**Table S1.** Chemical library.

| Compound | Class | Company |
|---|---|---|
| 3,4-Dihydroxybenzoic acid (protocatechuic acid) | Hydroxybenzoic acid | Sigma-Aldrich |
| γ-Valerolactone | Valerolactone | Sigma-Aldrich |
| Benzene-1,2-diol (catechol or pyrocatechol) | Benzene diol | Sigma-Aldrich |
| 1,5-Dicaffeoylquinic acid | Caffeoylquinic acid | Sigma-Aldrich |
| (4*R*)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone | Phenyl-γ-valerolactone | Toronto Research Chemicals |
| 2-Furoylglycine | *N*-containing compound | Sigma-Aldrich |
| 2-Piperidone (delta-Valerolactam) | *N*-containing compound | Sigma-Aldrich |
| 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (Dihydroferulic acid) | Phenylpropanoic acid | Sigma-Aldrich |
| 3-(4′-hydroxyphenyl)propanoic acid | Phenylpropanoic acid | Sigma-Aldrich |
| 3′,4′-Dihydroxyphenylacetic acid | Phenylacetic acid | Sigma-Aldrich |
| 3-Methoxytyrosine (3-*O*-methyldopa) | *N*-containing compound | Maybridge |
| 3-Methylglutaconic acid | Dicarboxylic acid | Vitas-M Laboratory, Ltd. |
| 4-Hydroxybenzoic acid | Hydroxybenzoic acid | Sigma-Aldrich |
| 2-Hydroxy-2-(4′-hydroxyphenyl)acetic acid (4′-hydroxymandelic acid) | Hydroxy-2-(phenyl)acetic acid | Ark Pharm, Inc. |
| 3,5-Dihydroxy-4-methoxybenzoic acid (4-*O*-methylgallic acid or 5-Hydroxyisovanillic acid) | Hydroxybenzoic acid | Extrasynthese |
| 5-(3′,4′-Dihydroxyphenyl)valeric acid (5-(3′,4′-Dihydroxyphenyl)pentanoic acid) | Phenylvaleric acid | ENAMINE |
| alpha-Hydroxyhippuric ((benzoylamino)hydroxyacetic acid) | *N*-containing compound | Sigma-Aldrich |
| 3′,4′-Dihydroxycinnamic acid (caffeic acid) | Cinnamic acid | Sigma-Aldrich |
| (+)-Catechin | Flavan-3-ol | Extrasynthese |
| (+)-Catechin-3-gallate | Flavan-3-ol | Sigma-Aldrich |
| 5-caffeoylquinic acid (Chlorogenic acid) | Caffeoylquinic acid | Sigma-Aldrich |
| 3-(3′,4′-Dihydroxyphenyl)propanoic acid (dihydrocaffeic acid) | Phenylpropanoic acid | Extrasynthese |
| (−)-Epicatechin | Flavan-3-ol | Extrasynthese |
| (−)-Epicatechin-3-gallate | Flavan-3-ol | Extrasynthese |
| (−)-Epigallocatechin-3-gallate | Flavan-3-ol | Extrasynthese |
| 4′-Hydroxy-3′-methoxycinnamic acid (ferulic acid) | Cinnamic acid | Extrasynthese |
| 3,4,5-Trihydroxybenzoic acid (gallic acid) | Hydroxybenzoic acid | Fluka |
| (+)-Gallocatechin | Flavan-3-ol | Sigma-Aldrich |
| (+)-Gallocatechin-3-gallate | Flavan-3-ol | Sigma-Aldrich |
| Glycitein 7-*O*-glucoside (glycitin) | Isoflavone | Extrasynthese |
| Hippuric acid | Hippuric acid | Extrasynthese |

