

Award Number:
W81XWH-06-1-0494

TITLE:
Malonyl-CoA Decarboxylase (MCD) as a Potential Therapeutic Target
for Breast Cancer

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REPORT DATE:
May 2010

TYPE OF REPORT:
Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

☒ Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE

May 1, 2010

3. REPORT TYPE AND DATES COVERED

Final 10 APR 2006 - 9 APR 2010

4. TITLE AND SUBTITLE

Malonyl-CoA Decarboxylase (MCD) as a Potential
Therapeutic Target for Breast Cancer

5. FUNDING NUMBERS

W81XWH-06-1-0494

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8. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

Abstract: Fatty acid synthesis (FAS) inhibition initiates selective apoptosis of cancer cells both *in vivo* and *in vitro*, which may involve malonyl-CoA metabolism. These findings led to exploration of malonyl-CoA decarboxylase (MCD) as a potential novel target for cancer treatment. MCD regulates the levels of cellular malonyl-CoA through the decarboxylation of malonyl-CoA to acetyl-CoA. Malonyl-CoA is both a substrate for FAS and an inhibitor of fatty acid oxidation acting as a metabolic switch between anabolic fatty acid synthesis and catabolic fatty acid oxidation. We now report that treatment of human breast cancer (MCF7) cells with MCD small interference RNA (siRNA) reduces MCD expression and activity, reduces ATP levels, and is cytotoxic to MCF7 cells, but not to human fibroblasts. In addition, we synthesized a small molecule inhibitor of MCD, 5-[(Morpholine-4-carbonyl)-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-amino]-pentanoic acid methyl ester (MPA). Similar to MCD siRNA, MPA inhibits MCD activity in MCF7 cells, increases cellular malonyl-CoA levels and is cytotoxic to a number of human breast cancer cell lines *in vitro*. Taken together, these data indicate that MCD-induced cytotoxicity is likely mediated through malonyl-CoA metabolism. These findings support the hypothesis that MCD is a potential therapeutic target for cancer therapy.

14. SUBJECT TERMS

Malonyl-CoA Decarboxylase, malonyl-CoA, siRNA

15. NUMBER OF PAGES

19

16. PRICE CODE

2

17. SECURITY CLASSIFICATION
OF REPORT

Unclassified

18. SECURITY CLASSIFICATION
OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION
OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

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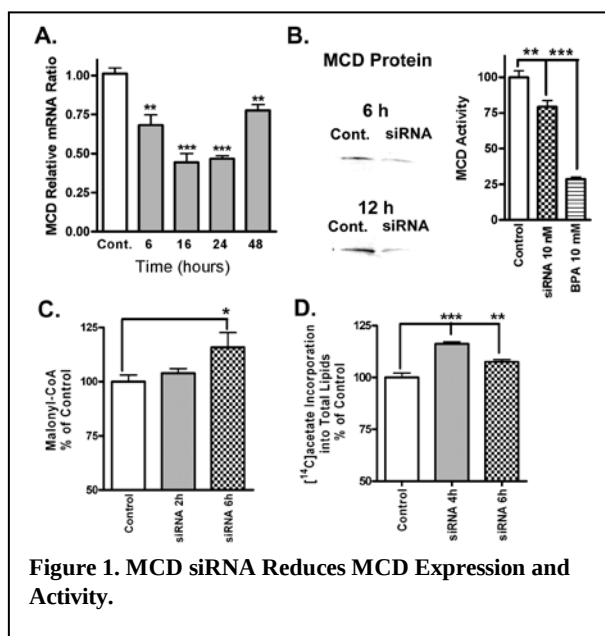
INTRODUCTION

Our studies of cancer cell metabolism led to the observation that transformed cells exhibit high levels of fatty acid synthase (FAS) expression and fatty acid synthesis (Kuhajda, 2006; Kuhajda et al., 1994). Cancer cells are dependent upon active lipogenesis, as FAS inhibition either through siRNA or pharmacological agents, leads to cancer cell death both *in vitro* and *in vivo* (Kuhajda, 2006; Swinnen et al., 2006). Following FAS inhibition, increased levels of malonyl-CoA, the substrate contributing most of the carbon for fatty acid synthesis (Wakil, 1989), have also been hypothesized as a possible trigger for cytotoxicity (Pizer et al., 2000; Zhou et al., 2003). As such, in lipogenic cells, malonyl-CoA levels are highly regulated by synthesis, utilization as substrate, and metabolism (Figure 1). Acetyl-CoA carboxylase (ACC), the rate limiting enzyme of fatty acid synthesis, produces malonyl-CoA from the ATP dependent carboxylation of acetyl CoA (Witters et al., 1994; Witters & Kemp, 1992). This step is highly regulated by a feed-forward allosteric activation by citrate, and inhibition by both AMPK activated kinase (AMPK) and long-chain acyl-CoA's. Malonyl-CoA is the substrate which provides the predominant carbon source for the synthesis of fatty acids by fatty acid synthase (FAS). Malonyl-CoA decarboxylase (MCD) (E.C. 4.1.1.9) acts to regulate malonyl-CoA levels through its decarboxylation back to acetyl-CoA (Fig. 1) (Goodwin & Taegtmeyer, 1999). Inhibition of MCD affords another strategy to rapidly increase malonyl-CoA levels through decreasing its catabolism. Using both siRNA and pharmacological inhibition of MCD, we report that MCD inhibition increases malonyl-CoA levels in cancer cells, induces cytotoxicity, and potentiates pharmacological FAS inhibition. These findings identify MCD as a potential target for cancer therapy development.

BODY

MCD siRNA Reduces MCD Expression and

Activity. To inhibit the expression and activity of MCD, we first utilized siRNA targeted to the human MCD sequence. MCD siRNA reduced both the expression and activity of MCD in MCF7 cells. As measured by quantitative RT-PCR, there was a significant reduction of MCD mRNA (Figure 1A) within 6 h, persisting through 48 h. Both protein expression by immunoblot (Figure 1B) and MCD activity



(Figure 1B) were substantially reduced within 6 h following MCD siRNA transfection. Since MCD siRNA rapidly reduced both MCD expression and activity in MCF7 cells, it was deemed a useful reagent to test the effects of MCD inhibition in cancer cells.

