Fatty acid synthase (E.C.2.3.1.85; FASN) is a lipogenic enzyme which catalyzes the de novo synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH precursors (1). FASN expression is generally low or undetectable in human tissues other than the liver and adipose tissue, and nonmalignant cells preferentially use circulating dietary fatty acids for the synthesis of new structural lipids. In contrast, high levels of FASN expression have been observed in breast cancer (2) and other human carcinomas (reviewed in ref. 3). Importantly, several reports have shown that FASN expression levels correlate with tumor progression, aggressiveness, and metastasis, and are found elevated in the serum of patients with cancer.
Translational Relevance

The enzyme fatty acid synthase (FASN) has been shown to be a key therapeutic target in cancer given its importance in tumor biology. The present study evaluated novel anticancer compounds that inhibited FASN activity and did not have effects on fatty acid oxidation or caused weight loss in experimental animals. These compounds also represent promising agents in preliminary in vivo studies. Here, we open a new therapeutic perspective providing preliminary in vitro and in vivo proof of evidence of a new generation of synthetic FASN inhibitors with low toxicity profile.

Materials and Methods

Synthesis of polyphenolic compounds 1–8. Target compounds 1–8 were synthesized via standard chemical procedures starting from conveniently protected gallic acid 17 (27), as described in Fig. 2 and in the Supplementary Data. Spectroscopic data of all synthesized compounds were consistent with the proposed structures. For series 1-8 and 9-16, we include the data of compounds 4, 6, 12, and 14. The rest of the compounds are described in the Supplementary Data.

1.3-Bis[3,4,5-trihydroxynaphthalene]benzene (4) was prepared following the general procedure, starting from intermediate 12. Yield: 60%; mp 194–195°C; IR (KBr): 3082, 2928, 1718, 1607, 1200; 13C-NMR (CD3OD) δ 71.3, 75.2, 109.7, 115.9, 123.4, 127.6, 128.0, 128.1, 128.2, 128.5, 136.5, 137.3, 143.2, 151.9, 152.7, 164.4.

3,4,5-Trimethyl-2-phenyltetrazolium bromide (MTT) were purchased from Sigma. Drug sensitivity was determined using a standard colorimetric MTT assay. Briefly, cells were plated out at a density of 7 × 10^4 cells/100 μL/well in 96-well microtiter plates, and allowed an overnight period for attachment. Then the medium was removed and cells were incubated for 48 h with fresh medium containing different concentrations of EGCG or the novel polyphenolic compound 1-8. Following treatment, drug-free medium (100 μL/well) and 10 μL of a 5 mg/mL MTT solution were added and cells were incubated for 3 h at 37°C. After careful removal of the supernatants, the MTT-formazan crystals formed by metabolically viable cells were dissolved in DMSO (100 μL), and the absorbance was measured at 570 nm in a multi-well plate reader (Model Anthos Labtec 210 1.7).
Lowry-based Bio-Rad assay (Bio-Rad Laboratories) and the adequate volume was diluted to a concentration of 1 μg/μL. One-hundred and twenty microliters of this particle-free supernatant were preincubated for 15 min at 37°C for temperature equilibration. The sample was then added to 150 μL of the reaction buffer followed by 30 μL of 500 μmol/L malonyl-CoA (FASN substrate), and the final volume of 0.3 mL of reaction mixture [200 mmol/L potassium phosphate buffer (pH 7.0), 1 mmol/L EDTA, 1 mmol/L DTT, 30 μmol/L acetyl-CoA, 0.24 mmol/L NADPH, and 50 μmol/L malonyl-CoA] was assayed for 10 min to determine FASN-dependent oxidation of NADPH. Before the addition of malonyl-CoA, the background rate of NADPH oxidation in the presence of acetyl-CoA was monitored at 340 nm for 3 min. FASN activity was expressed in nmol NADPH oxidized × min⁻¹ × mg protein⁻¹.

