**Title:** CYTOSTATIC EFFECTS OF FATTY ACID SYNTHASE INHIBITION

**Abstract:** This invention provides a method for treating an individual having a tumor by administering to the individual an inhibitor of fatty acid synthase (FAS) in an amount sufficient to retard growth of cells in the tumor. Preferably, the method of this invention is applied to an individual having a tumor comprising cells which do not overexpress FAS or a tumor comprising cells which are resistant to induction of apoptosis by inhibitors of FAS. Administration of an inhibitor of FAS according to this invention can induce a cellular response equivalent to a genotoxic stress response in the absence of substantial DNA damage. This invention also provides for use of a FAS inhibitor in the preparation of a medicament for treating a tumor in an individual whose tumor exhibits reduced p53 function.
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
CYTOSTATIC EFFECTS OF FATTY ACID SYNTHASE INHIBITION

This application is related to U.S. Provisional Application No. 60/268,680, filed February 15, 2001, which is incorporated herein by reference in its entirety.

5 BACKGROUND OF THE INVENTION

Field of the Invention

The present invention provides new methods for treating an individual having a tumor. In particular, the method comprises administering to the individual an inhibitor of fatty acid synthase (FAS) in an amount sufficient to retard growth of cells in the tumor.

Review of Related Art


The biological basis for this phenotypic alteration is not clear. However, altered fatty acid metabolism represents a novel target for anti-metabolite therapy, since pharmacological inhibition of FAS is selectively cytotoxic for tumor cells, triggering their programmed cell death (Pizer, E. S., Jackisch, C., Wood, F. D., Pasternack, G. R., Davidson, N. E., and Kuhajda, F. P., "Inhibition of Fatty Acid Synthesis Induces Programmed Cell Death in Human Breast Cancer Cells," *Cancer Research, 56*:2745-7, (1996b); Pizer, E. S., Wood, F. D., Heine, H. S., Romantsev, F. E., Pasternack, G. R.,

Cerulenin, (2R, 3S)-2,3-epoxy-4-oxo-7,10-trans,trans-dodecadienamide, a natural product of *Cephalosporium caerulens*, is a specific inhibitor of fatty acid synthase enzymes across a broad phylogenetic spectrum (Omura, S., "The Antibiotic Cerulenin, a Novel Tool for Biochemistry as an Inhibitor of Fatty Acid Synthesis," *Bacteriological Reviews, 40*:681-697, (1976); Vance, D., Goldberg, I., Mitsuhashi, O., and Bloch, K., "Inhibition of Fatty Acid Synthetases by the Antibiotic Cerulenin," *Biochemical & Biophysical Research Communications, 48*:649-656, (1972); Moche, M., Schneider, G., Edwards, P., Dehesh, K., and Lindqvist, Y., "Structure of the Complex Between the Antibiotic Cerulenin and its Target, b-ketoacyl-acyl Carrier Protein Synthase," *J. Biol. Chem., 274*:6031-6034, (1999)). Cerulenin irreversibly inhibits FAS by binding covalently to the active site cysteine of the beta keto acyl synthase moiety, which performs the condensation reaction between the elongating fatty acid chain and each successive acetyl or malonyl residue. In *Saccharomyces cerevisiae*, a point mutation in FAS that confers a 30-fold reduction in affinity of the enzyme for cerulenin also abolishes the drug's growth inhibitory effects accordingly, demonstrating that FAS is a critical target for the drug's cytotoxic effects (Inokoshi, J., Tomoda, H., Hashimoto, H., Watanabe, A., Takeshima, H., and Omura, S., "Cerulenin Resistant Mutants of Saccharomyces cerevisiae with an Altered Fatty Acid Synthase Gene, *Mol Gen Genet.*, 244*:90-96, (1994)). A novel small-molecule inhibitor of FAS has recently
been synthesized. It is an α-methylene-γ-butyrolactone with a C7 hydrocarbon side chain, called C-75, with inhibitory effects on fatty acid synthesis comparable to those seen with cerulenin (Kuhajda, F. P., Pizer, E. S., Li, J. N., Mani, N. S., Frehywot, G. L., and Townsend, C. A., "Synthesis and AntiTumor Activity of a Novel Inhibitor of Fatty Acid Synthase," Proceedings of the National Academy of Sciences, 97:3450-3454, (2000)).

**SUMMARY OF THE INVENTION**

Inhibitors of the enzyme fatty acid synthase (I-FAS) can be used therapeutically to treat cancer cells that overexpress fatty acid synthase (see US Pat. 5,759,837 and 5,981,575). By administering I-FAS, apoptosis may be induced in malignant cells overexpressing FAS. It has now been discovered that I-FAS may affect cells beyond its ability to induce apoptosis.

In contrast to the previously described apoptosis-inducing therapy, the present invention provides a method of treating malignancies by arresting cell growth. It has now been discovered that the presence of I-FAS impedes progression of cells through the cell cycle. Such an effect is limited to cells undergoing cell division, and therefore inherently avoids toxic effects on mature cells. Treatment of malignancies with I-FAS is broadly applicable for retarding progression of all types of tumors, in addition to eradication of neoplastic cells with p53 mutations and/or FAS overexpression.

The present invention provides new methods for treating an individual having a tumor. In particular, the method comprises administering to the individual an inhibitor of fatty acid synthase (FAS) in an amount sufficient to retard growth of cells in the tumor. In one embodiment, the individual treated by the method of this invention has a tumor comprising cells which do not overexpress FAS and/or the individual has a tumor comprising cells which are resistant to induction of apoptosis by inhibitors of FAS. In a preferred mode, the tumor is malignant. In a particularly preferred mode, the
inhibitor of FAS is administered in an amount sufficient to induce a cellular response equivalent to a genotoxic stress response in the absence of substantial DNA damage.

**BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 shows DNA content of RKO cells analyzed by flow cytometry after various time periods of exposure to cerulenin (10 µg/mL).

