Epigallocatechin 3-O-gallate Induces 67 kDa Laminin Receptor-Mediated Cell Death Accompanied by Downregulation of ErbB Proteins and Altered Lipid Raft Clustering in Mammary and Epidermoid Carcinoma Cells

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ABSTRACT: Since the administration of synthetic medicines is associated with drug resistance and undesired side effects, utilization of natural compounds could be an alternative and complementary modality to inhibit or prevent the development of tumors. Epigallocatechin 3-O-gallate (EGCG, 1), the major flavan component of green tea, and genistein (2), a soy isoflavonoid, are known to have chemopreventive and chemotherapeutic effects against cancer. This study demonstrated that both flavonoids inhibit cell proliferation, an effect enhanced under serum-free conditions. Compound 1, but not 2, induced downregulation of ErbB1 and ErbB2 in mammary and epidermoid carcinoma cells, and its inhibitory effect on cell viability was mediated by the 67 kDa laminin receptor (67LR). While 1 was superior in inducing cell death, 2 was more efficient in arresting the tumor cells in the G2/M phase. Furthermore, number and brightness analysis revealed that 1 decreased the homoclustering of a lipid raft marker, glycosylphosphatidylinositol-anchored GFP, and it also reduced the co-localization between lipid rafts and 67LR. The main conclusion made is that the primary target of 1 may be the lipid raft component of the plasma membrane followed by secondary changes in the expression of ErbB proteins. Compound 2, on the other hand, must have other unidentified targets.

Accumulating evidence supports the hypothesis that the consumption of diverse dietary constituents, e.g., epigallocatechin 3-O-gallate (EGCG, 1) from green tea or genistein (2) from soy beans, is associated with reduced risk of cancer1−4 and that these compounds have anti-inflammatory and antioxidant properties.5,6 The effect of these flavonoids on apoptosis, cell cycle progression, and the phosphorylation status of receptor tyrosine kinases (RTK) has been studied extensively in various cancer cell lines.7−10 However, their molecular mechanisms of action in cell lines overexpressing ErbB proteins still remain to be elucidated. ErbB proteins, a family of RTKs, play a central role in the development of various forms of cancer.11−14 The ErbB family comprises four members, ErbB1 to ErbB4, and their overexpression is associated with invasion, metastasis, and inhibition of apoptosis in cancer cells.12 ErbB1, the receptor for epidermal growth factor (EGF), forms homo- and heterodimers, triggers the activation of intracellular signaling pathways after ligand binding, and has been shown to be localized in lipid rafts.15,16 ErbB2, an oncprotein overexpressed in several human tumors, is a lipid raft-associated protein as well.17 Lipid rafts are transient cholesterol-, sphingolipid-, and ganglioside-enriched microdomains in the plasma membrane of eukaryotic cells in which glycosylphosphatidylinositol anchored proteins are known to reside.18,19 Lipid rafts have been implicated not only in the regulation of transmembrane signaling and membrane trafficking, but also in the mechanism of action of several drugs as well.20 In particular, previous studies showed the disruption of lipid rafts by flavonoids, especially by 1, in colon cancer cells.21 On the other hand, recent data have supported the hypothesis of lipid-raft clustering after treatment with 1 in multiple myeloma cells.9 However, the mechanism of interaction between the flavonoids and lipid rafts in tumor cells is not completely understood.