Immunoblot analysis of FASN, p185HER2/neu, phosphorylated p185HER2/neu, ERK1/2, phosphorylated ERK1/2, AKT, phosphorylated AKT Ser473, and poly(ADP-ribose) polymerase. The primary antibody for FASN immunoblotting was a mouse IgG1 FASN monoclonal antibody from BD Biosciences PharMingen. Monoclonal anti-β-actin mouse antibody (clone AC-15) was from Sigma. Rabbit polyclonal antibodies against poly(ADP-ribose) polymerase (PARP), AKT, phosphorylated AKT (Ser473), and mouse monoclonal antibodies against p185HER2/neu (clone Ab-3) and phosphorylated p185HER2/neu were from Cell Signaling Technology. Following treatment of SK-BR3 cells with EGCG or novel polyphenolic compounds at corresponding concentrations and time intervals, cells were harvested by treatment with trypsin-EDTA, washed twice with PBS, and stored at -80°C. Cells were lysed in lysis buffer [1 mmol/L EDTA, 150 mmol/L NaCl, 100 μg/mL phenylmethylsulfonyl fluoride, 50 mmol/L Tris-HCl (pH 7.5)] and kept at 4°C while they were routinely mixed every 2 min on the vortex for 30 min. A sample was taken for measurement of protein content by the Lowry-based Bio-Rad assay. Equal amounts of protein were heated in SDS sample buffer (Laemmli) for 5 min at 95°C, separated on a 3% to 8% SDS-polyacrylamide gel (FASN, p185HER2/neu, phosphorylated p185HER2/neu) or 4% to 12% SDS-polyacrylamide gel (AKT, phosphorylated AKT, ERK1/2, phosphorylated ERK1/2, and PARP), and transferred onto nitrocellulose membranes. Membranes were incubated for 1 h at room temperature in blocking buffer (2.5% powdered skim milk in tris-buffered solution with 0.05% Tween 20; TBS-T [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.05% Tween 20]) to prevent nonspecific antibody binding, and incubated with the corresponding primary antibody diluted in blocking buffer overnight at 4°C. After three 5-min washes in TBS-T, blots were incubated for 1 h with corresponding peroxidase-conjugated secondary antibody and developed using a commercial kit (West Pico chemiluminescent substrate). Blots were reprobed with an antibody against β-actin as control of protein loading and transfer.

Measurement of CPT-1 activity. CPT-1 activity assay was done using palmitoyl-CoA lithium salt from Sigma, fatty acid–free bovine serum albumin from Roche, l-carnitine hydrochloride from Sigma, and l-[methyl-3H]carnitine hydrochloride (82 Ci/mmol) from Amersham Biosciences. CPT-1 activity was assayed by the forward exchange method using l-[3H]carnitine as we previously described (17, 18). Briefly, reactions (total volume of 0.5 mL) consisted of the standard enzyme assay mixture with 200 μmol/L of the corresponding FASN inhibitor, 2 mmol/L of l-[3H]carnitine (~5,000 dpm/nmol), 80 μmol/L of...
palmitoyl-CoA, 20 mmol/L of HEPES (pH 7.0), 1% fatty acid–free albumin, and 40 to 75 mmol/L of KCl. Reactions were initiated by the addition of isolated intact yeast mitochondria. The reaction was linear up to 4 min and all incubations were done at 30°C for 3 min. Reactions were stopped by the addition of 6% perchloric acid and were then centrifuged at 2,500 rpm for 5 min. The resulting pellet was suspended in water and the product [3H]palmitoylcarnitine was extracted with butanol at low pH. After centrifugation at 2,300 rpm for 3 min, an aliquot of the butanol phase was transferred to a vial and counted by liquid scintillation.

Animal long-term weight loss experiments. Male mice C57BL/6J (12 wk, 23-25 g) were purchased from Harlan Laboratories (France), fed ad libitum with a standard rodent chow and housed in a light/dark 12 h/12 h cycle at 22°C for 1 wk. C75 was from Alexis Biochemicals. Animals were randomized into five groups of four animals each: control, C75-treated, EGCG-treated, and compounds 7– and 8-treated. All experiments were done in accordance with guidelines on animal care and use established by the University of Barcelona School of Pharmacy Institutional Ethic and Scientific Committee (Barcelona, Spain). Treatments were done as previously described (16, 28). Briefly, mice were fasted for 12 h during the dark cycle before treatment. Each group received a single i.p. injection (0.5 mL) of FASN inhibitor (30 mg/kg) or vehicle alone (DMSO), dissolved in RPMI 1640. After i.p. injections, mice were given free access to rodent chow for 24 h. At this time, the experiment finished and body weight was registered.