Figure 2A shows bromodeoxyuridine (BrdU) pulse/chase analysis of pulse labeled RKO cells chased for various time periods in the absence of FAS inhibitors.

Figure 2B shows BrdU pulse/chase analysis of pulse labeled RKO cells chased for various time periods in the presence of cerulenin (10 µg/mL).

Figure 3 shows cyclin A- and cyclin B1-associated kinase activities which were determined by an immunocomplex-kinase assay after RKO cells were exposed to FAS inhibitors for the indicated time periods. FAS inhibition induces a marked reduction of S- and G2/M-associated cdk activity during the early period of exposure.

Figure 4. shows accumulation of p53 and p21 induced in RKO colon carcinoma cells by pharmacological inhibitors of FAS. Cells were treated with cerulenin (10 µg/ml) (A) or C-75 (10 µg/ml) (B) for the stated exposure times, and analyzed by immunoblotting for p53 and p21 protein content, with actin as an internal control.

Figure 5. shows cerulenin- or C-75-treated MCF7 breast carcinoma cells subjected to alkaline single cell gel electrophoresis (comet assay). Olive tail moment indicates electrophoretic mobility of DNA induced by DNA damage.
Figure 6 shows RKO cells without or with a stably-transfected dominant negative mutant p53 gene which were subjected to multi-parameter flow cytometry after 24 h of exposure to cerulenin. Ungated two-dimensional analysis of DNA content versus MC540 fluorescence is displayed after no drug (A and B), cerulenin (5 μg/ml) (C and D), and cerulenin (10 μg/ml) (E and F). Apoptotic and non-apoptotic cells are in upper and lower boxes, respectively.

Figure 7. shows constitutive fatty acid synthesis pathway activity of parental and p53 deficient lines are similar (A). Cerulenin, C-75 and TOFA inhibit fatty acid synthesis to 60% or less of control levels at the doses used [μg/ml] (B). Apoptotic fraction of colon and breast carcinoma cells after 24 h exposure to FAS inhibitors, analyzed as in Figure 6 (C and E). Parallel determinations of sensitivity to FAS inhibitors were performed by clonogenic assay after a 6-h drug exposure. (D and F). SW480 is a colon carcinoma line with a naturally-occurring p53 mutation. SKBr3 is a breast carcinoma line with a naturally-occurring p53 mutation.

Figure 8 shows DNA content of RKO cells exposed to [cerulenin,10μg/ml] or [C-75,10μg/ml] for the indicated times, without or with 1 hour pretreatment with TOFA (5μg/ml to inhibit malonyl-CoA synthesis). FAS inhibitors (cerulenin or C-75) induced growth arrest independent of malonyl-CoA accumulation.

Figure 9 shows a comparison of FAS enzyme levels in non-transformed human cell line, IMR-90, and a panel of tumor lines.
**DETAILED DESCRIPTION OF THE EMBODIMENTS**

In previous inventions, cancer cells with high levels of fatty acid synthase (FAS) and fatty acid synthesis were shown to undergo apoptosis when treated with inhibitors of (FAS) (U.S. Patent No. 5,759,837; U.S. Patent No. 5,981,575). The present invention demonstrates that cancer cells with low levels of fatty acid synthase (FAS) and fatty acid synthesis, and intact p53 signaling, undergo growth arrest when treated with inhibitors of FAS, whereas those with loss of p53 function, undergo rapid, extensive apoptosis. A summary of the effect of FAS inhibitors on cells with varying levels of FAS expression and p53 function is shown in the accompanying Table.

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<th>High FAS Expression</th>
<th>Low FAS Expression</th>
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<tr>
<td>intact p53</td>
<td>apoptosis</td>
<td>growth arrest</td>
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<td>reduced p53 function</td>
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This invention provides a rationale to treat patients with inhibitors of FAS regardless of the rate of fatty acid synthesis or level of FAS expression, and it shows that FAS inhibitor therapy may be effective against the most virulent and treatment resistant human cancers that characteristically have reduced or absent p53 function.

This invention describes a novel, anti-tumor effect of FAS inhibitors in human cancer, namely growth inhibition. As disclosed herein, FAS inhibitors have anti-tumor effect regardless of the level of FAS expression or rate of fatty acid synthesis. Furthermore, this invention links p53 function to fatty acid synthesis perturbation in cancer cells; cancer cells with dysfunctional p53 signaling undergo
apoptosis when treated with FAS inhibitors. It is also disclosed that growth inhibition
induced by FAS inhibition is not dependent upon malonyl-CoA accumulation, but
rather from lipid product depletion.

As disclosed herein, FAS inhibition has an anti-tumor activity in human
cancer cells regardless of the level of FAS expression or fatty acid synthesis activity.
All human tumors may respond to FAS inhibitor therapy. This increases the scope of
FAS inhibitor therapy from patients whose tumors have high levels of fatty acid
synthesis to all patients. The subset of human tumors with high levels of fatty acid
synthesis and/or loss of p53 function will have a cytotoxic, apoptotic response to FAS
inhibition. The subset of human tumors with low levels of fatty acid synthesis and
intact p53 function will have a cytostatic response to FAS inhibition.

Cells having low levels of fatty acid synthesis can be identified by
immunoblotting using an antibody specific for FAS to develop the blot. Such
antibodies are disclosed in U.S. Patent No. 5,872,217, incorporated herein by reference.
Typically, such cells have FAS levels equal to or lower than the level of FAS detected
by immunoblot in RKO cells (see Example 9). Alternatively, FAS levels may be
characterized by comparison to IMR-90 cells, which express FAS at a level about four-
fold lower than RKO cells. IMR-90 cells may be obtained from the American Type
Culture Collection, Manassas, Virginia, USA, where they have been deposited under
ATCC Accession No. ________________.