As a consequence of MCD inhibition in MCF7 cells, malonyl-CoA levels were increased by approximately 120% 6 h following siRNA treatment (Figure 1C). Since MCF7 cells are known to express FAS and undergo lipogenesis, the increased malonyl-CoA levels also increased fatty acid synthesis as measured by [¹⁴C]-acetate incorporation into total lipids. Thus, MCD inhibition led to an increase in steady-state levels of malonyl-CoA, thereby enhancing fatty acid synthesis (Figure 1D). Since FAS is not a regulated step of the pathway, it is not surprising that increased substrate availability at the FAS step led to increased flux through the pathway.

MCD siRNA is cytotoxic to MCF7 cells but not MRC-5 fibroblasts. MCD inhibition with siRNA led

to rapid and selective cytotoxicity against MCF7 cells. Treated MCF7 cells exhibited morphological features of cell injury (Figure 2A, upper right panel) characterized by pyknotic and fragmented cells, while controls were unremarkable (Figure 2A, upper left panel). MRC-5 human fibroblasts (Figure 2A, lower right panel) and controls (Figure 2A, lower left panel) were essentially unaffected by siRNA treatment. Scrambled siRNA controls were similar to lipofectamine controls (data not shown). As further evidence of the cytotoxicity of MCD inhibition, MCD siRNA also reduced clonogenicity of MCF7 cells within 16h (Figure 2B). Evidence of apoptosis by positive TUNEL staining occurred within 2h (Figure 2C) and persisted through 6h.

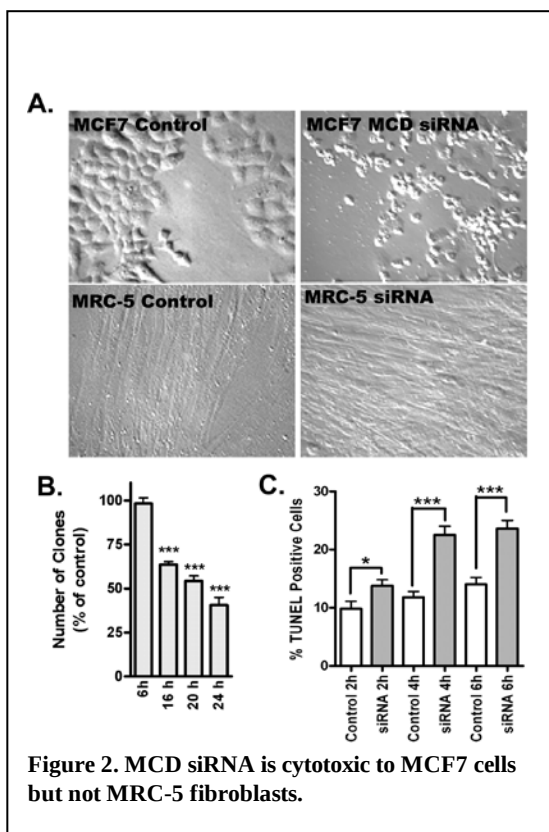


Figure 2. MCD siRNA is cytotoxic to MCF7 cells but not MRC-5 fibroblasts.

MPA, pharmacologically inhibits MCD in MCF7 cells.

The structure of MPA is shown in Figure 3A. It is a small molecule MCD inhibitor reported to inhibit MCD activity

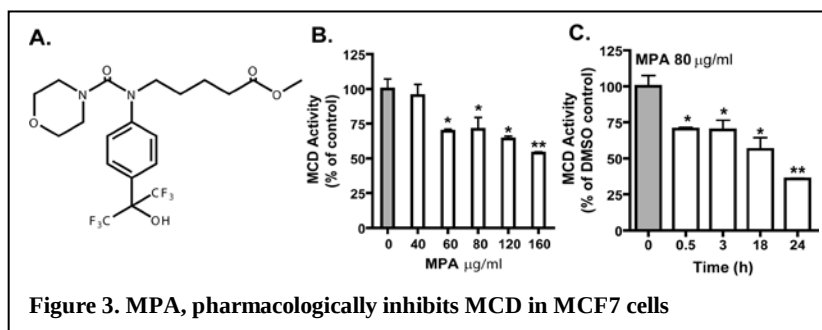
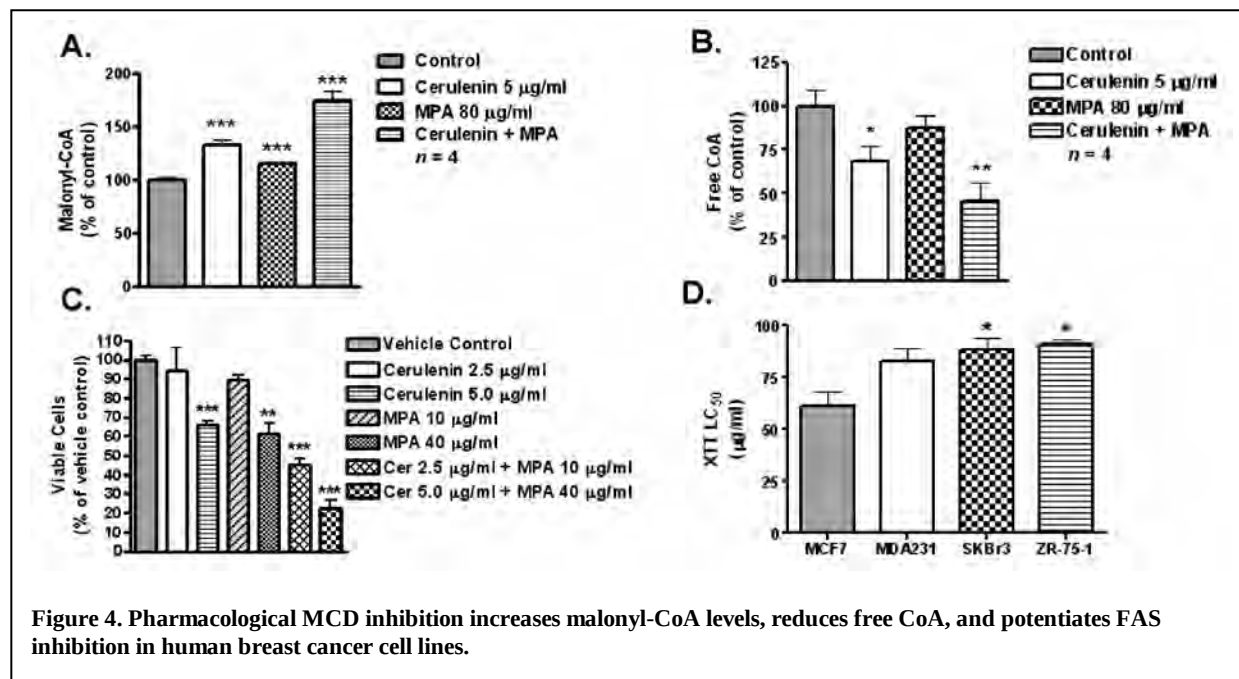