| | | |
|---|---|---|
| 4′-Hydroxy-3′-methoxyphenylacetic acid (homovanillic acid) | Phenylacetic acid | Extrasynthese |
| 3′-Hydroxy-4′-methoxycinnamic acid (isoferulic acid) | Cinnamic acid | Extrasynthese |
| Laricitrin | Flavonol | Extrasynthese |
| Luteolin | Flavone | Extrasynthese |
| Naringenin | Flavanone | Extrasynthese |
| 3-caffeoylquinic acid (neochlorogenic acid) | Caffeoylquinic acid | TransMIT |
| 2′-Hydroxycinnamic acid (*o*-coumaric acid) | Cinnamic acid | Sigma-Aldrich |
| 4′-Hydroxycinnamic acid (*p*-coumaric acid) | Cinnamic acid | Sigma-Aldrich |
| Phenylacetic acid | Phenylacetic acid | Sigma-Aldrich |
| Phloretin | Dihydrochalcone | Sigma-Aldrich |
| Phloretin-2′-*O*-glucoside (phloridizin) | Dihydrochalcone | Sigma-Aldrich |
| Benzene-1,3,5-triol (phloroglucinol) | Benzene triol | Sigma-Aldrich |
| Procyanidin B1 | Flavan-3-ol | Extrasynthese |
| Procyanidin B2 | Flavan-3-ol | Sigma-Aldrich |
| Benzene-1,2,3-triol (pyrogallol) | Benzene triol | Sigma-Aldrich |
| Quercetin | Flavonol | Fluka |
| Quercetin-3-glucuronide | Flavonol | Extrasynthese |
| Quinic acid | Sugar acid | Roth |
| Resveratrol | Stilbene | Sigma-Aldrich |
| Salvianolic acid A | Cinnamic acid | Sigma-Aldrich |
| 4′-Hydroxy-3′,5′-dimethoxycinnamic acid (sinapic acid) | Cinnamic acid | Fluka |
| Ursolic acid | Triterpenoid | Sigma-Aldrich |
| 4-Hydroxy-3-methoxybenzoic acid (vanillic acid) | Hydroxybenzoic acid | Extrasynthese |

**Table S2.** Extended Table 1 summarizing the spheroid integrity (average value) and the standard deviation (SD) for each compound. 3D spheroids were treated with all the compounds listed below at 50 µM, from at least four independent experiments. After 72h treatment, the spheroid integrity parameter was calculated as a percentage of the control (DMSO 0.05%).

| COMPOUND | Spheroid Integrity (Average) | Standard Deviation |
|---|---|---|
| (+)-Gallocatechin-3-gallate (GCG) | 1.0 | 1.1 |
| Benzene-1,2,3-triol (pyrogallol) | 1.5 | 1.6 |
| (−)-Epigallocatechin-3-gallate (EGCG) | 1.6 | 0.6 |
| Ursolic acid | 1.9 | 2.5 |
| (+)-Gallocatechin | 9.7 | 4.9 |
| 3,4,5-Trihydroxybenzoic acid (gallic acid) | 34.0 | 19.1 |
| **5-Fluorouracil** | 39.0 | 10.8 |
| (4*R*)-5-(3',4'-dihydroxyphenyl)-γ-valerolactone | 44.7 | 22.3 |
| Benzene-1,2-diol (catechol or pyrocatechol) | 50.7 | 16.3 |
| Naringenin | 54.7 | 6.3 |
| Resveratrol | 55.6 | 27.7 |
| Luteolin | 58.9 | 28.2 |
| 2'-Hydroxycinnamic acid (*o*-coumaric acid) | 63.8 | 36.5 |
| 3-(3',4'-Dihydroxyphenyl)propanoic acid (dihydrocaffeic acid) | 65.2 | 14.7 |
| 3',4'-Dihydroxyphenylacetic acid | 67.6 | 18.8 |
| 5-(3',4'-Dihydroxyphenyl)valeric acid | 68.7 | 7.9 |
| Glycitein 7-*O*-glucoside (glycitin) | 76.1 | 10.1 |
| 4'-Hydroxy-3'-methoxycinnamic acid (ferulic acid) | 80.1 | 13.4 |
| 2-Furoylglycine | 80.4 | 16.7 |
| 4'-Hydroxycinnamic acid (*p*-coumaric acid) | 83.7 | 10.3 |
| (−)-Epicatechin-3-gallate | 85.2 | 21.1 |
| 3-Methylglutaconic acid | 86.9 | 7.8 |
| Salvianolic acid A | 87.3 | 4.1 |
| 3,5-Dihydroxy-4-methoxybenzoic acid (4-*O*-methylgallic acid or 5-Hydroxyisovanillic acid) | 88.0 | 15.7 |
| Quinic acid | 89.9 | 10.0 |
| Phloretin-2′-*O*-glucoside (phloridizin) | 91.0 | 17.0 |
| 3'-Hydroxy-4'-methoxycinnamic acid (isoferulic acid) | 91.9 | 10.4 |
| Quercetin-3-glucuronide | 92.0 | 10.5 |
| 1,5-Dicaffeoylquinic acid | 92.8 | 14.1 |
| 3',4'-Dihydroxycinnamic acid (caffeic acid) | 93.0 | 18.5 |
| 3-caffeoylquinic acid (neochlorogenic acid) | 93.1 | 10.7 |
| 4'-Hydroxy-3'-methoxyphenylacetic acid (homovanillic acid) | 93.5 | 12.0 |
| 4-Hydroxy-3-methoxybenzoic acid (vanillic acid) | 94.0 | 7.4 |
| 4'-Hydroxy-3',5'-dimethoxycinnamic acid (sinapic acid) | 94.1 | 10.5 |