An FAS inhibitor (I-FAS) is a compound that specifically interferes with
the enzymatic activity of fatty acid synthase (FAS). The inhibition may be determined
by carrying out FAS assays in the presence and absence of the compound suspected to
be an inhibitor. Suitable assays are described in the Examples, although the skilled
artisan could readily devise alternative assays. FAS inhibitors according to this
invention are specific in that they do not indiscriminately directly inhibit the activities
of other enzymes, although cross-inhibition of a few related enzymes is not outside the
contemplation of this invention. The skilled artisan will recognize that pleiotropic effects of I-FAS on down-stream or ancillary pathways is to be expected. Exemplary compounds having the characteristics of I-FAS according to this invention include the antibiotic cerulenin and the novel compound C-75, as well as other compounds disclosed in U.S. Patent Nos. 5,759,837 and 5,981,575, incorporated herein by reference. The skilled artisan can readily determine whether a particular compound is an I-FAS.

Cells which over-express FAS are described in U.S. Patent No. 5,759,837, incorporated herein by reference. FAS is normally expressed in the liver and in adipose tissue, where it functions to convert dietary carbohydrate to fat, and in some specialized contexts, like lactating breast and the surfactant producing cells of the lung, but has little expression in most other normal adult tissues which predominantly utilize circulating sources of fatty acids. Detection of FAS expression in tissues that normally do not express it, by detecting mRNA encoding FAS or by detecting fatty acid synthesis in the cell (as described below in the Examples), is an indication that the cells expressing FAS may not be completely normal.

Genotoxic type stress response is a set of cellular events which mimic events that occur in cells containing damaged DNA. It is well established that DNA damage (for example, due to radiation) leads to growth arrest and accumulation of cells in G1 and G2/M. The genotoxic type stress response disclosed herein produces these cellular manifestations in cells without sufficient DNA damage to trigger the response.

Individuals that may be treated by the methods of this invention include animals, particularly mammals, more particularly humans. Typically, these individuals will be tumor bearing, and the tumors may be malignant or benign. While treatment of tumors with I-FAS was taught for tumors containing cells that overexpress FAS in U.S. Patent Nos. 5,759,837 and 5,981,575, the present invention is generally concerned with individuals bearing tumors with cells that do not overexpress FAS.
administration of I-FAS to such individuals will be analogous to that described in the cited patents.

In order to gain further insight into the biological role of the fatty acid synthetic pathway for tumor cells, and the nature of the growth inhibition resulting from inhibition of FAS, the present inventors examined the cellular events that follow inhibition of FAS and precede cell death. Two chemically distinct inhibitors of FAS were studied in parallel to provide a generic picture of the consequences of loss of FAS function. FAS inhibitors produce rapid, profound blocks of DNA replication and S-phase progression in human cancer cells (Pizer, E. S., Chrest, F. J., DiGiuseppe, J. A., and Han, W. F., "Pharmacological Inhibitors of Mammalian Fatty Acid Synthase Suppress DNA Replication and Induce Apoptosis in Tumor Cell Lines," Cancer Research, 58:4611-4615, (1998b)). Fatty acid synthesis inhibition occurred within 30 min and DNA synthesis inhibition occurred within 90 min of drug exposure, and induction of apoptosis followed several hours later. The suppressive effect of fatty acid synthesis inhibition on DNA replication was indirect, because expression of certain viral oncogenes alleviated it. The inventors further characterized the cellular response to FAS inhibition.

RKO colon carcinoma cells were selected for study because they undergo little apoptosis within the first 24 h after FAS inhibition. Instead, RKO cells exhibited a bi-phasic stress response, with a transient accumulation in S and G2 at 4 and 8 h that corresponds to a marked reduction in cyclin A- and B1-associated kinase activities, followed by accumulation of p53 and p21 proteins at 16 and 24 h, and growth arrest in G1 and G2. RKO cells stress response was marked by early loss of S phase and G2 cyclin-dependent kinase activity, and subsequent accumulation of p53 and p21 proteins may protect RKO cells from the cytotoxic effects of FAS inhibition. The delays in cell cycle progression with redistribution of cells into G1 and G2 after FAS inhibition were suggestive of cell cycle checkpoint activation by the tumor suppressor p53, as occurs after genotoxic or other cellular stresses (Meek, D. W., "Post-
Translational Modification of p53 and the Integration of Stress Signals," *Pathol Biol.*, 45:804-814, (1997)). While the response of RKO cells to FAS inhibition resembled a genotoxic stress response, but DNA damage did not appear to be an important downstream effect of FAS inhibition, since none was detected using the single cell gel electrophoresis assay (comet assay) to assess DNA damage.


Inhibition of FAS induced p53 and p21 protein accumulation and G1/G2 redistribution in RKO cells, which have an intact p53 pathway (and in other cell lines with wild type p53, not shown). However, many tumor lines with p53 mutations undergo apoptosis within 24 h of exposure to FAS inhibitors (Pizer et al., 1998b). The inventors determined the effect of p53 function on survival after FAS inhibition by comparing two pairs of isogenic cell lines with wild-type and altered p53 function. P53 function is probably important in protecting RKO cells from FAS inhibition, because RKO cells expressing a dominant negative mutant p53 gene underwent extensive apoptosis within 24 h after FAS inhibition, similar to many other tumor lines. Loss of p53 function substantially increased the sensitivity of tumor cells to FAS inhibitors. Sensitization of cells to FAS inhibitors by loss of p53 raises the possibility that these agents may be clinically useful against malignancies carrying p53 mutations.

Accumulation of malonyl-CoA, the committed substrate for fatty acid synthesis, is likely to participate in the cytotoxicity of FAS inhibition, since down
regulation of malonyl-CoA production alleviated the toxicity of cerulenin and C-75, and substantially reduced the apoptotic fraction at 24 hours (Pizer et al, 2000). However, while induction of apoptosis appeared related to accumulation of the substrate, malonyl-CoA, after FAS inhibition, the cytostatic effects were independent of malonyl-CoA accumulation, and may have resulted from product depletion.

