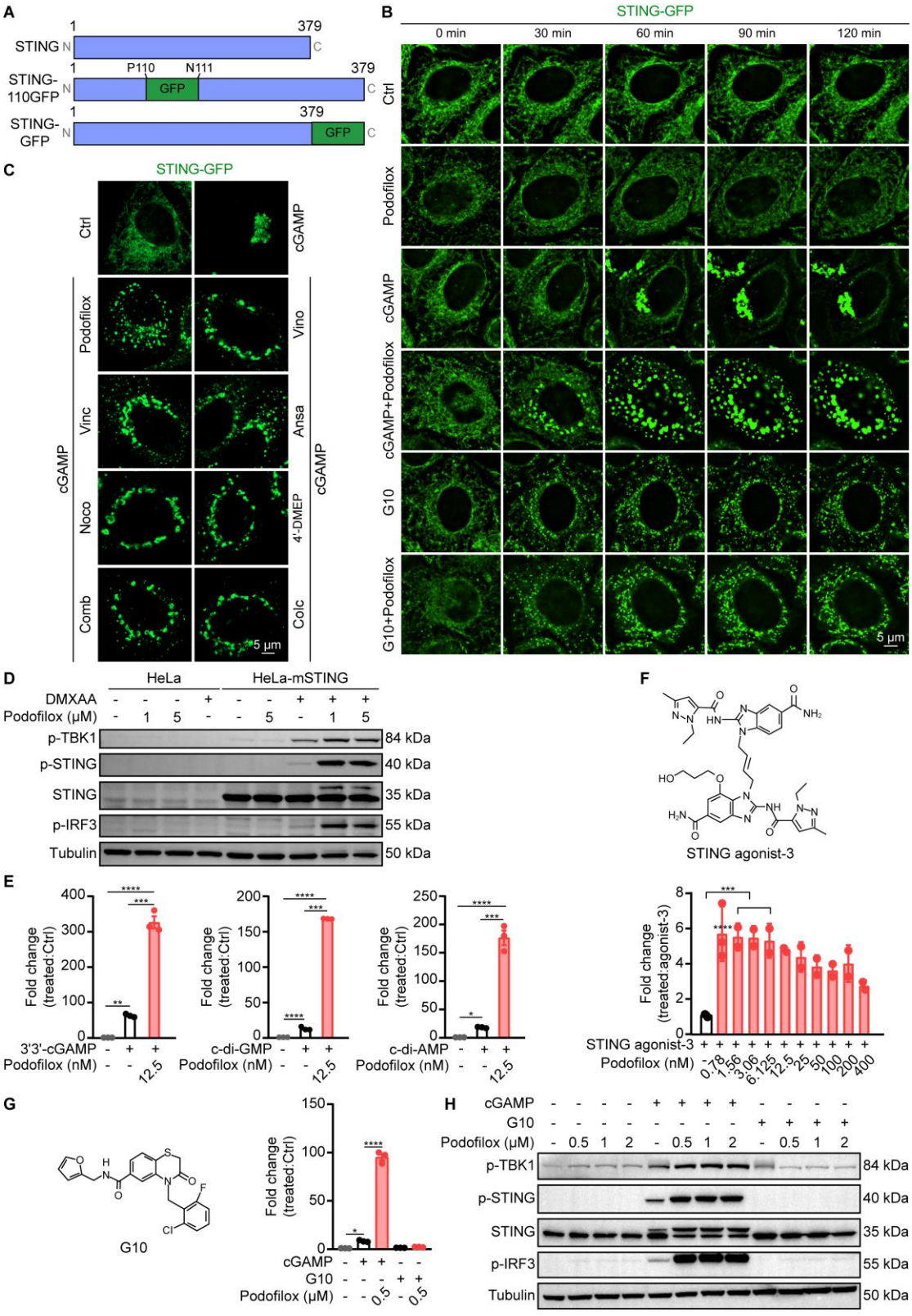


Supplementary Fig. S4. Podofilox enhanced the immune response in BMDM and ISG-THP1 cells induced by cGAMP.