Induction of apoptosis in cancer cell lines by flavonoids has been reported in multiple studies.9,22,23 These compounds modulate both the extrinsic and intrinsic apoptotic pathways by increasing caspase-3/7 and p53 expression levels while inhibiting the production of Bcl-2.22,23,24 Recent studies have demonstrated that the apoptotic activity of 1 is mediated by a 67 kDa laminin receptor (67LR) in multiple myeloma cells.9

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Moreover, another recent report has revealed that blocking the extracellular domain of 67LR by an anti-67LR antibody led to an almost complete inhibition of apoptosis in multiple myeloma cells, supporting the hypothesis that 67LR acts as a cancer-specific cell death receptor. 26 Flavonoid-mediated cell cycle arrest has also been reported: while 1 induced cell cycle arrest in the G₀/G₁ phase, 2 blocked cell cycle progression in the G₂/M phase. 27

In the present study the effects of two flavonoids, 1 and 2, were investigated in cell lines overexpressing ErbB1 or ErbB2. The results allowed us to conclude that rearrangement of lipid rafts is involved in the mechanism of action of 1 in tumor cell lines overexpressing ErbB proteins and that significant differences exist between the mechanisms of action of the two compounds.

Figure 1. Removal of serum from culture media enhanced the antiproliferative effect of 1 and 2 in tumor cell lines overexpressing ErbB proteins. Tumor cells (7 × 10³ cells/well) were plated in triplicate followed by treatment with different concentrations of 1 and 2 in complete medium (circles) and serum-free medium (triangles) for 24 h. Cells were allowed to proliferate for another 24 h in complete or serum-free medium. The relative cell numbers of SK-BR-3 (A) and A-431 cells (B) are plotted as a function of the concentration of the two flavonoids. Cell numbers were normalized to the number of cells in the untreated samples (DMSO) in the presence of serum.

Table 1. EC₅₀ Values of 1 and 2 in A-431 and SK-BR-3 Cells

<table>
<thead>
<tr>
<th>cell line</th>
<th>flavonoid</th>
<th>CM EC₅₀ (µM)ᵃ</th>
<th>SFM EC₅₀ (µM)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-BR-3</td>
<td>1</td>
<td>195 ± 38</td>
<td>100 ± 15</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>403 ± 37</td>
<td>97 ± 32</td>
</tr>
<tr>
<td>A-431</td>
<td>1</td>
<td>266 ± 40</td>
<td>38 ± 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>278 ± 19</td>
<td>108 ± 28</td>
</tr>
</tbody>
</table>

ᵃMean ± SEM (n = 3). bComplete medium. SFM-sulfate-free medium.

effect of 1 on cell proliferation in A-431 cells (Figure 2B). These results support the idea that 67LR is a molecule involved in the mechanism of action of 1 in tumor cell lines with ErbB protein overexpression.

The results demonstrated that mammary and epidermoid tumor cells with ErbB protein overexpression displayed significant resistance to 1 and 2 in complete medium, and serum withdrawal substantially enhanced the antiproliferative effect of these two flavonoids. The high EC₅₀ values measured in serum-containing medium are consistent with previous publications in which the inhibition of cell growth was reported at concentrations in the range 100–250 µM for both 1 and 2. 25,29,31 A possible explanation for the protective effect of serum against flavonoid-induced cytotoxicity in tumor cells could be related to the ability of different serum proteins, like albumin, to bind to the flavonoids. 32 Similar data on the protective effect of serum against cytotoxicity induced by flavonoids in human hepatoma cells were reported by Zhang et al. 33 The mechanisms responsible for the observed antiproliferative effects were also investigated. Compound 1 induced

![Figure 1](image-url)

**RESULTS AND DISCUSSION**

**Compound 1 Is More Effective in Inducing Tumor Cell Death Compared to 2, and the Effect Is Enhanced by Serum Starvation and Mediated by 67LR.** The effects of 1 and 2 (Figure 1, Table 1) on cell viability in breast tumor (SK-BR-3) and epidermoid adenocarcinoma (A-431) cells were evaluated. Both cell lines were relatively insensitive to these flavonoids with half-maximal effective concentrations (EC₅₀) well above 100 µM (circles in Figure 1, Table 1). When the tumor cells were treated with the flavonoids in the absence of serum, the EC₅₀ values were reduced 2–6-fold (triangles in Figure 1, Table 1), showing that serum starvation enhanced the cytotoxic effect of 1 and 2 on tumor cell lines overexpressing ErbB proteins.