**Growth arrest due to lipid product depletion.** Although not wishing to be bound by any particular mechanism, the inventors note that the bi-phasic stress response to FAS inhibition may result from lipid product depletion. The kinetics of the response of RKO cells to FAS inhibition illustrated in Examples 1 through 4 below suggests a rapid onset of a stress response. This response is characterized by a marked reduction in cyclin A- and B-associated kinase activities, an early suppression of DNA replication and an accumulation of cells in the S and G2 phases during the first 8 h of drug exposure, followed by enhanced expression of p53 and p21 proteins and growth arrest in G1 and G2 by 16 and 24 h.

While malonyl-CoA accumulation appears involved in triggering apoptosis after FAS inhibition, the growth arrest stress response produced by FAS inhibition may be due to altered lipid production, since ACC inhibition did not relieve it. Most of the fatty acids produced by tumor cells are incorporated into membrane phospholipids, and phospholipid synthesis is inhibited when fatty acid synthesis is inhibited (Pizer et al., 1996a; Jackowski et al., 2000). Phospholipid biosynthesis is greatest during the G1 and S phases, with doubling of the membrane mass occurring during S phase to prepare for cell division (Jackowski, S., Coordination of Membrane Phospholipid Synthesis with the Cell Cycle," *Journal of Biological Chemistry*, **269:**3858-3867, (1994)). It is possible, therefore, that limitation of phospholipid synthesis during the S phase affects DNA replication, or independently triggers late cell cycle delays similar to the pre-mitotic checkpoints of yeast (Thuriaux, P., Nurse, P., and Carter, B., "Mutants Altered in the Control Co-Ordinating Cell Division With Cell Growth in the Fission Yeast Schizosaccharomyces pombe," *Mol Gen Genet.*, **161:**215-

Notably, two ether lipids that specifically inhibit the CTP:phosphocholine cytidylyltransferase, an important enzyme in phospholipid synthesis, produce similar G2/M delays and are selectively cytotoxic to transformed cells (Boggs, K., Rock, C. O., and Jackowski, S., "The Antiproliferative Effect of Hexadecylphosphocholine Toward HL60 Cells is Prevented by Exogenous Lysophosphatidylcholine," *Biochimica et Biophysica Acta*, **1389**:1-12, (1998)). Studies in lower eukaryotes and prokaryotes have shown a requirement for active fatty acid synthesis at the time of cell division, either for simple mitosis, or for sporulation (Saitoh, S., Takahashi, K., Nabeshima, K., Yamashita, Y., Nakaseko, Y., Hirata, A., and Yanagida, M., "Aberrant Mitosis in Fission Yeast Mutants Defective in Fatty Acid Synthetase and Acetyl CoA Carboxylase," *Journal of Cell Biology*, **134**:949-961, (1996)). The defects in these systems appear related to chromatin configuration, or to transcriptional activation of key genes. In the FAS inhibition system discussed here, however, the specific mechanisms whereby cyclin A- and B-associated kinase activities decrease in RKO cells remain to be studied in detail.

**Role of tumor suppressor p53 in the response to FAS inhibitors.** The observation that FAS inhibitors induced the accumulation of p53 and p21 proteins might suggest that DNA damage is occurring, either as a direct effect of the drugs on the DNA molecule, or as a downstream effect of FAS inhibition. However, several other observations argue against DNA damage. First, the toxic effect of cerulenin was found to be dependent on its ability to inhibit FAS in yeast, thus ruling out a significant direct effect of cerulenin on DNA (Inokoshi et al., 1994). Secondly, toxicity in tumor cells is modulated by alterations in activity of the fatty acid synthesis pathway and substrate levels. Finally, no DNA damage was detected using the single cell gel electrophoresis (comet) screening assay, an assay that has been shown to be very
sensitive in detecting low levels of DNA damage. Consistent with these observations, no differences were detected in the sensitivity to FAS inhibitors of cells deficient in ATM (mutated in ataxia telangetasias) versus controls.


Whether the effects of FAS inhibition are observed as apoptosis or growth arrest clearly is influenced by p53 function. Since constitutive fatty acid synthesis activity, and inhibitor effects were similar between the paired parental and p53 deficient cells, it is unlikely that levels of malonyl-CoA accumulation were substantially different, however, the ability of the cell to survive malonyl-CoA accumulation may be greater in cells with intact p53. The relatively low fatty acid synthesis pathway activity of RKO cells (less malonyl-CoA) combined with intact p53 function may underlie the minimal apoptosis produced by FAS inhibitors in RKO cells, and in various non-transformed cells. It is likely that induction of p21 promotes growth arrest and exerts a protective effect after FAS inhibition, as it has been shown to do in a variety of other stress paradigms (Gorospe M, W. X., Holbrook NJ, "Functional Role of p21 During the Cellular Response to Stress," *GeneExpression*, 7:377-385, (1999)). The triggering of apoptosis after FAS inhibition is very rapid, and probably occurs before p21 induction. FAS inhibitors triggered comparable apoptotic responses in the majority of tumor lines with mutant p53 status that have been studied. The predominant pattern of sensitization by loss of p53 function suggests that endogenous fatty acid synthesis will hold special appeal as an experimental therapeutic target. FAS inhibitors combine the target specificity for cancer cells afforded by both elevated fatty acid synthesis and loss of p53 function.
EXAMPLES

Example 1. FAS inhibitors induce delays in cell cycle progression as shown by DNA content of treated cells.