A, mRNA expression levels of CCL5, IL-6, CXCL10, and IFITM1 in BMDMs (WT and *Sting*$^{gt/gt}$) activated with cGAMP (0.5 μM) and/or podofilox for 6 h (n = 3 biological replicates). **B-F,** ISG-THP1 (WT) or ISG-THP1 (STING KO) cells were treated with Sendai virus (SeV, MOI=0.1), LPS (1 μg), Poly(I:C) (10 μg), cGAMP (0.5 μM), or RO8191 (2.5 μM)

with or without various doses of podofilox for 24 h. Normalized values for ISRE luciferase activity induced by cGAMP (SeV, LPS, Poly(I:C), or RO8191) along with podofilox are shown in comparison to the activity triggered by DMSO. Data are representative of three independent experiments; each bar represents mean ± SEM. One-way ANOVA was applied for more than two datasets, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. **G, I, J,** Immunoblotting analysis of indicated proteins in WT, Stat1 KO, and Stat3 KO ISG-THP1 cells were treated with cGAMP (0.5 μM), RO8191 (2.5 μM) for 4 h with or without podofilox. **H,** Stat1 was analyzed by immunoblotting in ISG-THP1 cells (WT and Stat1 KO).

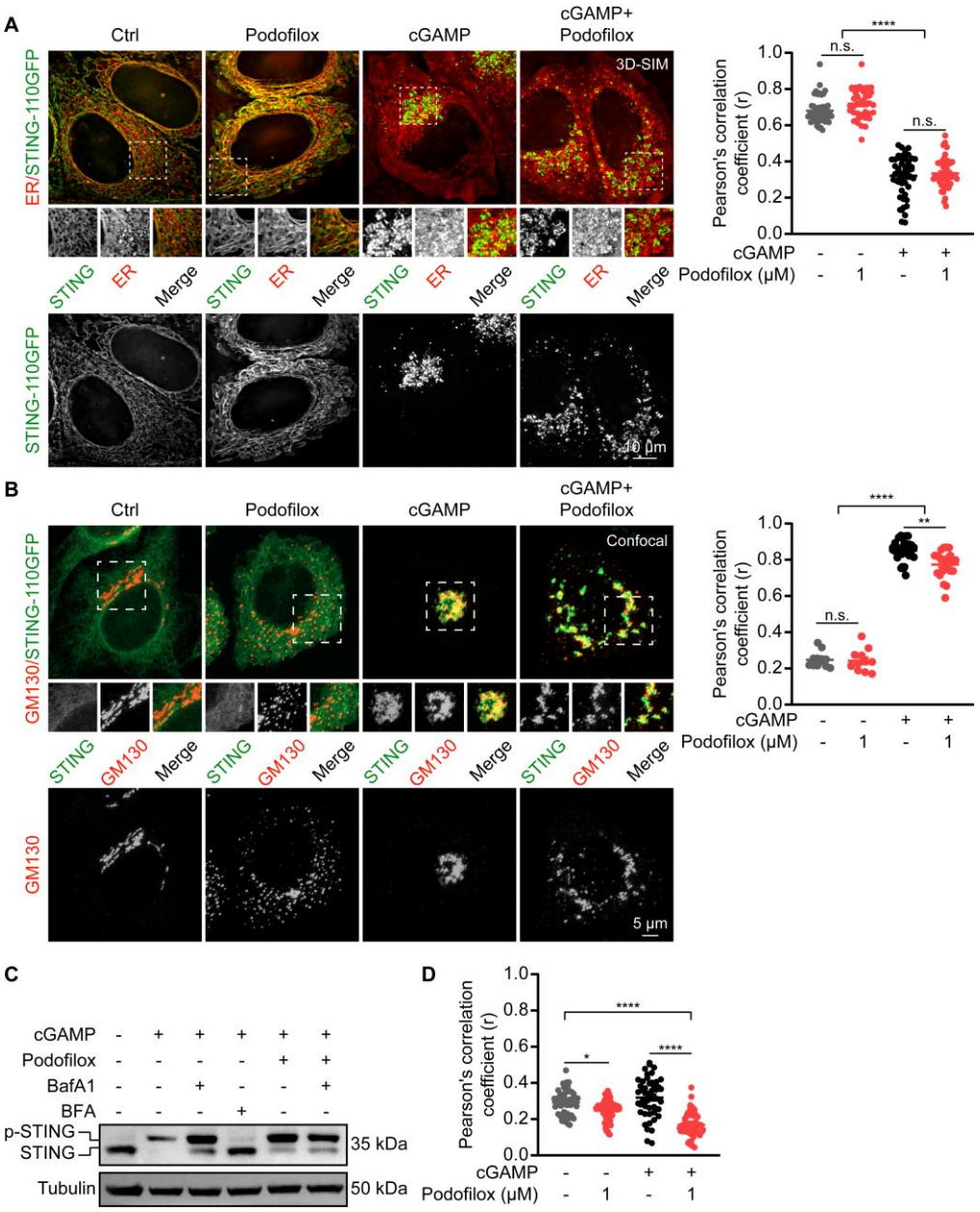
Supplementary Figure S5



Supplementary Fig. S5. Podofilox enhanced the innate immunity induced by multiple STING agonists.