In order to check the mechanism of cell death induced by 1 and 2, SK-BR-3 and A-431 cells were stained with annexin V-FITC and 7-AAD (Figure 2; Supporting Information, Figures S1 and S2). Compound 1 was more effective in inducing cell death in both cell lines with 75 µM of this flavonoid driving ~80% of A-431 cells in ~40% of SK-BR-3 cells into late apoptosis or necrosis (Figure 2A). Compound 2 also induced late apoptosis and necrosis in A-431 cells, while it had no significant effect on SK-BR-3 cells. The fact that 1 had more potent cytotoxic activity than 2 is in agreement with its lower EC₅₀ values determined in the cell proliferation experiments. These experiments also showed that A-431 cells were more sensitive to the cytotoxic effect of the two flavonoids investigated.

Since previous reports indicated that 67LR is a receptor for 1, the possible influence of an anti-67LR receptor antibody on effects of this compound was investigated. 26 The cell lines were pretreated with 20 µg/mL anti-67LR antibody followed by incubation with 1. The incubation with anti-67LR reduced the

![Figure 2](image-url)
apoptosis and necrosis supported by the increase in phosphatidylserine externalization (measured by annexin V binding) and increased nonspecific membrane permeability in both SK-BR-3 and A-431 cells without significant effects on the cell cycle distribution of cells. These findings are in agreement with previous studies demonstrating that 80–100 μM of 1 induced early and late apoptotic/necrotic events in colorectal and cervical cancer cell lines. 33 67LR is a non-integrin laminin receptor that can bind 1. 28 It displays tumor-specific overexpression with very low or no expression under physiological conditions. 9,26 The overexpression of 67LR in tumor cells is associated with tumor aggressiveness and metastasis. 34 The fact that an antibody against 67LR diminished the antiproliferative effect of 1 suggests that 67LR is involved in the mechanism of action of this flavonoid. These findings are in agreement with previous reports in multiple myeloma cells. 9

Figure 2. Induction of late apoptosis/necrosis in cancer cell lines overexpressing ErbB proteins. (A) SK-BR-3 and A-431 cells were treated with 50 and 75 μM of 1 and 2 for 72 h in complete medium followed by staining with annexin V-FITC and 7-AAD for flow cytometric measurement of apoptosis. Ten-thousand events were recorded and analyzed by quadrant gating in triplicate. The stacked bars indicate the percentage of cells in the viable (annexin V-FITC−/7-ADD−), early apoptotic (annexin V-FITC+/7-ADD−), late apoptotic (annexin V-FITC+/7-ADD+), and necrotic (annexin V-FITC+/7-ADD+) regions. (B) Cells pretreated with anti-67LR for 3 h were incubated in the presence of the indicated concentrations of 1 for 24 h. Cell proliferation was measured in 24 h after removing 1 using the WST-1 reagent and spectrophotometric measurements (n = 3, means ± SEM). The asterisks indicate a significant difference compared to control (**p < 0.01, ***p < 0.001). The number sign (#) indicates a significant difference between antibody-treated and untreated samples (p < 0.01).

Figure 3. Cell cycle analysis of SK-BR-3 and A431 cells treated with 1 and 2. SK-BR-3 and A-431 tumor cells were treated with 50 and 75 μM of 1 and 2 for 36 h. Cell cycle distribution was determined by flow cytometric DNA content analysis after propidium iodide staining and by measuring 10 000 events/sample (n = 3). The asterisks indicate a significant difference compared to control (**p < 0.05, ***p < 0.001).
Compound 2 Causes Cell Cycle Arrest of Tumor Cells Overexpressing ErbB Proteins. Since the previous data indicated that 1 and 2 inhibit the growth of tumor cells, the effect of these flavonoids on cell cycle progression was also investigated. Compound 2 was more potent in inducing cell cycle arrest, with 50 and 75 μM of this isoflavonoid arresting 50–55% of SK-BR-3 cells in the G2/M phase (Figure 3; Supporting Information, Figure S3A). The increase in the proportion of cells in the G2/M phase consistently correlated with a decrease in the percentage of cells in the G0/G1 phase. A-431 cells showed a similar response to 2, although the magnitude of the response was smaller (Figure 3; Supporting Information, Figure S3B). The effect of 1 on cell cycle progression in both cell lines was not as pronounced as in the case of 2 (Figure 3).