| | | |
|---|---|---|
| 4-Hydroxybenzoic acid | 94.9 | 5.1 |
| 3,4-Dihydroxybenzoic acid (protocatechuic acid) | 95.6 | 7.4 |
| (+)-Catechin-3-gallate | 95.8 | 9.5 |
| Laricitrin | 96.2 | 9.2 |
| Hippuric acid | 96.7 | 7.1 |
| Benzene-1,3,5-triol (phloroglucinol) | 97.2 | 24.6 |
| γ-Valerolactone | 98.3 | 2.6 |
| Phenylacetic acid | 98.4 | 8.7 |
| 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid (Dihydroferulic acid) | 99.4 | 9.3 |
| **DMSO (0.05%)** | 100 | 9.1 |
| 2-Hydroxy-2-(4'-hydroxyphenyl)acetic acid (4'-hydroxymandelic acid) | 100.1 | 5.5 |
| Quercetin | 100.1 | 22.6 |
| Procyanidin B2 | 101.8 | 8.3 |
| 2-Piperidone (delta-valerolactam) | 103.1 | 4.0 |
| Procyanidin B1 | 104.2 | 11.0 |
| 5-caffeoylquinic acid (Chlorogenic acid) | 104.5 | 7.3 |
| alpha-Hydroxyhippuric ((benzoylamino)hydroxyacetic acid) | 104.9 | 6.4 |
| (−)-Epicatechin | 105.0 | 4.1 |
| (+)-Catechin | 106.3 | 6.5 |
| Phloretin | 114.5 | 21.2 |
| 3-(4'-hydroxyphenyl)propanoic acid | 115.9 | 13.9 |
| 3-Methoxytyrosine (3-*O*-methyldopa) | 118.0 | 6.8 |

**Figure S1.** (A) Growth curves of HCT116 wild-type (red) and HCT116 EGFP (green) cell lines grown as monolayers in presence of vehicle (DMSO 0.05%) quantified as normalized cell index by *xCELLigence* System. (B) Dose response curves of HCT116 wild-type (red) and HCT116 EGFP (green) cell lines grown as monolayers for different concentration levels of 5-FU (1, 10, 100µM) for 72h, quantified as normalized cell index by *xCELLigence* System. Cell index was normalized to time point 24h corresponding to drug or vehicle addition. Dose-response curve of 3D multicellular spheroids (C) Dose-response curve of 3D multicellular spheroids HCT116 wild-type (red) and HCT116 EGFP (green) at different concentration levels of 5-FU (10, 30, 100µM) for 72h. Spheroid integrity was quantified by High Content Imaging (Perkin Elmer) and normalized to vehicle control (DMSO 0.05%). Data are expressed as mean ± SD obtained. All experiments were performed in triplicates. (D) Images of HCT116 wild-type (grey) and HCT116 EGFP (green) grown as monolayers (2D) and spheroids (3D). Images were acquired by High Content Imaging System Operetta (Perkin Elmer) with 10XLWD objective in brightfield and green fluorescent channels. Scale bars, 200 µm.