A, Schematic diagram of plasmid construction. **B,** STING-GFP vesicle trafficking. Time-lapse live cell microscopy recording was started 0 min after cGAMP (8 μM, with 50 ng/ml perfringolysin O) or G10 (20 μM) with or without podofilox (1 μM) treatment. Selected frames from the movie are shown in **B**. **C,** HeLa STING-GFP cells were treated with cGAMP (8 μM, with 50 ng/ml perfringolysin O) alone, or cGAMP in combination with podofilox, vincristine sulfate, nocodazole, combretastatin A4, vinorelbine detartrate, ansamitocin P-3, 4'-Demethylepipodophyllotoxin (4'-DMEP), or colchicine for 2 h. **D,** Western blot analysis of indicated proteins was performed in HeLa cells stably expressing mouse STING stimulated with DMXAA (a small molecule agonist of mouse STING, 5 μM) and/or podofilox for 2 h. **E-G,** ISG-THP1 cells were stimulated with cyclic dinucleotides (2 μM 3'3'-cGAMP, 15 μM c-di-GMP, 10 μM c-di-AMP), STING agonist-3 (extracted from patent WO2017175147A1-example 10, a selective and non-nucleotide small-molecule STING agonist, 1 nM), or G10 (human-specific STING agonist, 10 μM) in the presence of podofilox or not for 24 h. Fold change of luminescence was normalized to DMSO or STING agonist-3 treated cells. **H,** Western blot analysis of indicated proteins in ISG-THP1 cells stimulated with podofilox along with cGAMP (0.5 μM) or G10 (10 μM) for 6 h. Data are representative of three independent experiments; each bar represents mean ± SEM. One-way ANOVA was applied for more than two datasets, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supplementary Figure S6