Contradictory data have been reported about 1 blocking the cell cycle in the G0/G1 phase. While some results suggest such an effect, other researchers have failed to observe this.35 The data presented in this paper are in agreement with the inability of 1 to influence the cell cycle distribution of cells. On the other hand, the antiproliferative effect of 2 seems to be caused almost exclusively by its ability to cause cell cycle arrest in the G2/M phase. These findings are in agreement with previous data, in which 2 was shown to arrest prostate cancer cells in the G2/M phase correlated with p21WAF1/Cip1 upregulation and cyclin B1 downregulation and with other reports in which the antiproliferative effect of 2 and its derivatives have been shown to be associated with their interaction with microtubules.36–38

Compound 1, but Not 2 Induces Downregulation and Internalization of ErbB Proteins. Since 1 has been implied to induce downregulation of EGFR,39 the effect of 1 and 2 on the expression level of two ErbB family members, ErbB1 and ErbB2, was investigated. Cells, serum-starved for 24 h, were treated with 50 μM of 1 or 2 for another 24 h in serum-free medium and then processed for flow cytometry. The expression levels of ErbB1 and ErbB2 were measured in A-431 and SK-BR-3 cells, respectively, since they are expressed at sufficiently large levels in the respective cell lines. Compound 1 induced a ~30% downregulation of ErbB1 and ErbB2, whereas 2 did not have any significant effect (Figure 4A). The aforementioned effect of 1 was not observed if cells were treated in the presence of serum, indicating that the effect of this flavon on the expression level of ErbB proteins was enhanced by serum starvation (data not shown). Since one-day incubation with 2 did not have any significant effect on the expression levels, a detailed microscopic analysis of the short-term effects was only undertaken for 1. The tumor cells were incubated with 200 μM of 1 for 30 min at 37 °C and stained for ErbB proteins with primary fluorescent antibodies. Confocal microscopy confirmed the flow cytometric results revealing a marked reduction in ErbB1 and ErbB2 expression levels within 30 min of incubation (Figure 4B; Supporting Information, Figure S4). Conspicuous intracellular vesicles containing ErbB2 immunofluorescence were observed, most likely corresponding to endosomes or lysosomes containing ErbB2 removed from the plasma membrane (Supporting Information, Figure S4).

Several studies have investigated the effect of 1 on receptor tyrosine kinases since 1997, when the inhibition of EGF binding to ErbB1 in A-431 cells mediated by this flavonoid was reported for the first time.40 The absorption spectrum of 1 with a peak below 400 nm did not interfere with the spectrum of fluorescently labeled antibodies.41 The data obtained indicated that short-time incubation with 1 leads to downregulation of ErbB1 and ErbB2. These results regarding ErbB1 expression are consistent with previous reports, but 1-induced ErbB2 internalization and downregulation from the plasma membrane have not been shown previously.39–42 The importance of this finding lies in the fact that ErbB2 is known to be an internalization-resistant receptor; therefore agents capable of removing it from the membrane have therapeutic potential.43 The exact mechanism behind the downregulation of ErbB1 and ErbB2 is unknown. Weinstein and his co-workers found that 1 did not inhibit EGF binding when assays were performed at 4 °C, suggesting the involvement of a metabolic process rather than evidence for competition between 1 and EGF.39 Additional data from the same investigators showed that 1 achieves ErbB1 downregulation by reversible internalization without degradation.39