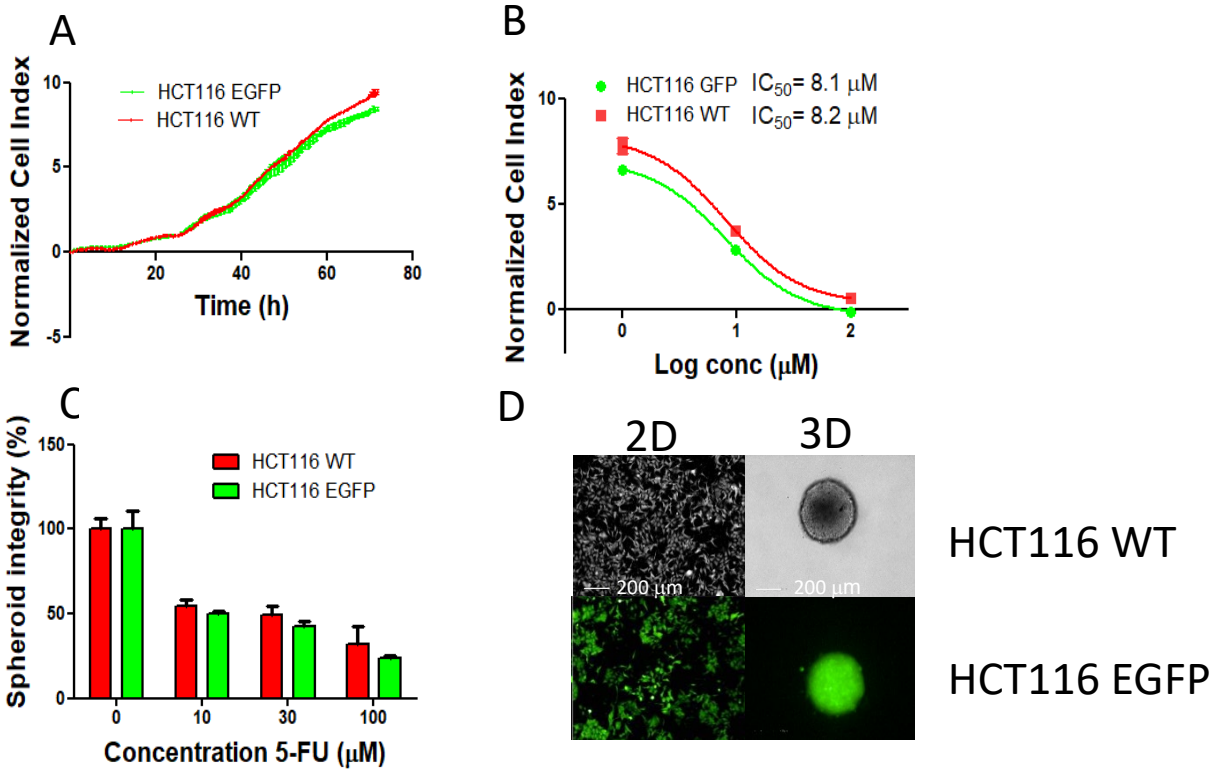

**Figure S2.** Z' factor was evaluated treating 3D HCT116 EGFP spheroids (n=27) with DMSO 0.05% (negative control) and 5-FU (50 μM, positive control). Z' factor was calculated using the formula below ($\sigma_p$= the standard deviation of the positive control, $\sigma_n$= the standard deviation of the negative controls, $\mu_p$= the mean of the positive control, and $\mu_n$= the mean of the negative control). Spheroids were imaged on High Content Imaging System Operetta (Perkin Elmer) with 10XLWD objective in brightfield and green fluorescent channels (merged images). Scale bars, 200 μm.