Figure 3. MPA, pharmacologically inhibits MCD in MCF7 cells

in rat heart (Cheng et al., 2006). Figure 3B shows a concentration dependent inhibition of MCD activity by MPA in MCF7 cells after 2 h treatment. MPA inhibited human MCD by 30% at 80 μg/mL with 50% inhibition at approximately 160 μg/mL. Although there is a dose dependence, there is not

a substantial time dependence of the inhibition at 80 $\mu\text{g/mL}$ as shown in Figure 3C. MPA rapidly inhibits MCD activity within 30 minutes by about 30% increasing to 65% after 24 h. The 24 h reduction is more likely due to cellular consequence of MCD inhibition rather than direct effects of MPA upon MCD.

Pharmacological inhibition of both MCD and FAS increases cellular malonyl-CoA levels while reducing free CoA. MCF7 cells were treated with MPA (80 $\mu\text{g/mL}$) or cerulenin (5 $\mu\text{g/mL}$), an FAS



inhibitor (Funabashi et al., 1989) for 6 h, after which malonyl-CoA levels were measured by HPLC (Figure 4A). Inhibiting FAS with cerulenin increased malonyl-CoA levels by 123% while MCD inhibition with MPA increased malonyl-CoA to 110% of control. Thus, blocking the utilization of malonyl-CoA with FAS inhibition or malonyl-CoA metabolism with MCD inhibition both increased malonyl-CoA levels. Blocking both MCD and FAS led to a 162% increase in malonyl-CoA levels. To achieve this, cells were treated with cerulenin one hour prior to the addition of MPA. Conversely, as malonyl-CoA levels increased, free CoA levels were reduced. Figure 4B demonstrate that cerulenin reduced free CoA by 32%, MPA by 13 %, and combined cerulenin and MPA by 54%.

Acetyl-CoA levels were not substantially affected by either FAS or MCD inhibition (data not shown). Thus, both FAS and MCD inhibition increase malonyl-CoA levels seemingly at the expense of cellular free CoA reserves.

Pharmacological MCD inhibition is cytotoxic to human breast cancer cells and potentiates FAS inhibition induced cytotoxicity. MCF7 cells treated with MPA at 40 µg/mL for 24 h induced substantial cytotoxicity (Figure 4C), which was similar to cerulenin treatment at 5 µg/mL. Neither MPA at 10 µg/mL nor cerulenin at 2.5 µg/mL alone were significantly cytotoxic, however, when combined, 55% of the cells were no longer viable. The combination of MPA at 40 µg/mL and cerulenin at 5 µg/mL were more cytotoxic than either compound alone. Figure 4D illustrates the LC₅₀ for MPA against a panel of human breast cancer cell lines which include estrogen receptor (ER) positive / HER2 unamplified MCF7, ER positive / HER2 amplified ZR-75-1, ER negative / HER2 amplified SKBr3, and ER negative / HER2 unamplified MDA231 (Menendez et al., 2004; Pegram et al., 2004; Shiu et al., 2008).

KEY RESEARCH ACCOMPLISHMENTS

[1] Reduction of MCD activity by both siRNA reducing its expression, or by pharmacological inhibition was selectively cytotoxic to human breast cancer cells.

[2] Exploration of the mechanism of action of MPA, the pharmacological FAS inhibitor, showed that it both inhibited MCD activity, and increased malonyl-CoA levels in cells. This demonstrates that the compound inhibits MCD *in vitro* in human cancer cells.

[3] Pharmacological MCD inhibition potentiated pharmacological FAS inhibition consistent with malonyl-CoA involvement in its mechanism of action.

[4] Pharmacological MCD inhibition reduced free CoA levels in cells which may also contribute to its mechanism of action.

With the exception of xenograft studies which are in the planning stages, all of the specific aims of the grant were achieved.

REPORTABLE OUTCOMES

[1] Presented these data at the Era of Hope meeting sponsored by the DOD in Baltimore, MD in 2007.

[2] A manuscript containing these data has been published in Oncogene. Zhou W, Tu, Y, Simpson, PJ, Kuhajda, FP. "Malonyl-CoA Decarboxylase Inhibition is Selectively Cytotoxic to Human Breast Cancer Cells". *Oncogene* 28: 2979-2987, 2009.

CONCLUSIONS

[1] MCD is a potential therapeutic target for breast cancer therapy.

[2] Increased cellular levels of malonyl-CoA is likely a key mediator of both MCD and FAS inhibition.

REFERENCES

- Cheng JF, Chen M, Wallace D, Tith S, Haramura M, Liu B, Mak CC, Arrhenius T, Reily S, Brown S, Thorn V, Harmon C, Barr R, Dyck JR, Lopaschuk GD and Nadzan AM. (2006). *J Med Chem*, 49, 1517-25.
- Funabashi H, Kawaguchi A, Tomoda H, Omura S, Okuda S and Iwasaki S. (1989). *J Biochem (Tokyo)*, 105, 751-5.
- Goodwin GW and Taegtmeyer H. (1999). *Am J Physiol*, 277, E772-7.