Compound 1 Causes Reduction in the Clustering of Lipid Rafts and in Their Co-localization with 67LR. Flavonoids, in particular 1, have already been reported to disrupt lipid rafts.21 Therefore, number and brightness analysis, a recently developed technique, was used to investigate whether 1 causes any modification in the clustering of lipid rafts. To this end, cells were transfected with GFP-GPI, a protein known to partition to lipid rafts.18 Number and brightness analysis carried out in untreated cells suggested that GFP-GPI proteins homoassociate (Table 2). Comparison of the results obtained in control cells to the molecular brightness of soluble
monomeric GFP (0.13 ± 0.013) revealed partial dimerization or trimerization of GFP-GPI with the cluster sizes somewhat larger in A-431 cells. Treatment of both cell types with 1, but not with 2, significantly reduced the molecular brightness values, suggesting a reduction in the clustering of GFP-GPI.

These results implied that the organization of lipid rafts is altered by 1. Since results presented in previous sections showed that 67LR may be involved in the mechanism of action of 1, the possible common background of these findings was investigated in more detail. To this end, GM1 gangliosides, known to reside in lipid rafts, and 67LR were labeled fluorescently in A-431 cells, revealing ~75% co-localization between the labels (Figure 5). Treatment with 1 for 30 min reduced the co-localization between 67LR and lipid rafts to ~47%. Taken together, these results suggest that alterations in the structure of the plasma membrane in general, or the clustering of lipid rafts in particular, may be partially responsible for the effects of 1 on tumor cells. The co-localization experiments were performed only for 1, since 67LR is known to be a receptor for 1, and not for 2.

The plasma membrane contains small dynamic micro-domains rich in sphingomyelin and cholesterol called “lipid rafts”, which are involved in signal transduction and membrane trafficking in general and in the signaling of ErbB proteins in particular. GPI-anchored proteins and GM1 gangliosides were used as markers of lipid rafts. Two important findings may be presented about the role of lipid rafts in the action of 1: (i) this flavonoid induces disruption of GPI-anchored protein clusters; (ii) it removes 67LR from lipid rafts. A previous study has already implied that 1 decreases lipid order and the quantity of lipid rafts. However, while the aforementioned publication showed only indirectly the altered structure of lipid rafts, the number and brightness experiments in this study convincingly established that the clustering of lipid rafts or raft-associated proteins is abolished by 1. Although Tsukamoto et al. claimed to have found evidence for 1-induced increased clustering of lipid rafts, their conclusions are based on noncalibrated FRET measurements, and caution should be taken in interpreting these kinds of data. Another characteristic of the mechanism of action of 1 at the plasma membrane level involved the removal of 67LR from lipid rafts. The data in this study suggest that 1 might induce the disruption of the integrity of lipid rafts in tumor cells, resulting in a loss of specific and ordered distribution required for proper functioning. In this regard, previous studies have reported that the disruption of lipid rafts by cholesterol depletion resulted in downregulation of 67LR expression in human basophiles. This feature of action of 1 could be of biological interest for other plasma membrane receptors associated with lipid rafts.

**Biomedical Aspects and Conclusions.** Although receptor-oriented cancer therapy against ErbB1 and ErbB2, overexpressed in a wide range of human cancers, offers several advantages over conventional chemotherapy, alternative therapeutic and preventive strategies are still required against ErbB-overexpressing tumors. Flavonoids are a group of plant secondary metabolites studied intensively for their chemopreventive and therapeutic effect on cancer cells and low cytotoxicity in normal cells. Compound 1, a green tea catechin, and 2, an isoflavonoid from soy, were shown to exert an inhibitory effect on different intracellular pathways, including protein tyrosine kinases, leading to pro-apoptotic and cytotoxic effects in tumor cells. In the present study, it was shown that (i) the antiproliferative effect of both flavonoids is enhanced by serum-free conditions; (ii) while 1 has more potent cytotoxic effects, 2 is superior in inducing cell cycle arrest; (iii) the antiproliferative effect of 1 is blocked by an antibody against 67LR; (iv) 1 causes downregulation of both ErbB1 and ErbB2 from the plasma membrane; (v) 1 induces disassembly of clusters of a lipid raft-associated protein, pointing to changes in the structure of lipid rafts; and (vi) the localization of 67LR to lipid rafts was reduced by 1. This is the first time that the effect of 1 on ErbB2 internalization and GPI-anchored proteins has been reported.