Human breast tumor xenograft experiments. Female athymic nude BALB/c mice (4-5 wk old) were purchased from Harlan Laboratories (France) and housed in a pathogen-free facility. Tumor xenograft was established by s.c. injection of 10 × 10⁶ BT474 cells mixed in Matrigel (BD Bioscience) into the female’s flank. Tumors were allowed to increase 150 to 250 mm³ size, and the mice were randomized into groups of six animals each. Mice were treated by i.p injection daily with 40 mg/kg of compound 7 for 45 days. Tumors were measured daily with electronic calipers, and tumor volumes were calculated by the formula: π/6 × (v₁ × v₂ × v₂), where v₁ represents the largest tumor diameter, and v₂ the smallest one. Weight of control and treatment groups was registered daily. At the end of the experiment, all mice were euthanized, and tumors were stored at -80°C. All experiments were conducted in accordance with guidelines on animal care and use established by Biomedical Research Institute of Bellvitge (IDIBELL) Institutional Animal Care and Scientific Committee.

Table 1. Structure and biological activity of novel polyphenolic compounds I(1-8)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cyclic system</th>
<th>Cancer cell cytotoxicity, IC₅₀ (μmol/L)</th>
<th>Inhibition of FASN activity (% of control)</th>
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<tr>
<td></td>
<td></td>
<td>SK-Br3 (++++)</td>
<td>MCF-7 (+)</td>
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<tr>
<td>EGCG</td>
<td></td>
<td>149 ± 20</td>
<td>205 ± 7</td>
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<tr>
<td>1</td>
<td></td>
<td>186 ± 34</td>
<td>&gt;250</td>
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<tr>
<td>2</td>
<td></td>
<td>179 ± 49</td>
<td>&gt;250</td>
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<tr>
<td>3</td>
<td></td>
<td>125 ± 5</td>
<td>165 ± 8</td>
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<td>4</td>
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<td>42 ± 7</td>
<td>162 ± 18</td>
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<td>25 ± 4</td>
<td>&gt;250</td>
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<td>21 ± 7</td>
<td>46 ± 18</td>
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<tr>
<td>8</td>
<td></td>
<td>29 ± 0.3</td>
<td>44 ± 16</td>
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</table>

*ns, no significant inhibition of FASN activity at a concentration equal to the cytotoxicity IC₅₀ value of the compound in SK-Br3 cells.

Statistical analysis. Results were analyzed by Student’s t test or by one-way ANOVA using a Tukey test as a post-test. P < 0.05 was considered statistically significant.

Further details. For details concerning apoptosis, and malignant and nonmalignant cell proliferating assays, see Supplementary Data.
relationships in SK-Br3 cells could be drawn: (a) the cyclic system linking both galloyl moieties must be aromatic because cyclohexane derivatives 1 and 2 are poorly cytotoxic (IC50 > 150 μmol/L) whereas all the aromatic analogues (3-8) exhibit moderate to high cytotoxic activity (21-125 μmol/L); (b) in general, compounds with a naphthalene ring in the aromatic system were more potent than analogues containing a benzene ring. This effect is specially notable when we observe that compound 7 (IC50 = 21 μmol/L) is twice more potent than analogue 4 (IC50 = 42 μmol/L); and (c) with respect to the relative position of the galloyl moieties, the general trend is the increase in cytotoxicity as one is farther from the other within the cyclic system. Thus, a progressive decrease in IC50 values is observed when we compare 1,2-, 1,3-, and 1,4-disubstituted benzene derivatives (179, 125, and 42 μmol/L, respectively). In addition, the presence of hydroxyl groups in the molecule seems to be a key feature for the inhibition of cell proliferation, as concluded from the inactivity of all the assayed benzyl precursors (data not shown).

One important observation drawn from this set of data is the strong correlation between FASN expression and cell growth inhibition. In general, compounds are less potent as FASN levels decrease in the studied cell lines. This trend is particularly important in the case of compound 7, the most potent compound within the series, with IC50 values for SK-Br3, MCF-7, and MDA-MB-231 of 21, 46, and 79 μmol/L, respectively (Table 1). Taken together, these data suggest that direct FASN inhibition plays a predominant role in the cytotoxicity of the compounds. To further characterize the compounds, we determined the cytotoxic effects of compounds 7 and 8 in two types of nonmalignant cells (fibroblasts and adipocytes), and we observed no significant effects in cell death or morphology using doses up to 60 μmol/L. Under the same conditions, EGCG caused massive cell death in the nonmalignant cells that we studied (Supplementary Fig. S1). Moreover, we also evaluated the cytotoxicity of compounds 7 and 8 in a panel of additional cell lines (Supplementary Table S1), and observed that cytotoxicity was not correlated with the organ of origin, but rather with the expression levels of FASN.