- Kuhajda FP. (2006). *Cancer Res*, 66, 5977-80.
- Kuhajda FP, Jenner K, Wood FD, Hennigar RA, Jacobs LB, Dick JD and Pasternack GR. (1994). *Proc Natl Acad Sci U S A*, 91, 6379-83.
- Menendez JA, Mehmi I, Atlas E, Colomer R and Lupu R. (2004). *Int J Oncol*, 24, 591-608.
- Pegram MD, Konecny GE, O'Callaghan C, Beryt M, Pietras R and Slamon DJ. (2004). *J Natl Cancer Inst*, 96, 739-49.
- Pizer ES, Thupari J, Han WF, Pinn ML, Chrest FJ, Frehywot GL, Townsend CA and Kuhajda FP. (2000). *Cancer Res*, 60, 213-8.
- Shiu LY, Liang CH, Huang YS, Sheu HM and Kuo KW. (2008). *Cell Biol Toxicol*, 24, 1-10.
- Swinnen JV, Brusselmans K and Verhoeven G. (2006). *Curr Opin Clin Nutr Metab Care*, 9, 358-65.
- Wakil S. (1989). *Biochemistry*, 28, 4523-4530.
- Witters LA, Gao G, Kemp BE and Quistorff B. (1994). *Arch Biochem Biophys*, 308, 413-9.
- Witters LA and Kemp BE. (1992). *J Biol Chem*, 267, 2864-7.
- Zhou W, Simpson PJ, McFadden JM, Townsend CA, Medghalchi SM, Vadlamudi A, Pinn ML, Ronnett GV and Kuhajda FP. (2003). *Cancer Res*, 63, 7330-7.

APPENDIX

Zhou W, Tu Y, Simpson J, Kuhajda FP. Malonyl-CoA Decarboxylase (MCD) Inhibition is selectively cytotoxic to human breast cancer cells. *Oncogene* 28: 2979-2987, 2009. See next page for the beginning of the published manuscript.

ORIGINAL ARTICLE

Malonyl-CoA decarboxylase inhibition is selectively cytotoxic to human breast cancer cells

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Fatty acid synthase (FAS) inhibition initiates selective apoptosis of cancer cells both *in vivo* and *in vitro*, which may involve malonyl-CoA metabolism. These findings have led to the exploration of malonyl-CoA decarboxylase (MCD) as a potential novel target for cancer treatment. MCD regulates the levels of cellular malonyl-CoA through the decarboxylation of malonyl-CoA to acetyl-CoA. Malonyl-CoA is both a substrate for FAS and an inhibitor of fatty acid oxidation acting as a metabolic switch between anabolic fatty acid synthesis and catabolic fatty acid oxidation. We now report that the treatment of human breast cancer (MCF7) cells with MCD small interference RNA (siRNA) reduces MCD expression and activity, reduces adenosine triphosphate levels, and is cytotoxic to MCF7 cells, but not to human fibroblasts. In addition, we synthesized a small-molecule inhibitor of MCD, 5-[(Morpholine-4-carbonyl)-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-amino]-penta-noic acid methyl ester (MPA). Similar to MCD siRNA, MPA inhibits MCD activity in MCF7 cells, increases cellular malonyl-CoA levels and is cytotoxic to a number of human breast cancer cell lines *in vitro*. Taken together, these data indicate that MCD-induced cytotoxicity is likely mediated through malonyl-CoA metabolism. These findings support the hypothesis that MCD is a potential therapeutic target for cancer therapy.

Oncogene (2009) 28, 2979–2987; doi:10.1038/onc.2009.160; published online 22 June 2009

Keywords: malonyl-CoA decarboxylase; apoptosis; fatty acid metabolism; malonyl-CoA; fatty acid synthase

Introduction

One of the most consistent biochemical changes associated with cancer is its predilection for aerobic

glycolysis described in the 1920s by Otto Warburg (Warburg *et al.*, 1924). Despite access to adequate oxygen, cancer cells continue to rely on glycolysis over respiration to generate adenosine triphosphate (ATP) (Elstrom *et al.*, 2004). Recently, the convergence of molecular biology and biochemistry has refocused interest on cancer metabolism as an area of new targets for cancer treatment. Our studies of cancer cell metabolism led to the observation that transformed cells exhibit high levels of fatty acid synthase (FAS) expression and fatty acid synthesis (Kuhajda *et al.*, 1994; Kuhajda, 2006). Cancer cells are dependent on active lipogenesis, as FAS inhibition either through small interference RNA (siRNA) or pharmacological agents, leads to cancer cell death both *in vitro* and *in vivo* (Kuhajda, 2006; Swinnen *et al.*, 2006).

Fatty acid synthesis and FAS expression occur in the liver and adipose tissue as a means to store energy (Girard *et al.*, 1997). During times of excess calorie consumption, FAS converts excess carbon from carbohydrates to fatty acids for eventual storage as triglycerides (Girard *et al.*, 1997). Cancer cells, in contrast to the liver and adipose tissues, do not synthesize or accumulate triglyceride for energy storage. Instead, our studies indicate that transformed cells undergo lipogenesis as a means to maintain redox and energy balance. As such, we recently observed rapid redox imbalance and increased AMP/ATP ratio following FAS inhibition in human ovarian cancer cells, which resulted in the activation of AMP-activated protein kinase (AMPK) (Zhou *et al.*, 2007).