The following model is presented to account for the experimental data. The primary targets of 1 are lipid rafts for which the clustering is decreased by the flavonoid, leading to secondary effects including downregulation of ErbB1 and ErbB2 as well as removal of 67LR from lipid rafts. The raft localization may be important for the survival-promoting effect of 67LR known to be present in dimeric form in the membrane, which may be abolished by dislocation from lipid rafts. In addition, the connection between 1, apoptosis, and lipid rafts supports the hypothesis that lipid rafts could be considered as “floating islands of death and survival”. Further studies will be required to investigate the effect of 1 in combination with other test compounds, to assess the impact on tumors overexpressing ErbB proteins in animal models, and to carefully evaluate the clinical applicability considering its therapeutic index.

### EXPERIMENTAL SECTION

**Chemicals and Reagents.** Epigallocatechin 3-O-gallate (1) and genistein (2) were purchased from Sigma-Aldrich (St. Louis, MO, USA; catalog numbers 50299 and G6649, respectively) and dissolved

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**Table 2. Molecular Brightness Values of GFP-GPI**

<table>
<thead>
<tr>
<th></th>
<th>SK-BR.3*</th>
<th>A-431*</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>0.207 ± 0.023</td>
<td>0.372 ± 0.106</td>
</tr>
<tr>
<td>1</td>
<td>0.114 ± 0.011b</td>
<td>0.162 ± 0.037b</td>
</tr>
<tr>
<td>2</td>
<td>0.188 ± 0.028</td>
<td>0.355 ± 0.098</td>
</tr>
</tbody>
</table>

*Molecular brightness values ± SEM of untreated and treated tumor cells are shown. One-way ANOVA separately carried out for the two cell lines indicated significant difference between the means (p < 0.05). bPost hoc analysis by Tukey’s HSD test indicated that the EGCG-treated samples significantly differed from the other two (p < 0.05).*

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**Figure 5.** Effect of 1 on 67LR and GM1 on the surface of A-431 cells. A-431 cells serum-starved for 24 h were incubated for 30 min with 1 at 37 °C and fixed and labeled for GM1 ganglioside with fluorescent cholera toxin and 67LR with a monoclonal antibody. Confocal images of untreated and treated A-431 cells were taken, and images recorded in the channels corresponding to the immunofluorescence of 67LR (green) and GM1 (red). The overlay of the images at the top of cells is shown in the figure. (C = Pearson correlation coefficient; scale bar = 5 μm.)
in DMSO. HPLC analysis by the manufacturer indicated a purity of ≥97% for both compounds. The anti-67LR antibody (MLuC5) was obtained from Abcam (Cambridge, UK), while the anti-ErbB2 antibody (Herceptin) was purchased from Roche (Budapest, Hungary). Monoclonal antibody 528 (Mab 528, against ErbB1) was produced by the hybridoma cell line 528 (HB-BS09, ATCC), and the antibodies were purified from hybridoma supernatants. The primary antibodies were labeled with AlexaFluor (Invitrogen) dyes, according to the manufacturer’s specifications. The secondary antibody Alexa488-GaMlg (H+L) against the light chain of mouse IgG was purchased from Invitrogen.