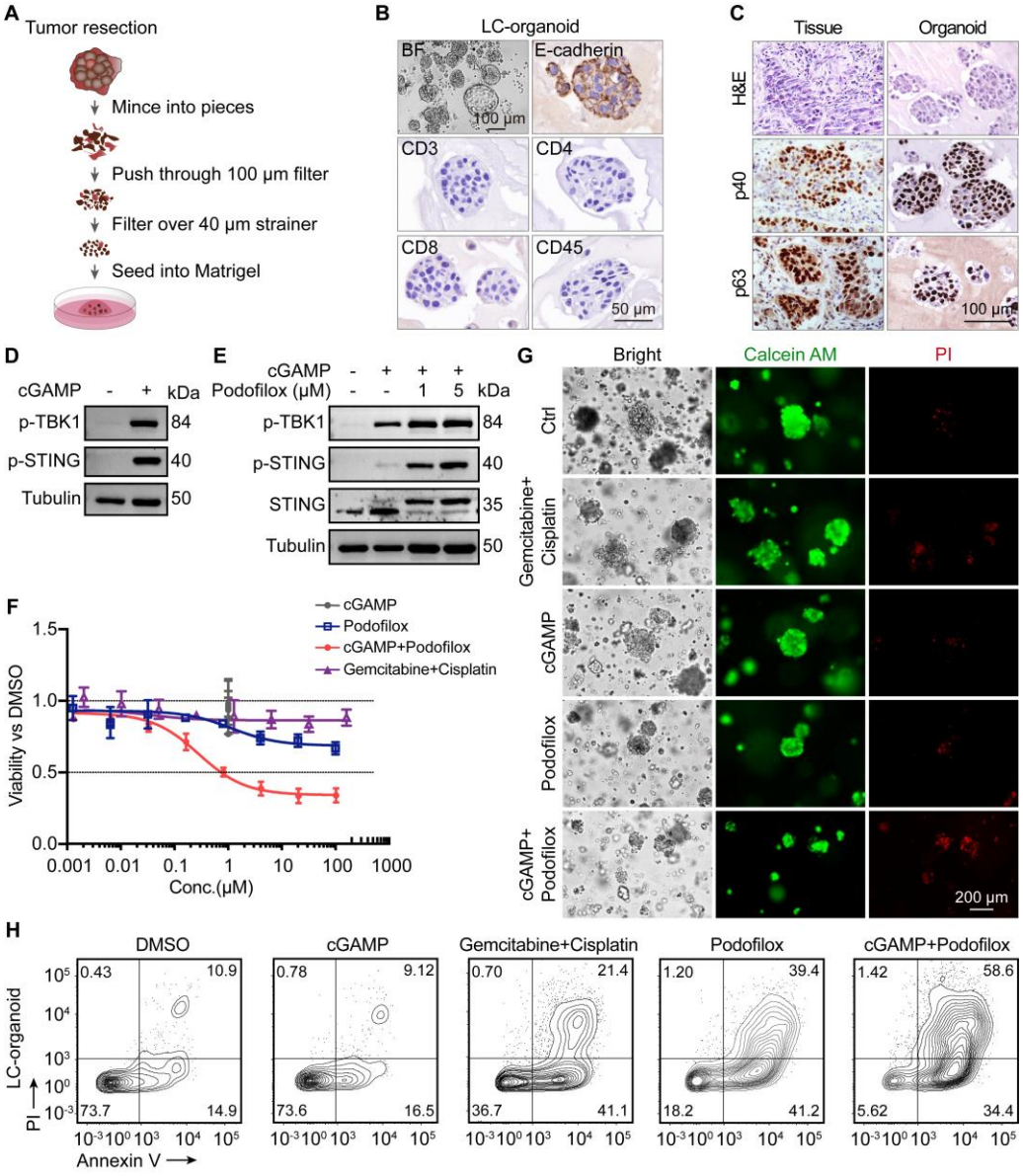


Supplementary Fig. S6. Podofilox switched the trafficking pattern of STING.

A and **B,** HeLa STING-110GFP cells were stimulated with cGAMP (2 μM, with 50 ng/ml perfringolysin O) and/or podofilox (1 μM) for 2 h, stained for ER-Tracker™ Red and GM130 (a Golgi protein, red). Scale bars, 10 μm or 5 μm. Quantitation of colocalization was calculated as Pearson's correlation coefficient (r), shown on the right of each row of images (n = 50 in **A**, or n=15 in **B**). Dashed white box in each main image indicates enlarged area of interest shown below. All 3D-SIM and confocal images are z-stack images. **C,**

Immunoblotting analysis of STING degradation in ISG-THP1 cells treated with cGAMP (8 μM) with or without podofilox (1 μM, pre-treated for 30 min), bafilomycin A1 (BafA1, 100 nM), or brefeldin A (BFA, 1 μM) for 8 h. **D**, The Pearson's correlation coefficient (r) of STING puncta and lysosomes in **Fig. 3E** (n = 50).