Following FAS inhibition, increased levels of malonyl-CoA, the substrate contributing most of the carbon for fatty acid synthesis (Wakil, 1989) have also been hypothesized as a possible trigger for cytotoxicity (Pizer *et al.*, 2000; Zhou *et al.*, 2003). As such, in lipogenic cells, malonyl-CoA levels are highly regulated by synthesis, utilization as substrate and metabolism (Figure 1). Acetyl-CoA carboxylase (ACC), the rate limiting enzyme of fatty acid synthesis, produces malonyl-CoA from the ATP-dependent carboxylation of acetyl CoA (Witters and Kemp, 1992; Witters *et al.*, 1994a). This step is highly regulated by a feed-forward allosteric activation by citrate, and inhibition by both AMPK-activated kinase (AMPK) and long-chain

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Received 28 January 2009; revised 13 April 2009; accepted 14 May 2009; published online 22 June 2009

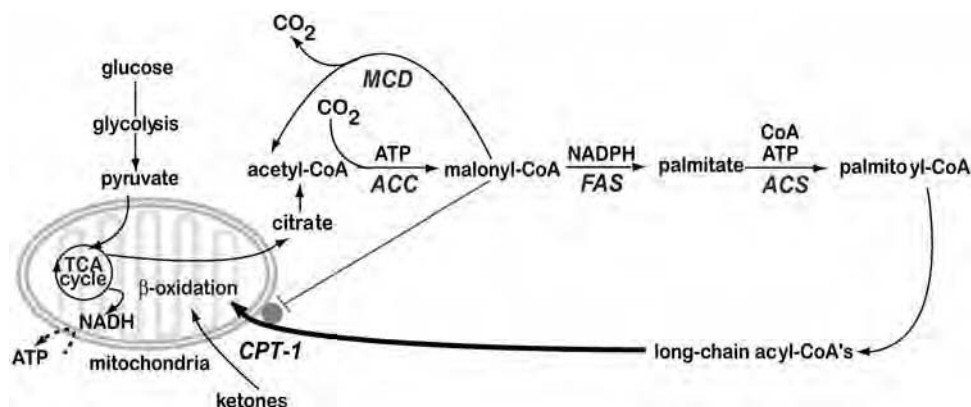


Figure 1 Malonyl-CoA decarboxylase (MCD) acts to regulate cellular malonyl-CoA levels. Acetyl-CoA, generated by the action of citrate lyase, is carboxylated by acetyl-CoA carboxylase (ACC) to malonyl-CoA. In lipogenic cells, malonyl-CoA is the predominant substrate for FAS. In muscle cells, which are devoid of FAS, malonyl-CoA acts to regulate fatty acid oxidation. MCD is poised to regulate either fatty acid synthesis or oxidation depending upon the tissue and its metabolic status.

acyl-CoA's. Malonyl-CoA is the substrate, which provides the predominant carbon source for the synthesis of fatty acids by FAS. Malonyl-CoA decarboxylase (MCD) (E.C. 4.1.1.9) acts to regulate malonyl-CoA levels through its decarboxylation back to acetyl-CoA (Figure 1) (Goodwin and Taegtmeyer, 1999). Inhibition of MCD affords another strategy to rapidly increase malonyl-CoA levels through decreasing its catabolism. Using both siRNA and pharmacological inhibition of MCD, we report that MCD inhibition increases malonyl-CoA levels in cancer cells, induces cytotoxicity and potentiates pharmacological FAS inhibition. These findings identify MCD as a potential target for cancer therapy development.

Results

MCD siRNA reduces MCD expression and activity

To inhibit the expression and activity of MCD, we first used siRNA targeted to the human MCD sequence. MCD siRNA reduced both the expression and activity of MCD in MCF7 cells. As measured by the quantitative reverse transcription-PCR, there was a significant reduction of MCD mRNA (Figure 2a) within 6 h, persisting through 48 h. Both protein expression by immunoblot (Figure 2b) and MCD activity (Figure 2b) were substantially reduced within 6 h following MCD siRNA transfection. As MCD siRNA rapidly reduced both MCD expression and activity in MCF7 cells, it was deemed a useful reagent to test the effects of MCD inhibition in cancer cells.

As a consequence of MCD inhibition in MCF7 cells, malonyl-CoA levels were increased by approximately 120% 6 h after siRNA treatment (Figure 2c). As MCF7 cells are known to express FAS and undergo lipogenesis, the increased malonyl-CoA levels also increased fatty acid synthesis as measured by [14 C]-acetate incorporation into total lipids. Thus, MCD inhibition led to an increase in steady-state levels of malonyl-CoA, thereby

enhancing fatty acid synthesis. As FAS is not a regulated step of the pathway, it is not surprising that increased substrate availability at the FAS step led to increased flux through the pathway.

MCD siRNA is cytotoxic to MCF7 cells but not MRC-5 fibroblasts

Malonyl-CoA decarboxylase inhibition with siRNA led to rapid and selective cytotoxicity against MCF7 cells. Treated MCF7 cells exhibited morphological features of cell injury (Figure 3a, upper right panel) characterized by pyknotic and fragmented cells, while controls were unremarkable (Figure 3a, upper left panel). MRC-5 human fibroblasts (Figure 3a, lower right panel) and controls (Figure 3a, lower left panel) were essentially unaffected by siRNA treatment. Scrambled siRNA controls were similar to lipofectamine controls (data not shown). As further evidence of the cytotoxicity of MCD inhibition, MCD siRNA also reduced clonogenicity of MCF7 cells within 16 h (Figure 3b). Evidence of apoptosis by positive TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) staining occurred within 2 h (Figure 3c) and persisted through 6 h.

MPA pharmacologically inhibits MCD in MCF7 cells

The structure of 5-[(Morpholine-4-carbonyl)-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-amino]-pentanoic acid methyl ester (MPA) is shown in Figure 4a. It is a small-molecule MCD inhibitor reported to inhibit MCD activity in the rat heart (Cheng et al., 2006). Figure 4b shows a concentration-dependent inhibition of MCD activity by MPA in MCF7 cells after 2 h treatment. MPA inhibited human MCD by 30% at 80 μ g/ml with 50% inhibition at approximately 160 μ g/ml. Although there is dose dependence, there is not a substantial time dependence of the inhibition at 80 μ g/ml as shown in Figure 4c. MPA rapidly inhibits MCD activity within 30 min by about 30% increasing to 65% after 24 h. The 24 h reduction is more likely because of