Investigation by flow cytometric analysis of serial samples taken after FAS inhibition demonstrated a bi-phasic effect on the cell cycle progression of RKO colon carcinoma cells. Cells were cultured in DMEM with 10% fetal bovine serum (HyClone). Cells were screened periodically for mycoplasma contamination (Genprobe). Cerulenin (Sigma) C-75 and TOFA, dissolved in DMSO, were added from 5 mg/ml stock solutions; the final concentration of DMSO in cultures was at or below 0.2%. Cells were exposed to cerulenin or C-75 for the indicated doses and time intervals, then detached from plastic with trypsin for flow cytometry analysis. DNA content was measured by multiparameter flow cytometry using a FACStarPlus flow cytometer equipped with argon and krypton lasers (Becton Dickinson).

When proliferating cells were exposed to 10 μg/ml cerulenin, there was a redistribution of cells into S phase and G2/M during the early time points, at 5 and 8 h, compatible with inhibited progression through these cell cycle phases (Figure 1). Later, at 16 and 24 h, the S-phase fraction decreased substantially, with a redistribution of cells into G1 and G2/M. This effect was characteristic of both cerulenin and C-75 treatment on RKO cells, as well as on other cell lines that had limited apoptotic responses to FAS inhibitors (not shown).

Example 2. Delays induced in Cell Cycle Progression by FAS Inhibitors.

A similar experiment measured cell cycle progression by pulse/chase labeling with bromodeoxyuridine (BrdU, Figure 2). The BrdU-positive S-phase fraction at time zero progresses through the cell cycle at later time points. The progress of BrdU pulse-labeled RKO cells through the cell cycle was monitored over 24
h without inhibition of FAS, or during exposure to cerulenin (10 μg/ml) or C-75 (10 μg/ml).

**Bromodeoxyuridine Detection by Laser Scanning Cytometry:** Dual-parameter detection of Bromodeoxyuridine (BrdU) labeling and DNA content was performed using a laser scanning cytometer (Compucyte Corp.). Cell cultures were pulse-labeled for 20 min with 10 μM BrdU, and chased for the indicated times in the absence or presence of drug, then detached from plastic with trypsin, ethanol-fixed and applied to glass slides. Cells were subjected to standard heat-induced epitope retrieval (DAKO) before staining with anti-BrdU antibody (DAKO) and FITC-conjugated goat anti-mouse antibody (CALTAG, DAKO Autostainer™). DNA content was assessed after staining with 0.5% propidium iodide. Data were collected and analyzed using WinCyte software (Compucyte Corp.).

A 20-min exposure of proliferating cells to BrdU labeled the S-phase population at time zero. Chase samples were collected at 4, 8, 16 and 24 h. In control cultures (Figure 2A), the BrdU-labeled population progressed through G2/M, first reappearing in the G1 population in the 8 h chase sample. By 16 h the BrdU-labeled population was in G1 and S phase again, indicating a complete cell cycle traverse time of approximately 16 h for RKO cells. By 24 h labeled and unlabeled populations were distributed throughout the cell cycle, indicating continued progression and loss of synchronization (not shown).

RKO cells treated with FAS inhibitors demonstrated substantial delays in cell cycle progression that corresponded with the flow cytometry single-parameter cell cycle results (Figure 1). The cerulenin-treated samples are shown in Figure 2B; C-75-treated populations exhibited a similar response (not shown). The treated 8-h chase sample showed no BrdU-labeled cells yet reappearing in G1, in agreement with our observation that cells redistribute into the S and G2/M phases seen in Figure 1. By 16 h, most of the BrdU-labeled cells had reentered G1, but very few had entered S phase,
and by 24 h most cells, labeled and unlabeled, were in G1 or G2/M, and were still synchronized, indicating that cell cycle progression had slowed down substantially.

Example 3. FAS inhibition induces a marked reduction of S- and G2/M-associated cdk activity during the early period of exposure.

The effect of FAS inhibitors on the activity of cyclin/cdk complexes in RKO cells was determined in a time course analysis. After RKO cells were exposed to FAS inhibitors for the indicated time periods, cyclin A- and cyclin B1-associated kinase activities were determined by an immunocomplex-kinase assay.

Immunoprecipitation and Immunocomplex-Kinase Assay: Five x $10^6$ RKO cells per 100-mm plate were treated with 10 μg/ml cerulenin or C-75 for the indicated time intervals. The control cells received equivalent amounts of DMSO. After drug treatment, the plates were washed once and lysed with immunoprecipitation (IP) buffer (150 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM ethyleneglyco-bis-tetraacetic acid, 0.2 mM sodium vanadate, and 0.2 mM phenylmethylsulfonyl fluoride). Protein concentration was measured using the BCA Protein Assay Kit (Pierce). One hundred μg of protein from each sample was incubated at 4°C for 1 h with 1 μg of primary antibody (anti-human cyclin A rabbit polyclonal antibody or anti-human cyclin B1 monoclonal antibody, Santa Cruz) and then overnight after addition of Protein A or protein G-Sepharose (Santa Cruz). The immunoprecipitates were washed twice with IP buffer and once with kinase buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl$_2$ and 0.5 mM DTT) and resuspended in 40 μl of kinase buffer containing 1 μg of histone H1, 25 μM of ATP, and 2.5 μCi of γ-$^{32}$P-ATP. Following a 30-min incubation at 30°C, the reaction was terminated by adding 40 μl of 2x Laemmli sample buffer. Samples were resolved by electrophoresis through 12% SDS-polyacrylamide gels and quantitated on a Storm 820 system (Molecular Dynamics). All samples were run in duplicate, and each experiment was performed at least twice. Means and standard errors of one representative experiment are shown in Figure 3B.
As shown (Figure 3), the kinase activity associated with immunoprecipitated complexes containing cyclin A decreased to less than 40% of control levels at 4 and 8 h after exposure to either cerulenin or C-75, then increased moderately at later time points. The kinase activity associated with immunoprecipitated cyclin B decreased to less than 5% of control levels by 4 and 8 h after exposure to either cerulenin or C-75, then increased to greater than 80% of control levels at 16 and 24 h. These changes in S and G2 cdk activity correlated well with the bi-phasic pattern of cell cycle distribution demonstrated in Figures 1 and 2. Immunoblots of cyclin A and B levels performed in parallel with the experiment in Figure 3, demonstrate that unlike the associated kinase activities, the cyclin levels do not decrease until 24 h (not shown).