Supplementary Figure S7

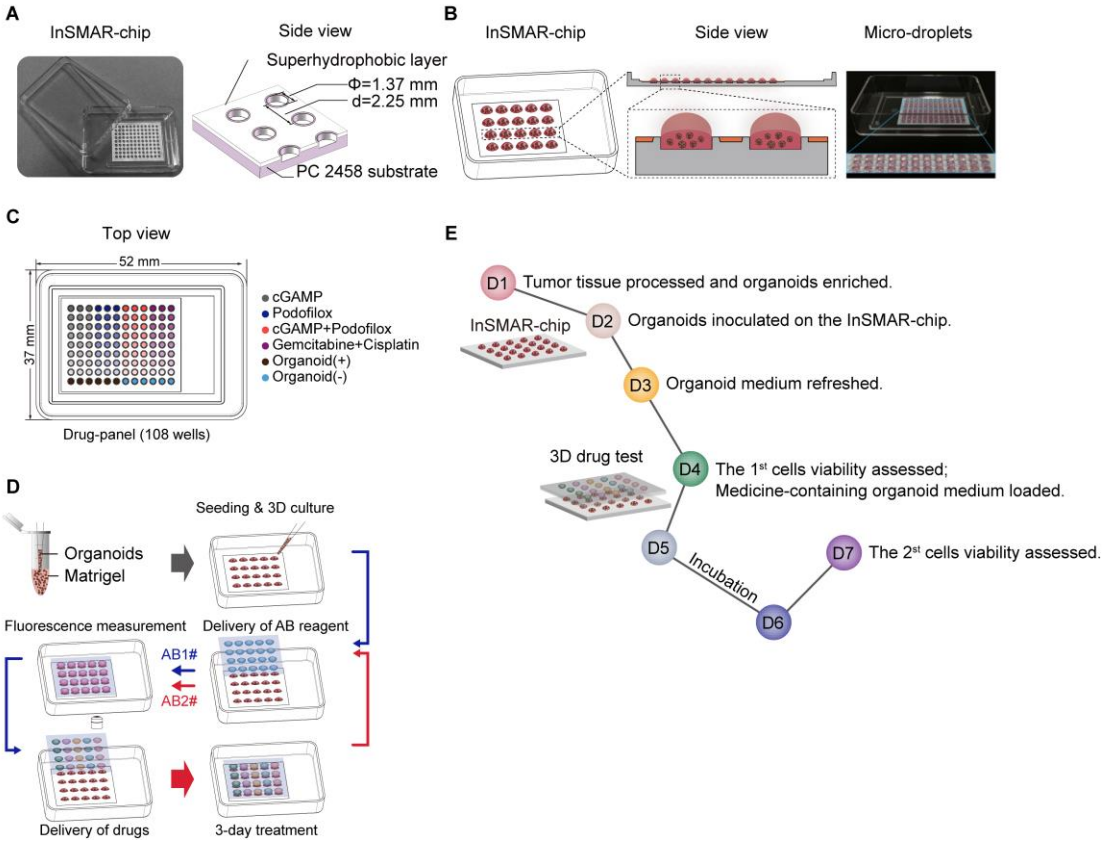


Supplementary Fig. S7. Therapeutic activity of cGAMP combined with podofilox in tumor organoids

A, Diagram of the process of establishing tumor organoids from patient tumor tissues. **B,** Bright-field and immunohistochemical images of lung cancer organoid (LC-organoid). Organoids are displayed by positive staining of E-cadherin and negative staining of immune cell markers CD3, CD4, CD8, and CD45. Scale bars, 50 μm. **C,** H&E and immunohistochemical staining images of lung cancer tissues and derived organoids. The

organoid retained the tumor cell organization and expression patterns of the characteristic markers (p40 and p63 for lung squamous cell carcinoma). **D** and **E,** Immunoblotting was performed to examine the phosphorylation levels of STING and TBK1 in LC-organoids. cGAMP was delivered by perfringolysin O (final concentration, 30 ng/ml). **F,** (as in **H**) Fitted dose-response curves illustrating results of the on-chip screening of drugs in LC-organoids. Each data point represents the average value of 3 repeats. The viabilities at 1 μM concentrations of drugs were compared with the unpaired two-tailed Student's *t*-test. **G,** (as in **H**) Bright-field images of LC-organoids, and fluorescent images showing the viability of organoids after drug treatment (green: live cells, red: dead cells). Gemcitabine-Cisplatin is a first-line chemotherapeutic for the treatment of patients with lung cancer. **H,** Annexin-V/PI analysis of apoptotic cells from tumor organoids by flow cytometry. LC-organoids were either untreated (DMSO) or treated for 72 h with indicated drugs, cGAMP (10 μM, with 30 ng/ml perfringolysin O) and/or podofilox (10 μM), Gemcitabine (10 μM) +Cisplatin (10 μM). Indicated drugs were tested under the same perfringolysin O (final concentration, 30 ng/ml) background. Quadrant assignments: lower left, viable cells; lower right, the early stage of apoptosis; upper right, late stage of apoptosis; and upper left, necrosis. The fluorescence from 10,000 cells was acquired by a BD FACSCalibur™.