Compounds inhibit FASN activity. We next evaluated the capacity of the new compounds to block FASN activity in intact cells. SK-Br3 cells were incubated in the presence of a concentration of the compound equal to its cytotoxic IC50 value in these cells for 6, 12, and 24 hours. FASN activity decreased in a time-dependent manner, and maximal inhibition at 24 hours were 90 ± 4% and 69 ± 16% for compounds 7 and 8, respectively (Table 1). We showed that this effect was not related to downregulation of FASN expression levels by immunoblot analysis (Fig. 3B). Because compounds 7 and 8 fulfill the sought profile of FASN inhibition and breast cancer cell cytotoxicity, they were selected for further studies with the objective of determining their mechanism of action.
Compounds 7 and 8 induce apoptosis and downregulate the phosphorylation of HER2, AKT, and ERK1/2. Study of cell death and signaling mechanisms were carried out using SK-BR3 as the optimal breast cancer cell line model due to its high constitutive FASN expression and activity. First, we studied the cell death mechanism. Apoptosis and induction of caspase activity were confirmed by Western blotting analysis showing cleavage of PARP. Treatment of SK-BR3 cells for 48 hours with 30 μmol/L of compounds 7 and 8 induced a marked increase in the levels of the PARP cleavage product (89 kDa band) in a time-dependent manner (Fig. 3A). To validate these results, the apoptotic effects of compounds 7 and 8 were also analyzed and quantified by flow cytometry using the Annexin V-Alexa Fluor 488 staining (Supplementary Fig. S2). We previously reported that FASN inhibitors (cerulenin, C75, and EGCG) induced apoptosis, and blocked the activation of the oncogene HER2 and their downstream signal transduction pathways ERK1/2 and PI3K/AKT in breast cancer cells (13, 18). Therefore, here we sought to examine the effects of FASN inhibitors 7 and 8 on HER2, AKT, and ERK1/2 activation. Incubation of cells with 30 μmol/L of compounds 7 and 8 dramatically decreased the levels of the phosphorylated forms of HER2 (p-HER2), that occurred as soon as 2 hours after treatment (data not shown), and became basically complete at 6 hours after treatment (Fig. 3B). During this period, there was no significant change in the total level of HER2 protein, as assessed by either Western blotting analysis (Fig. 3B) or HER2-specific ELISA (data not shown). Phosphorylated forms of ERK1/2 (p-ERK1/2) and AKT (p-AKT) were also noticeably decreased after 6 hours of exposure to compounds 7 and 8 (Fig. 3B). Overall, the two compounds induce a sustained blockade of the HER2 signaling pathway, at least throughout the whole time interval analyzed (24 hours). During this period, no significant change neither in the total level of the corresponding proteins (HER2, AKT, and ERK1/2) nor in FASN level was detected (Fig. 3B). Remarkably, under the same culture conditions, EGCG did not induce apoptosis (Fig. 3A) and did not block the activation of HER2 oncogene and its downstream signal transduction pathways, ERK1/2 and PI3K/AKT (Fig. 3B). Only significantly higher concentrations of EGCG (150 μmol/L) reached effects comparable to those observed with compounds 7 and 8 (18).

Compounds 7 and 8 do not stimulate CPT-1 activity in vitro and do not induce short-term weight loss in vivo. To evaluate the specificity of compounds 7 and 8 for FASN, we analyzed their effects on CPT-1 activity (Fig. 4A). Intact yeast mitochondria were assayed and neither EGCG, nor compounds 7 and 8 exerted any significant effect on CPT-1 (88%, 89%, and 90% CPT-1 activity of control, respectively) in sharp contrast to C75 (included for comparative purposes) which, as previously reported, produced a substantial activation of CPT-1 activity (129 ± 6%, with respect to controls). Consistently with the in vitro results, healthy mice treated with a single dose of 30 mg/kg administered i.p. of compounds 7 or 8 for 24 hours did not show any significant weight loss (97% and 95% of the control group, respectively). Food intake was similar to controls and altered behavior or signs of suffering or distress were not observed (data not shown). These effects are especially significant when compared with animals treated with the same i.p. dose of C75, in which a 21% weight loss (Fig. 4B), as well as signs of suffering, were observed. Altogether, our findings indicate that the new synthetic FASN inhibitors do not induce weight loss in vivo.