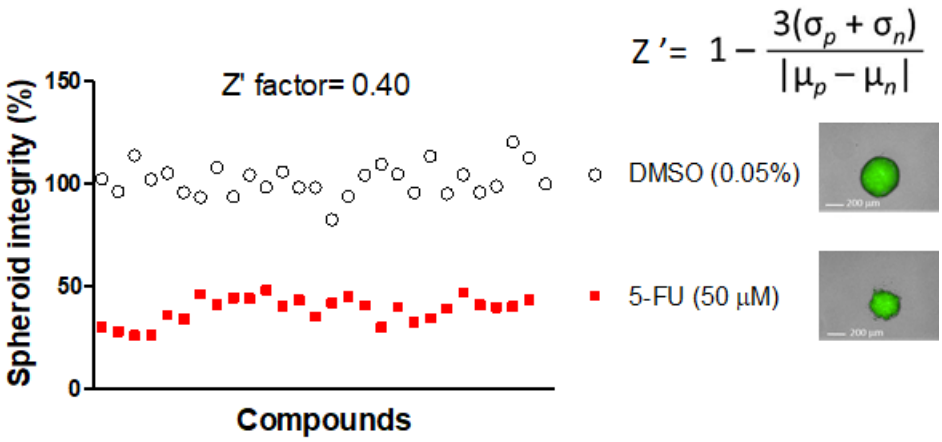

**Figure S3.** Results of the screening on 3D HCT EGFP forming spheroids treated with the chemical library at 50 µM for 72h. Round white dots represent positive (5-FU) and negative (vehicle) (DMSO 0,05%) controls. The classification of the compounds in metabolites and native is reported in the legend. Spheroid integrity (SI) was quantified by High Content Imaging System Operetta (Perkin Elmer) and expressed as a percentage of SI of vehicle-treated controls (DMSO 0.05%). Molecules affecting the spheroid integrity between 0-80% (red line) were selected as primary hits (green dots), and they are also reported in Table 1. The data are the mean ± standard deviation from at least four independent experiments.

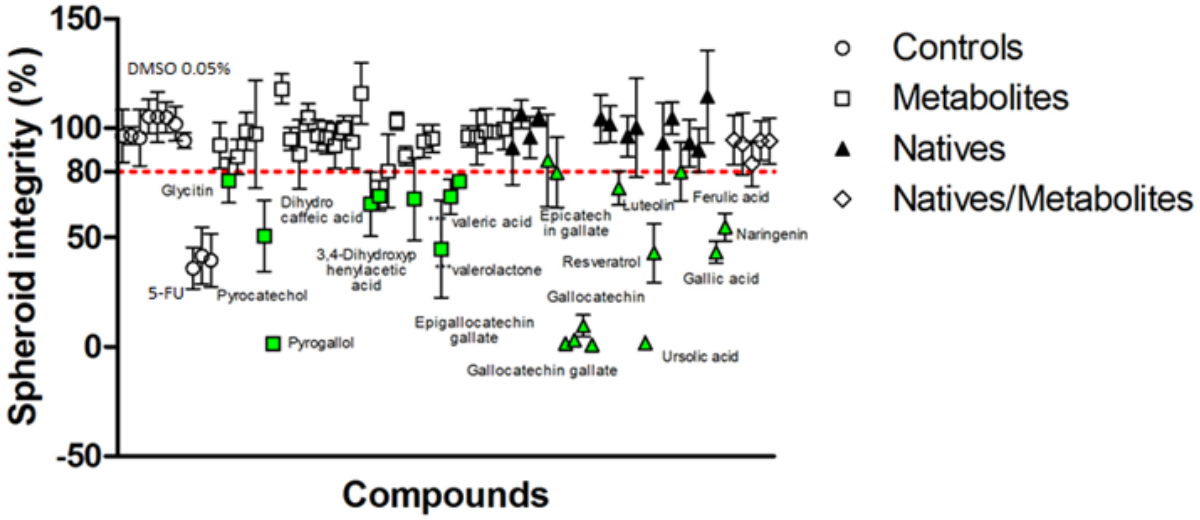

**Figure S4**. Seven-point dose-response curve of butyric acid (ranging from 100 to 2000μM) on HCT116 EGFP forming spheroids (A), and formed spheroids (B) for 72h of incubation. Spheroid integrity (SI) was quantified by High Content Imaging System Operetta (Perkin Elmer) and expressed as a percentage of SI of vehicle-treated controls (DMSO 0.05%). $IC_{50}$ was calculated applying dose-response-inhibition nonlinear regression analysis. Data are expressed as mean ± SD. All experiments were performed from at least three independent experiments. HCT116 EGFP spheroids were imaged on High Content Imaging System Operetta (Perkin Elmer) with 10XLWD objective in brightfield and green fluorescent channels (merged images) at different concentrations. Scale bars, 200 μm.