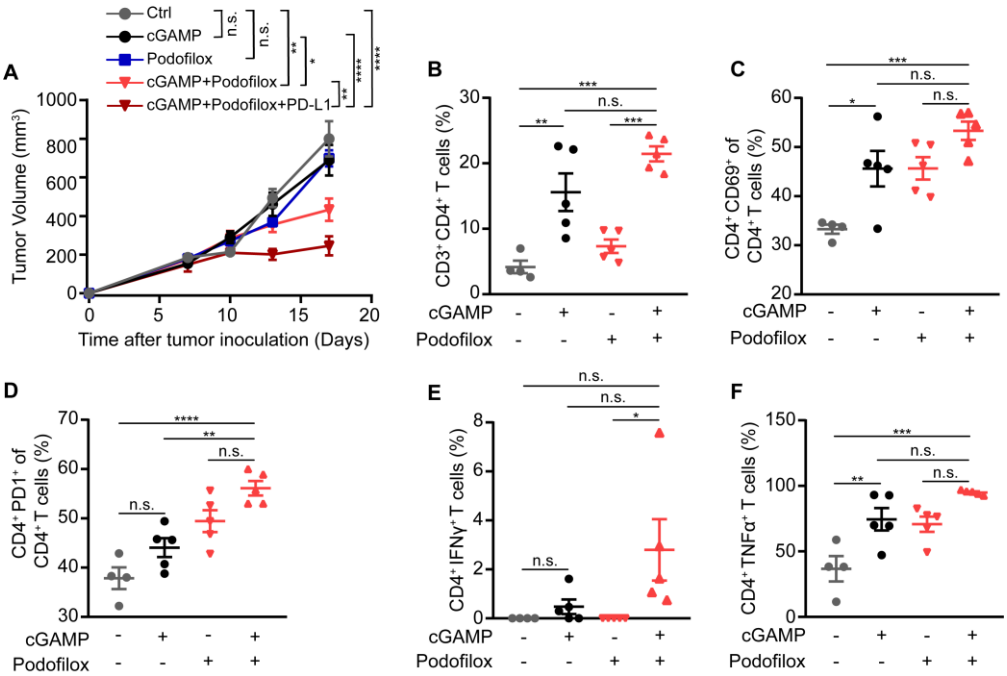
Supplementary Figure S8



Supplementary Fig. S8. Characterization of the InSMAR-chip and drug sensitivity on-chip.

A, Schematics of the InSMAR-chip (left panel), and cross-sectional view of the chip (right panel). **B,** Photographs of droplets in the microwells. (Middle) cross-sectional view of the droplet array of Matrigel mixed organoids overlaid with culture medium. (Right) Photograph of an InSMAR-chip with a micro-droplet array in the microwells. **C,** Top view of the drug panel on InSMAR-chip. **D,** Outline of the medicine susceptibility test for organoids on InSMAR-chip. **E,** Diagram illustrating the procedure of the 3D drug test performed on the InSMAR-chip. AB1#: cell viability test with alamarBlue TM before drug treatment, AB2#: cell viability test after drug treatment.

Supplementary Figure S9



Supplementary Fig. S9. Podofilox enhanced the antitumor effects of cGAMP *in vivo*.

A, WT Balb/c mice (n = 5) were inoculated with 1 × 10^6 4T1 tumor cells and treated with vehicle, cGAMP (10 μg/mice), podofilox (0.7 mg/kg), or cGAMP along with podofilox by intratumoral injection on days 7, 10, and 13. Anti-PD-L1 (200 μg/mice) was given on days 7, 10, and 13 by intraperitoneal injection. Tumor growth was measured every 3 days. Data are shown as means ± SEM and analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. **B-F,** B16F10 tumor cells were inoculated and treated with vehicle, cGAMP (10 μg/mice), podofilox (0.7 mg/kg), or cGAMP combined with podofilox by intratumoral injection on days 7, 10, and 13 (n=5). Cells were isolated from the tumor on day 18 and analyzed by flow cytometry. **B,** Population of $CD3^+CD4^+$ T cells in $CD45^+$ leukocytes was analyzed. **C** and **D,** CD69 and PD1 expression on $CD4^+$ T cells were assessed by flow cytometry. **E** and **F,** IFNγ and TNFα production in $CD4^+$ T cells were tested. Data were shown as dot plots and analyzed by one-way ANOVA followed by

Tukey’s multiple comparisons test (**B-F**). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.