Compound 7 inhibits the growth of human breast cancer xenografts without causing long-term animal weight loss. We next explored the potential effectiveness of compound 7 for breast cancer treatment in vivo. Figure 5 illustrates the antitumor effect of compound 7 on subcutaneous xenografts of BT474 human breast cancer cells in athymic mice. The BT474 cell line was selected for in vivo experiments of this study due to its high constitutive FASN expression (30). In the BT474 xenograft-bearing mice, the six informative compound 7–treated animals substantially reduced tumor growth compared with the vehicle-treated control animals (Fig. 5A). One of the compound-treated animals had no identifiable residual tumor and one of them died due to traumatic i.p. injections. Importantly, no significant weight loss or anorexia was identified after 45 days of treatment in the experimental animals (Fig. 5B). At the conclusion of the
study, control animals weighed 6.69 ± 2.01% of pretreatment weight compared with 4.90 ± 1.26% for the compound 7-treated animals. Furthermore, histologic examination of liver, heart, kidney, lung, and brain showed no microscopic evidence of drug-induced toxicity (data not shown).

Discussion

In this study, we characterize the in vitro biochemical, enzymatic, and anticancer effects of novel synthetic FASN inhibitors related to green tea epigallocatechin. FASN has been suggested as a valuable molecular target for anticancer drug development (7, 11, 18, 22, 31, 32), although cross-activation of β-oxidation and in vivo body weight loss (16, 17, 33, 34) have limited its development in oncology. We describe in our report that the new compounds do not decrease weight loss in experimental animals, although it does have in vivo anticancer effects, thereby highlighting their potential applicability.

Two of the new polyphenolic compounds showed marked cytotoxicity (IC50 < 30 μmol/L) on the tested cancer cell lines, and were simultaneously FASN inhibitors (compounds 7 and 8). This effect was not due to a downregulation of the FASN protein levels because protein expression did not vary in immunoblot analyses from equally treated samples. There was a correlation between the cytotoxic effects and the cellular protein levels of FASN, observing more pronounced effects on the SK-BR3 cell line which expresses high levels of FASN. Compound 5, which had a cytotoxic potency in SK-BR3 cells comparable to compounds 7 or 8, but did not inhibit FASN, suggests that additional mechanisms apart from inhibition of FASN activity may be responsible for the cytotoxicity. Compounds 7 and 8 were characterized as potent FASN inhibitors with cytotoxic activity against breast cancer cell lines and therefore were selected for in-depth molecular studies. The novel FASN inhibitors induced apoptosis in cancer cells, and a time-response analysis revealed the appearance of PARP cleavage as early as 12 hours. The inhibition of FASN enzymatic activity by the new compounds was accompanied by changes in cell growth and proliferation signaling pathways. The active phosphorylated form of HER2 (p-HER2) decreased as soon as 2 hours after exposure, and was completely abolished after 6 hours of exposure. Similarly, phosphorylated forms of ERK1/2 (p-ERK1/2) and AKT (p-AKT) were also markedly decreased. It is remarkable that comparable concentrations of EGCG, even with prolonged exposure (24, 48, and 72 hours), had no detectable effect on apoptosis or on inhibition of pHER2, pERK, or pAKT. We have reported that 5-fold higher concentrations of EGCG (150 μmol/L) are needed to detect apoptosis or p-HER2 inhibition (18), indicating that the novel compounds are more active than the parent molecule.

An important result of our study concerns the specificity of the new compounds. A key feature of the novel synthetic compounds is that they do not act on CPT-1 activity in vitro and, consistently with this, they do not induce weight loss in experimental animals. The FASN inhibitors cerulenin and C75, in contrast, stimulate CPT-1 and accelerate fatty acid β-oxidation, which has been related to the severe decrease of food intake and induction of weight loss in rodents (16–18, 33, 34). In this first set of experiments, which follow the reports of Thupari et al.
In vivo therapeutic potential in different cancer animal models. These results will enable the definitive validation of FASN as a therapeutically useful option for cancer treatment.

In summary, we have developed a new class of polyphenolic compounds that induce apoptotic cancer cell death, inhibit FASN without showing concomitant adverse effects on body weight, and display antitumor activity in vivo, therefore, holding promise for further target-directed anticancer drug studies either alone or coadministered with other antitumor drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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