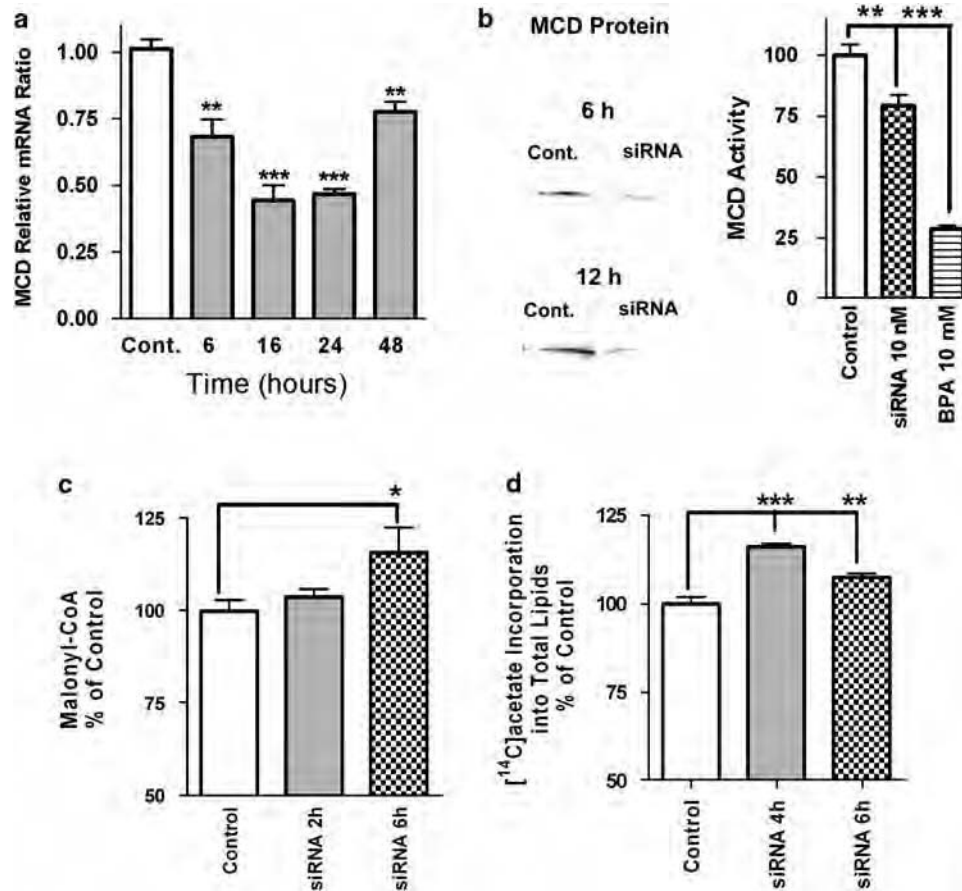


Figure 2 Malonyl-CoA decarboxylase (MCD) small interfering RNA (siRNA) reduces MCD activity. In MCF7 cells, MCD siRNA reduces MCD mRNA (a), protein expression, and enzyme activity (b). As a consequence, cellular malonyl-CoA levels are increased (c) along with fatty acid synthesis as measured by [¹⁴C]acetate incorporation into lipids (d). Error bars represent standard error of the mean (two-tailed *t*-tests **P*<0.05; ***P*<0.01; ****P*<0.001) (Graph Pad Software, San Diego, CA, USA).

the cellular consequence of MCD inhibition rather than direct effects of MPA upon MCD.

Pharmacological inhibition of both MCD and FAS increases cellular malonyl-CoA levels while reducing free CoA

MCF7 cells were treated with MPA (80 µg/ml) or cerulenin (5 µg/ml), an FAS inhibitor (Funabashi *et al.*, 1989) for 6 h, after which malonyl-CoA levels were measured by high-pressure liquid chromatography (Figure 5a). Inhibiting FAS with cerulenin increased malonyl-CoA levels by 123%, whereas MCD inhibition with MPA increased malonyl-CoA to 110% of control. Thus, blocking the utilization of malonyl-CoA with FAS inhibition or malonyl-CoA metabolism with MCD inhibition both increased malonyl-CoA levels. Blocking both MCD and FAS led to a 162% increase in malonyl-CoA levels. To achieve this, cells were treated with cerulenin 1 h before the addition of MPA. Conversely, as malonyl-CoA levels increased, free CoA levels were reduced. Figure 5b shows that cerulenin reduced free CoA by 32%, MPA by 13%, and combined cerulenin and MPA by 54%. Acetyl-CoA levels were not

substantially affected by either FAS or MCD inhibition (data not shown). Thus, both FAS and MCD inhibition increase malonyl-CoA levels seemingly at the expense of cellular free CoA reserves.

Pharmacological MCD inhibition is cytotoxic to human breast cancer cells and potentiates FAS inhibition induced cytotoxicity

MCF7 cells treated with MPA at 40 µg/ml for 24 h induced substantial cytotoxicity (Figure 5c), which was similar to cerulenin treatment at 5 µg/ml. Neither MPA at 10 µg/ml nor cerulenin at 2.5 µg/ml alone were significantly cytotoxic; however, when combined, 55% of the cells were no longer viable. The combination of MPA at 40 µg/ml and cerulenin at 5 µg/ml were more cytotoxic than either compound alone. Figure 5d illustrates the LC₅₀ for MPA against a panel of human breast cancer cell lines, which include estrogen receptor (ER) positive/HER2 unamplified MCF7, ER positive/HER2 amplified ZR-75-1, ER negative/HER2 amplified SKBr3 and ER negative/HER2 unamplified MDA231 (Menendez *et al.*, 2004; Pegram *et al.*, 2004; Shiu *et al.*, 2008).