**Cell Culture and Transfection.** The human breast cancer cell line SK-BR-3 and the human epidermoid carcinoma cell line A-431 were obtained from the American Type Culture Collection (Rockville, MD, USA) and were grown according to their specifications. For flow cytometry, the cells were harvested by trypsinization. Transfection of A-431 cells was carried out by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, using 0.4 μg of GFP-GPI plasmid and 0.8 μL of transfection agent for a single well of an eight-well chamber (Nunc, Roskilde, Denmark). Transfection of SK-BR-3 cells with GFP-GPI was performed using an Amaxa instrument with Cell Line Nucleofector kit C and program E-009. The GFP-GPI plasmid was a kind gift from Jennifer Lippincott-Schwartz (NIH, Bethesda, MD, USA).

**Viability Assay and Anti-67LR Treatment.** SK-BR-3 and A-431 cells were seeded in triplicate at a density of 7 × 10^4 cells/sample in 96-well flat-bottom microwells 24 h prior to the experiments and incubated 24 h with flavonoid 1 and 2. The viability assay was performed in triplicate using the cell proliferation reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany), based on the reduction of tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to formazan by mitochondrial dehydrogenases. The absorbance of formazan was measured at 450 nm and corrected at 620 nm using a 96-well spectrophotometer (ELISA reader). The plot of absorbance against time was fitted to the following equation using SigmaPlot 10 (Systat Software, Chicago, IL, USA):

\[
A_{\min} + \frac{A_{\max} - A_{\min}}{1 + (x/EC_{50})^n}
\]

(1)

where \(A_{\min}\) and \(A_{\max}\) are the lowest and highest absorbance values, respectively, \(n\) is the Hill slope, and \(x\) is the concentration. For treatment with the anti-67LR antibody cells seeded in 96-well plates were incubated for 3 h with 20 μg/mL MLuC5 antibody and maintained in the presence of 100 and 200 μM of 1 for another 24 h in serum-free medium followed by the WST-1 viability assay.

**Apoptosis Assay.** Cells were collected by trypsinization and washed twice in PBS, and 10^5 cells/sample were double stained with annexin V-FITC (Beckman Coulter, Brea, CA, USA) and 7-AAD (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions in each case. The samples were analyzed by flow cytometry within 1 h after staining using a FACSCalibur instrument (Becton Dickinson, San Jose, CA, USA). Both FITC and 7-AAD were instructed in each case. The samples were analyzed by flow cytometry within 1 h after staining using a FACSCalibur instrument (Becton Dickinson, San Jose, CA, USA). Both FITC and 7-AAD were stained immediately and stained with primary antibodies against ErbB1 (528-Alexa Fluor 488), ErbB2 (Herceptin-Alexa Fluor 488), and 67LR (MLuC5) in 1% BSA/0.1% Triton X-100/PBS for 30 min on ice followed by secondary labeling for 67LR. Lipid rafts were labeled with 8 μg/mL fluorescently-labeled subunits of cholera toxin (AlexaFluor647-CTX: Molecular Probes, Eugene, OR) for 30 min on ice. The fluorescent images were recorded with an Olympus FV1000 inverted confocal microscope (Olympus Corp., Tokyo, Japan) equipped with three lasers emitting at 488, 532, and 633 nm using a 63× objective (N.A. = 1.35).

**Calculation of Image Cross-Correlation.** Co-localization \(C\) between two different fluorescent labels was calculated according to Pearson’s formula:

\[
C = \frac{\sum (S_1 - S_{1av})(S_2 - S_{2av})}{\sqrt{\sum (S_1 - S_{1av})^2 \sum (S_2 - S_{2av})^2}}
\]

(2)

where \(S_1\) and \(S_2\) are the intensities of the ith pixel in the first and second image, respectively, and \(S_{1av}\) and \(S_{2av}\) are the average fluorescence intensities of the first and second image, respectively. Values close to 1 and 0 indicate a high and low degree of co-localization, respectively. \(C\) values were computed using a custom-written software written in LabView (National Instruments, Austin, TX, USA).