Example 4. Accumulation of p53 and p21 is induced in RKO colon carcinoma cells by pharmacological inhibitors of FAS.

Accumulation of p53 protein, and the p53-regulated cdk inhibitor p21WAF1/CIP1, were assayed by immunoblotting in a parallel time course after inhibition of FAS (Figure 4). Cells were treated with cerulenin (10 μg/ml) (A) or C-75 (10 μg/ml) (B) for the stated exposure times, and analyzed by immunoblotting for p53 and p21 protein content, with actin as an internal control. One million cells per 60-mm plate were treated with 10 μg/ml cerulenin or C-75 in duplicate for the indicated time intervals; control cells received equivalent amounts of DMSO. After drug treatment, cells were lysed with 200 μl Laemmli sample buffer and boiled. Ten μl of each lysate per lane was separated by SDS-PAGE, transferred to nitrocellulose, and exposed to antibodies against p53 (Pab1801, Oncogene Research Products), p21 (6B6, PharMingen) or actin (I-19, Santa Cruz), followed by horseradish peroxidase-conjugated goat anti-mouse or rabbit anti-goat antibody (Pierce), enhanced chemiluminescence (Amersham) and autoradiography.

p53 and p21 protein levels were unchanged or decreased during the early period of FAS inhibitor exposure. However, treatment with 10 μg/ml of either
cerulentin or C-75 induced accumulation of p53 and p21 protein at 16 and 24 h in RKO cells (Figure 4). Of note, p21 mRNA levels did not show increases of the same magnitude, suggesting translational and/or post-translational mechanism(s) regulating p21 accumulation (not shown).

5 Example 5. FAS inhibitors do not induce DNA damage.

To determine whether significant DNA damage occurred after FAS inhibitor exposure, alkaline single cell gel electrophoresis (comet assay) was performed on MCF7 breast cancer cells after exposure to concentrations of cerulentin and C-75 which resulted in 75% survival (Figure 5). This assay detects DNA strand breaks, and a spectrum of alkali-labile DNA damage at low levels (Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L., "A simple Technique for Quantitation of Low Levels of DNA Damage in Individual Cells," Experimental Cell Research, 175:184-191, (1988); Plappert, U., Raddatz, K., Roth, S., and Fiedler, T. M., "DNA-Damage Detection in Man After Radiation Exposure--the Comet Assay--Its Possible Application for Human Biomonitoring," Stem Cells, 13 Supplement 1:215-222, (1995)).

Cerulenin- or C-75-treated MCF7 breast carcinoma cells were subjected to alkaline single cell gel electrophoresis (comet assay). MCF7 breast cancer cells were treated with cerulentin or C-75 for 3 h at doses bracketing 75% survival at 24 h. All experiments were repeated three times and duplicate slides from each experiment were prepared and scored. The comet assay was performed under alkaline conditions, essentially as described (Singh et al., 1988), with some modifications. In brief, cells were suspended in 0.5% low melting point agarose (LMA) (Trevigen) and spread on glass microscope slides precoated with 1% normal agarose. After immersion in lysis solution (Trevigen) at 4°C for a minimum period of 1 h to remove cellular proteins, the slides were immersed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH>13) for unwinding DNA, and subjected to electrophoresis (25 V, 300 mA) for 20 min. Neutralized, dehydrated slides were stained with ethidium bromide (2 ng/ml) and
comets scored under a Nikon fluorescence microscope (with TRITC filters) coupled to a KOMET 4.0 software (Kinetic Imaging Ltd).

Olive tail moment indicates electrophoretic mobility of DNA induced by DNA damage. The comet parameters, ‘Olive Tail Moment’ (OTM), ‘Tail Length’ (DNA migration) and ‘percentage DNA in the tail’ were used as indicators of DNA damage. One hundred consecutive cells were scored from the middle of each slide, and the means calculated. The final results were expressed as the (mean of the individual means) ± (standard deviation of the means). Lymphoblasts exposed to 0 or 1 Gy gamma irradiation had olive tail moments of 0.9 ± 0.3 and 7.1 ± 0.8 in this experiment. Exposure to 5 cGy gamma irradiation typically produces an olive tail moment of twice the control.

Neither cerulenin nor C-75 induced olive tail moments over background values for untreated control cells, indicating that DNA damage was not induced by either agent at doses previously shown to induce inhibition of DNA synthesis and reduce clonogenic activity (Pizer et al., 2000; Pizer et al., 1998b). This suggests that C-75 and cerulenin induced cytotoxic, not genotoxic damage to cells in an assay that under similar conditions readily detected DNA damage induced by 5 cGy of gamma irradiation or 25 μm H₂O₂. A similar absence of DNA damage was seen after drug treatment of GM1310B human lymphoblasts (not shown).

Example 6. Loss of p53 function substantially increased the sensitivity of tumor cells to FAS inhibitors.