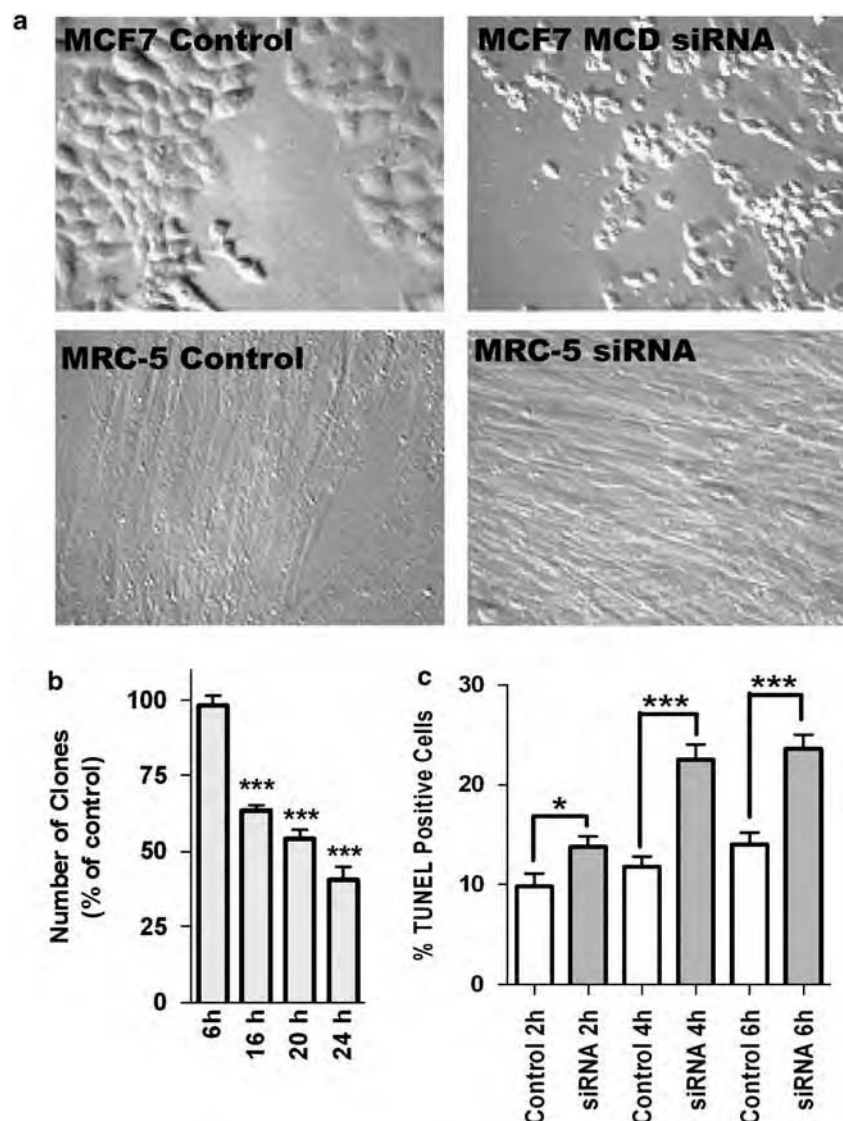


Figure 3 Malonyl-CoA decarboxylase (MCD) small interfering RNA (siRNA) is selective cytotoxic to MCF7 cells. Morphological changes ($\times 200$) of cell injury are identified 48 h post-treatment in MCF7 cells (**a**) (upper right panel) compared with siRNA control (upper left panel). MRC-5 fibroblasts are unaffected by MCD siRNA (lower panels). (**b**) MCD siRNA (10 nM) is cytotoxic to MCF7 cells in a clonogenic assay. Times indicate the length of exposure to the siRNA. (**c**) Apoptosis was also demonstrated by TUNEL assay beginning at 2 h post-treatment. Error bars represent standard error of the mean (two-tailed *t*-tests * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) (Graph Pad Software, San Diego, CA, USA).

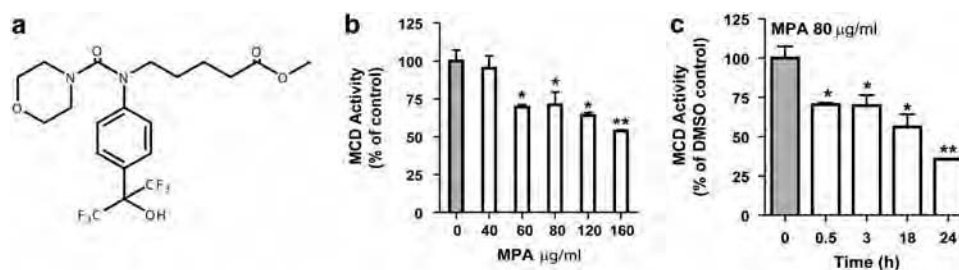


Figure 4 MPA (5-((Morpholine-4-carbonyl)-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-amino)-pentanoic acid methyl ester), a pharmacological malonyl-CoA decarboxylase (MCD) inhibitor, reduces MCD activity in MCF7 cells. (**a**) MPA is a small-molecule MCD inhibitor (486 GMW). Treatment of MCF7 cells shows more a (**b**) concentration than (**c**) time dependence upon enzyme inhibition. Error bars represent standard error of the mean (two-tailed *t*-tests * $P < 0.05$; ** $P < 0.01$) (Graph Pad Software, San Diego, CA, USA).

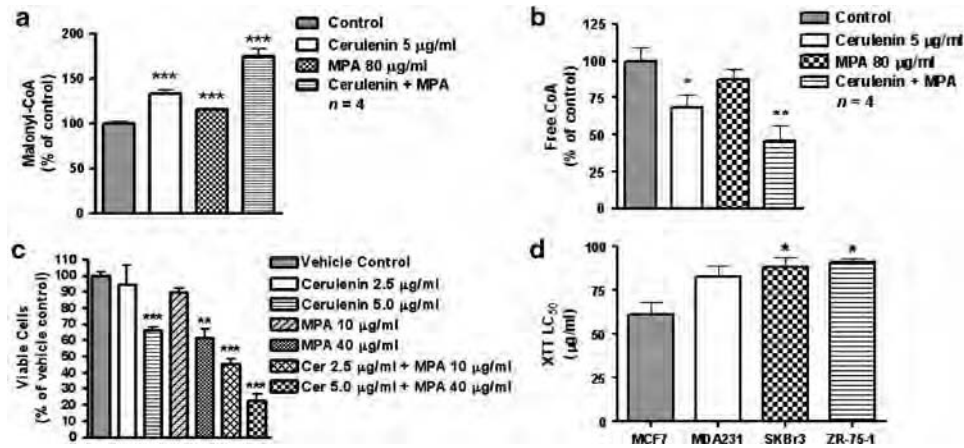


Figure 5 Pharmacological inhibition of both malonyl-CoA decarboxylase (MCD) and fatty acid synthase (FAS) increases cellular malonyl-CoA, reduces free CoA, and are synergistic against MCF7 cells. (a) The highest cellular malonyl-CoA levels are achieved with a combination of FAS and MCD inhibition (horizontal stripes). (b) Similarly, free-CoA levels are reduced. (c) Sublethal concentrations of cerulenin (solid white) and MPA (diagonal stripes) when combined (hashed lines) are significantly cytotoxic to MCF7 cells. Increased concentrations of cerulenin and MPA show concentration-dependent cytotoxicity. (d) MPA cytotoxicity (LC₅₀) in a panel of human breast cancer cell lines. Error bars represent standard error of the mean (two-tailed *t*-tests **P* < 0.05; ***P* < 0.01; ****P* < 0.001) (Graph Pad Software, San Diego, CA, USA).