**Number and Brightness Analysis.** An Olympus FV1000 confocal microscope running in pseudo-photon-counting mode was used to carry out number and brightness analysis according to Digman et al. To investigate the clustering of GPl-anchored proteins, cells serum-starved overnight were incubated with 100 μM of 1 or 2 for 12 h in complete medium. Live cells were analyzed at room temperature in Hank’s buffer supplemented with 10 mM glucose and 0.1% BSA. Image series of 100 optical slices of the cell membrane adjacent to the coverslip were acquired with a pixel size of 82 nm and pixel dwell time of 10 μs. The image stack was analyzed with a custom-written Matlab (Mathworks, Inc., Natick, MA, USA) program incorporating functions of the DipImage toolbox (University of Technology, Delft, The Netherlands). The images were first registered (i.e., corrected for lateral shift) followed by calculating the mean and variance of every pixel. The apparent brightness was calculated according to the following equation:

\[
B = \frac{\sigma^2}{k} = x + 1
\]

(3)

where \(\sigma^2\) and \(k\) are the variance and the mean, respectively, of a given pixel. The molecular brightness \(x\) characterizes the clustering state of a fluorescent molecule by giving the number of photons detected from a single diffusing unit during the pixel dwell time. Comparison of the molecular brightness determined in cells to that of soluble monomeric GFP reveals the clustering state of proteins, since

**Immunofluorescence Staining for Flow Cytometry.** Trypsinized cells, serum-starved for 24 h, were incubated at 37 °C for another 24 h in the presence of various concentrations of the compounds in serum-free medium followed by immunofluorescent staining. Primary antibodies against ErbB2 (Herceptin-Alexa Fluor 488), ErbB1 (528-Alexa Fluor 488), and 67LR (MLuC5) as well as secondary antibodies were used at a concentration of 10−20 μg/mL. Incubation with the primary antibodies was carried out on ice for 30 min followed by washing in cold PBS and fixation in 4% formaldehyde or by secondary staining in the case of MLuC5 antibody. The fluorescence of 20,000 cells was recorded with a FACSCalibur instrument (Becton Dickinson).
the molecular brightness is linearly proportional to the number of molecules in a homocentric.

## ASSOCIATED CONTENT

### Supporting Information

Figures S1–S4 are available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

P. Nagy and J. Szöllösi contributed equally to the work.

### Notes

The authors declare no competing financial interest.

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Supporting Information

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**Figure S1.** Induction of cell death in SK-BR-3 mammary cancer cells by 1 and 2. SK-BR-3 cells were incubated in the presence of the indicated concentration of 1 and 2 for 72 h followed by staining with annexin V-FITC and 7-AAD. The figures show representative dot plots used for the determination of viable, apoptotic, late apoptotic and necrotic cells whose quantitative evaluation is displayed in Fig. 2.
Figure S2. Induction of early and late apoptosis in A431 epidermoid cancer cells by 1 and 2. A-431 cells were incubated in the presence of the indicated concentration of 1 and 2 for 72 h followed by staining with annexin V-FITC and 7-AAD. The figures show representative dot plots used for the determination of viable, apoptotic, late apoptotic and necrotic cells whose quantitative evaluation was displayed in Fig. 2.
Figure S3. Representative DNA histograms of control cells and samples treated with compound 2. SK-BR-3 and A-431 cells were treated with 75 µM of 2 for 36 h and stained with propidium iodide. The DNA histograms of treated cells alongside with those of control samples are displayed in the figure.
Figure S4. Compound 1 reduces the expression of ErbB proteins in mammary and epidermoid tumor cells lines. Immunofluorescent images of untreated samples (DMSO) and A-431 cells treated with 200 µM of 1 for 30 min at 37 °C stained with an anti-ErbB1 monoclonal antibody. The confocal image shows a plane in the middle of the cells. Control SK-BR-3 cells and those treated with compound 1 were labeled with an anti-ErbB2 monoclonal antibody revealing downregulation and internalization of ErbB2 after 30 min of treatment. The arrows on the panel point to putative endosomes and lysosomes.