The effect of p53 function on survival after FAS inhibition was investigated by comparing two pairs of isogenic cell lines with wild-type and altered p53 function. RKO cells were rendered p53-mutant by stable transfection with a dominant-negative mutant p53 gene (RKO-p53); the human breast carcinoma cell line MCF7 was rendered p53-deficient by constitutive expression of the human papilloma virus 16 E6 gene (MCF7-E6) (Fan, S., Smith, M. L., Rivet, D. J., 2nd, Duba, D., Zhan,

Fatty acid synthesis was compared in cells were plated at 5x10^4/well in 1 ml in 24 well plates and incubated overnight. Fatty acid synthesis was assayed with a 2 hour pulse of [U-14C]-acetic acid, 1μCi/ well, followed by Folch extraction and scintillation counting (Pizer et al., 1996a). The fatty acid synthetic pathway activity in these paired lines was very similar, so loss of p53 function had no discernible effect on fatty acid synthesis level (Figure 7A). For determination of residual pathway activity after FAS inhibitor exposure (Figure 7B) a 3 hour pulse of [U-14C]-acetic acid, 1μCi/ well, was performed after 2 hours of drug exposure. All determinations were in triplicate. Data are presented as mean values with bars showing the standard error. Calculations and graphing were performed in Prism 2.0 (GraphPad). Cerulenin, C-75 and TOFA inhibit fatty acid synthesis to 60% or less of control levels at the doses used ((Pizer et al., 2000; Pizer et al., 1998b), see also Figure 7B). FAS inhibitors produced comparable reduction of pathway activity in the paired lines (Figure 7B).

Cells were exposed to cerulenin or C-75 for the indicated doses and time intervals, then detached from plastic with trypsin for flow cytometry analysis. Apoptosis was measured by multiparameter flow cytometry using a FACStarPlus flow cytometer equipped with argon and krypton lasers (Becton Dickinson). Apoptosis was quantified using 10 μg/ml merocyanine 540 (Sigma), which detects altered plasma membrane phospholipid packing that occurs early in apoptosis (Pizer et al., 1998b; Reid, S., Cross, R., and Snow, E. C., "Combined Hoechst 33342 and merocyanine 540 Staining to Examine Murine B Cell Cycle Stage, Viability and Apoptosis," Journal of Immunological Methods, 192:43-54, (1996); Mower, D. A., Jr., Peckham, D. W., Illera, V. A., Fishbaugh, J. K., Stunz, L. L., and Ashman, R. F., "Decreased Membrane Phospholipid Packing and Decreased Cell Size Precede DNA Cleavage in Mature Mouse B Cell Apoptosis," Journal of Immunology, 152:4832-4842, (1994); Castedo,
M., Hirsch, T., Susin, S. A., Zamzami, N., Marchetti, P., Macho, A., and Kroemer, G., Sequential Acquisition of Mitochondrial and Plasma Membrane Alterations During Early Lymphocyte Apoptosis," Journal of Immunology, 157:512-521, (1996)). Merocyanine 540-positive cells were identified using 488-nm excitation from an argon laser and a 575-nm DF26 bandpass filter for collection of events with increased red fluorescence. Data were collected and analyzed using CellQuest software (Becton Dickinson). Figures show representative results of at least two independently performed experiments.

RKO cells without or with a stably-transfected dominant negative mutant p53 gene were subjected to multi-parameter flow cytometry after 24 h of exposure to cerulenin. Ungated two-dimensional analysis of DNA content versus MC540 fluorescence is displayed in Figure 6 after no drug (A and B), cerulenin (5 μg/ml) (C and D), and cerulenin (10 μg/ml) (E and F). Apoptotic and non-apoptotic cells are in upper and lower boxes, respectively.

Loss of p53 function sensitized RKO and MCF7 cells to the cytotoxic effect of FAS inhibition. There was a large, dose-dependent increase in apoptosis after cerulenin exposure in RKO-p53 cells compared to the parent RKO line (Figure 6). The cell cycle distribution of the non-apoptotic (lower boxes) and apoptotic (upper boxes) sub-populations of RKO cells after 24 h of exposure to 5 or 10 μg/ml cerulenin was determined by multi-parameter flow cytometry. Cell cycle position (DNA content) was determined with HO33342 dye, and apoptosis was detected by bright staining with merocyanine 540 (MC540), which detects conformational changes in the plasma membrane that occur early during apoptosis (Reid et al., 1996; Mower et al., 1994; Castedo et al., 1996). The validity of MC540 staining as a measure of entry into apoptosis has been confirmed in our experimental system by evaluation of morphology, change in light scatter parameters and "TUNEL" DNA end-labeling in parallel experiments ([Pizer et al., 2000; Pizer et al., 1998b) and data not shown]. Entry into apoptosis after FAS inhibition by cerulenin occurred from G1, S and G2/M without
increased sensitivity in any subpopulation. Apoptosis with lack of cell cycle phase specificity was typical of many experiments with several cell lines (not shown).

Example 7. Loss of p53 function sensitizes colon and breast carcinoma cells to FAS inhibitor cytotoxicity.

A similar apoptotic response was seen with 3 independent RKO-p53 clones and with MCF7-E6, and was seen after exposure to C-75 (Figures 7C and E and data not shown). Apoptotic fraction of colon and breast carcinoma cells after 24 h exposure to FAS inhibitors, analyzed as in Example 6 (Figure 6 C and E).

The cytotoxic effects of the FAS inhibitors on these paired lines were also tested by clonogenic assay, as well as SW480, a colon carcinoma line with a naturally-occurring p53 mutation, and SKBr3 is a breast carcinoma line with a naturally-occurring p53 mutation (see Fig. 7 D and F). Parallel determinations of sensitivity to FAS inhibitors were performed by clonogenic assay after a 6-h drug exposure. Subconfluent cells were exposed to the indicated drug concentrations for 6 h, then were detached from plastic with trypsin, counted and replated for colony formation. Clones were fixed, stained with crystal violet [0.1%] (Sigma) and counted one week later. Data are presented as mean values with bars showing the standard error. Calculations and graphing were performed in Prism 2.0 (GraphPad).