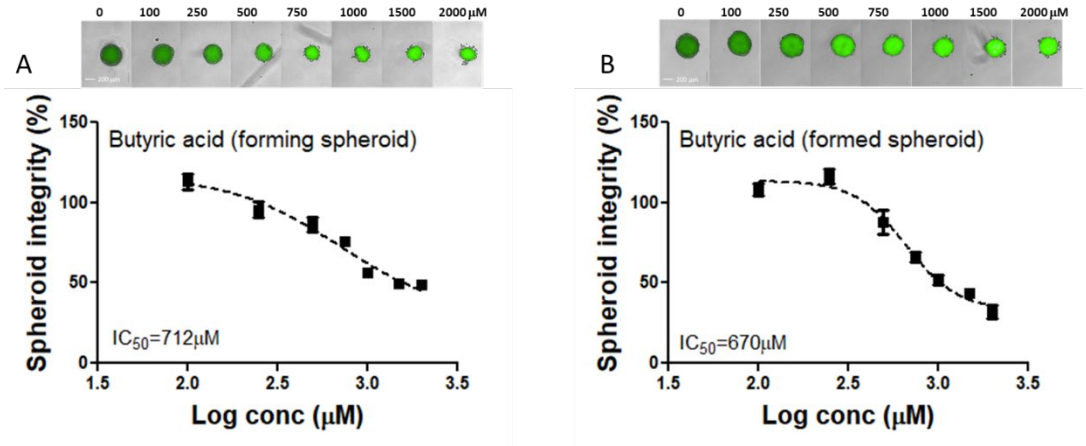

**Figure S5.** Six-point dose-response curves of GICs forming spheroids (from 500 cell) were calculated for etoposide (A), and an EGCG (B  temozolomide (C) at different concentrations increasing from 0.3 to 1000 µM. Drug treatment was carried out in GravityPLUS™ microplate for four days and repeated after transfer in GravityTRAP™ plate for other four days with fresh medium Spheroid integrity (SI) was quantified by High Content Imaging System Operetta (Perkin Elmer) and expressed as a percentage of SI of vehicle-treated controls (DMSO 0.05%). $IC_{50}$ was calculated applying dose-response-inhibition nonlinear regression analysis. Data are expressed as mean ± SD obtained. All experiments were performed from at least three independent experiments. GICS spheroids were imaged on the High Content Imaging System Operetta (Perkin Elmer) with 10XLWD objective in brightfield and red fluorescent channels (merged images) at different compound concentrations. Scale bars, 200 µm.

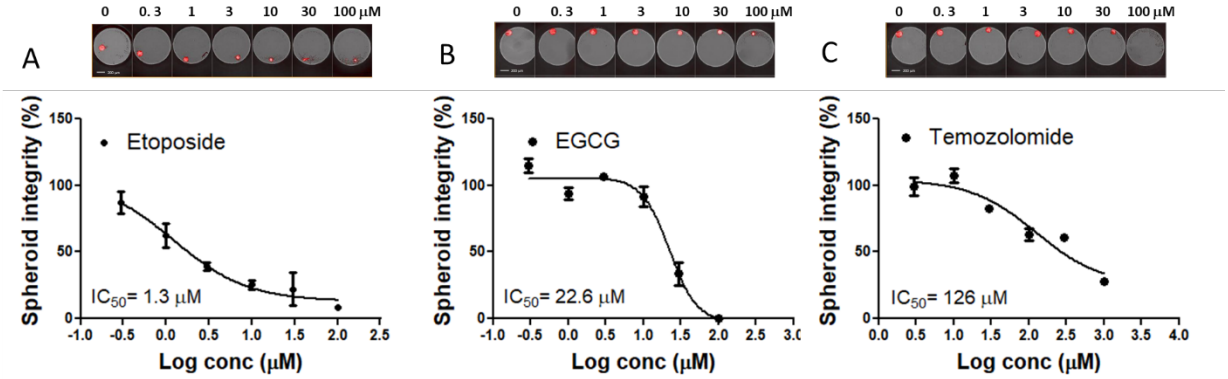

**Figure S6.** Seven-point dose-response curves of GICs forming spheroids (from 2000 cells) treated with (A) etoposide, (B) quercetin, (c) isorhamnetin and (D) quercetin-3-glucuronide at different concentrations (0 to 100 µM). Drug addition was carried out in GravityPLUS™ microplate for four days and repeated after transfer it into a GravityTRAP™ plate for other four days with fresh medium. Spheroids were imaged in red and green channel by High Content Imaging System Operetta (Perkin Elmer) to evaluate spheroid reduction (spheroid integrity SI) and apoptosis, respectively. These parameters were expressed as a percentage of vehicle-treated controls (DMSO 0.05%). Dose-response inhibition nonlinear regression analysis was applied. Data are expressed as mean ± SD obtained. All experiments were performed from at least three independent experiments. GICS spheroids were imaged on High Content Imaging System Operetta (Perkin Elmer) with 10XLWD objective in brightfield, green and red fluorescent channels (merged images) at different concentrations. Scale bars, 200 µm.