Comparison of the two cytotoxicity assays shows that inhibition of FAS causes a reduction in the number of clonable RKO and MCF7 cells that is not detected by the apoptosis assay. The clonogenic assay probably detects subpopulations undergoing growth arrest and potentially other growth inhibitory processes in addition to those undergoing rapid apoptosis. However, it appears that the early apoptosis associated with loss of p53 function illustrated in Example 6 further reduces the clonable fraction, resulting in sensitivity to FAS inhibitors that is comparable to that seen with other lines bearing naturally-occurring p53 mutations (SW480 colon carcinoma and SKBr3 breast carcinoma cells).
Example 8. **FAS inhibitor induced growth arrest is independent of malonyl-CoA accumulation.**

In order to determine the role of malonyl-CoA accumulation in delaying cell cycle progression, RKO cells were analyzed by flow cytometry after 8 or 24 hours of FAS inhibitor exposure, without or with pretreatment for 1 hour with the acetyl-CoA carboxylase (ACC) inhibitor, 5-(tetradecyloxy)-2-furoic acid (TOFA), which blocks the carboxylation of acetyl-CoA to form malonyl-CoA (Figure 8). RKO cells were exposed to [cerulenin,10μg/ml] or [C-75,10μg/ml] for the indicated times, without or with 1 hour [TOFA, 5μg/ml] pretreatment to inhibit malonyl-CoA synthesis. DNA content was determined as described in Example 1. Determination of the percentages of cells in G1, S and G2/M was done with Multicycle software.

Cells pretreated with TOFA, followed by cerulenin or C-75, showed similar or greater cell cycle delays to cells exposed only to the FAS inhibitors. Of note, however, TOFA pretreatment did rescue FAS inhibitor mediated apoptosis in RKO-p53 cells, similar to earlier results (Pizer et al., 2000), indicating that the effects of FAS inhibitors on cell cycle progression are distinct from those mediating apoptotic cell death.

Example 9. **Comparison of FAS enzyme levels.**

The level of FAS enzyme was measured in non-transformed human cell line, IMR-90, and a panel of tumor lines. FAS enzyme levels in immortalized, non-transformed control cells, IMR-90 (fetal lung), and for tumor lines; HCT116, RKO (colon), SKBr3, ZR75-1 and MCF-7 (breast) were quantitated by immunoblot. The levels of enzyme were adjusted to total cellular protein, and the values obtained were normalized to IMR-90. As shown in Figure 9, the breast cancer cell lines tested in this comparison have at least eight-fold more FAS than IMR-90, while the colon cancer lines showed 3-5-fold greater FAS.
Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims. Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in medicine, immunology, hybridoma technology, pharmacology, and/or related fields are intended to be within the scope of the following claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All such publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.
CLAIMS

1. A method for treating an individual having a tumor, said method comprising administering to the individual an inhibitor of fatty acid synthase (FAS) in an amount sufficient to retard growth of cells in the tumor.

2. The method of claim 1, wherein the individual has a tumor comprising cells which do not overexpress FAS.

3. The method of claim 1, wherein the individual has a tumor comprising cells which are resistant to induction of apoptosis by inhibitors of FAS.

4. The method of claim 1-3 wherein the tumor is malignant.

5. The method of claim 1, wherein the inhibitor of FAS is administered in an amount sufficient to induce a cellular response equivalent to a genotoxic stress response in the absence of substantial DNA damage.

6. The method of claim 1, wherein cells in the tumor express FAS at a level equal to or less than four-fold higher than IMR-90 cells.

7. Use of a FAS inhibitor in the preparation of a medicament for treating a tumor in an individual whose tumor exhibits reduced p53 function.
FIGURE 1
FIGURE 2
FIGURE 3
FIGURE 5
FIGURE 6
FIGURE 7
FIGURE 8
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**Colon**  **Breast**

**FIGURE 9**
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

| IPC | A61K45/00 |

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

| Minimum documentation searched (classification system followed by classification symbols) |
| IPC 7 A61K |

| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched |

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

| EPO-Internal, BIOSIS, WPI Data |

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>US 5 759 837 A (KUHAJDA FRANCIS P ET AL) 2 June 1998 (1998-06-02) claims 1,6,8,21</td>
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<td>WO 94 02108 A (UNIV JOHNS HOPKINS) 3 February 1994 (1994-02-03) claims 1,3,33,48</td>
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<td>US 5 539 132 A (ROYER GARFIELD P ET AL) 23 July 1996 (1996-07-23) column 5, line 66 - column 6, line 10 column 6, line 29 - line 40</td>
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Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:

  **"A"** document defining the general state of the art which is not considered to be of particular relevance

  **"E"** earlier document but published on or after the international filing date

  **"L"** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

  **"O"** document referring to an oral disclosure, use, exhibition or other means

  **"P"** document published prior to the international filing date but later than the priority date claimed

  **"T"** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

  **"X"** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

  **"Y"** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

  **"R"** document member of the same patent family

**Date of the actual completion of the international search**

28 June 2002

**Date of mailing of the international search report**

12/07/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016

Authorized officer

Beranová, P
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<td>PIZER E S ET AL: &quot;INHIBITION OF FATTY ACID SYNTHESIS DELAYS DISEASE PROGRESSION IN A XENOGRAFT MODEL OF OVARIAN CANCER&quot; CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 56, no. 6, 15 March 1996 (1996-03-15), pages 1189-1193, XP001026211 ISSN: 0008-5472 page 1190, left-hand column (Results and Discussion) page 1190, right-hand column (first paragraph) page 1193, left-hand column (second paragraph)</td>
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### Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **X** Claims Nos.: 
   - because they relate to subject matter not required to be searched by this Authority, namely:
     - Although claims 1 – 6 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.

2. **X** Claims Nos.: 
   - 1 – 7
   - because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
     - see FURTHER INFORMATION sheet PCT/ISA/210

3. **☐** Claims Nos.: 
   - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. **☐** As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. **☐** As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. **☐** As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. **☐** No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- **☐** The additional search fees were accompanied by the applicant's protest.
- **☐** No protest accompanied the payment of additional search fees.
Continuation of Box I.2

Claims Nos.: 1 - 7

Present claims 1 - 7 relate to an extremely large number of possible compounds/methods ("inhibitor of fatty acid synthase"). Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds/methods claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the use of cerulenin or C-75 for the treatment of tumors/cancer.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.
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