Discussion

Earlier studies from our laboratory and others have shown that the increased malonyl-CoA levels following the inhibition of FAS are proapoptotic in human breast cancer cells (Pizer *et al.*, 2000; Bandyopadhyay *et al.*, 2006). As an effort to search for novel metabolic targets in cancer treatment, inhibition of MCD affords another strategy to rapidly increase malonyl-CoA levels through decreasing its catabolism. Utilizing both a siRNA and a small-molecule pharmacological inhibitor against MCD, we now report that the reduction of MCD activity induced increased levels of malonyl-CoA and displayed significant cytotoxicity against cultured human breast cancer cells.

Malonyl-CoA decarboxylase inhibition has been best characterized in non-lipogenic tissues, such as skeletal or cardiac muscle (Dyck and Lopaschuk, 2002; Saha and Ruderman, 2003; Dyck *et al.*, 2004). Muscle cells use malonyl-CoA as a means to rapidly and tightly regulate fatty acid oxidation. As such, malonyl-CoA levels are controlled through the reciprocal action of two key enzymes, ACC which synthesizes malonyl-CoA through the ATP-dependent carboxylation of acetyl-CoA, and MCD which decarboxylates malonyl-CoA back to acetyl-CoA (Saha and Ruderman, 2003). Pharmacological MCD inhibition increases malonyl-CoA levels, which indirectly inhibits fatty acid oxidation through malonyl-CoA inhibition of carnitine palmitoyl-transferase-1 (CPT-1). CPT-1 functions to transport long-chain acyl-CoAs into the mitochondria and is the rate limiting enzymatic step of mitochondrial fatty acid oxidation (Figure 1) (McGarry and Brown, 1997). Indeed, the 10-fold lower *K_i* (0.07 µM) of the muscle CPT-1 isoform for malonyl-CoA is in keeping with this function (McGarry and Brown, 1997). Recent *in vivo* studies of pharmacological MCD inhibition

in cardiac tissue has shown a benefit of reduced fatty acid oxidation during cardiac ischemia (Dyck *et al.*, 2004).

Malonyl-CoA decarboxylase inhibition in lipogenic tissues, such as the liver or human cancer, has not been well studied. In muscle cells, MCD is the predominant enzyme for malonyl-CoA disposal. In lipogenic tissues, however, malonyl-CoA metabolism is more complex. In addition to elimination by MCD decarboxylation, malonyl-CoA is the predominant substrate for fatty acid synthesis. FAS incorporates seven moles of malonyl-CoA per mole of the 16-carbon saturated fat, palmitic acid, synthesized. Similar to muscle cells, the high steady-state levels of malonyl-CoA in lipogenic tissues serves to inhibit CPT-1 activity and fatty acid oxidation, thereby preventing a futile cycle of fatty acid synthesis and oxidation. As FAS could consume malonyl-CoA, it was not clear if MCD inhibition would lead to cytotoxicity in lipogenic cancer cells.

We began our investigation of MCD inhibition in cancer cells using MCD siRNA to reduce MCD expression and activity. Treatment of MCF7 cells with MCD siRNA reduced expression and overall MCD activity compared with controls. Consequently, malonyl-CoA levels increased, similar to what was observed in muscle cells. Within 6 h of MCD siRNA treatment of MCF7 cells, steady state malonyl-CoA levels rose above control cells by 20%. Acetyl-CoA levels were similarly increased (data not shown). As a result of the dysregulated metabolism following MCD inhibition, MCD siRNA was significantly cytotoxic to MCF7 cells. Within 24 h of siRNA application, MCF7 cells exhibited extensive cytotoxicity by morphology, clonogenic assays and TUNEL assays. In contrast, MRC-5 human fibroblasts were unaffected. The cytotoxic effect was also rapid, with evidence of increased TUNEL positivity within 2 h after siRNA application.

To more directly explore the effect of rapid MCD inhibition without changing enzyme levels with siRNA, we synthesized a small-molecule pharmacological MCD inhibitor, MPA. Although MPA has been shown to inhibit MCD activity in muscle cells (Dyck *et al.*, 2004), it has not been studied in lipogenic human cancer cells such as MCF7. In MCF7 cells, MPA significantly inhibited MCD activity within 30 min of treatment. Similar to MCD siRNA, MPA elevated malonyl-CoA levels in MCF7 cells at the levels similar to FAS inhibition with cerulenin. MPA also produced substantial cytotoxicity in MCF7 cells. The cytotoxic effect of MPA was not restricted to ER-positive HER2 unamplified MCF7 cells. MPA induced a cytotoxic response in three additional human breast cancer cell lines encompassing ER positive and negative and HER2 amplified and unamplified phenotypes. These data suggest that the cytotoxic effect of MCD inhibition is independent of ER or HER2 status.

As FAS and MCD are the two primary enzymes utilizing malonyl-CoA in cancer cells, inhibiting either enzyme alone allows malonyl-CoA to be consumed by the other pathway. In the next series of experiments, we studied a combination of both MCD and FAS inhibition with MPA and cerulenin, respectively. Blocking both the consumption and metabolism of malonyl-CoA should elevate malonyl-CoA levels higher than blocking either alone. Indeed, the combination of MPA and cerulenin elevated malonyl-CoA levels greater than with either agent alone. As FAS and MCD inhibition both elevate malonyl-CoA levels, we hypothesized that the cellular pool of free CoA would be diminished. This was indeed the case. Similar to malonyl-CoA, combined FAS and MPA inhibition reduced free CoA levels greater than either alone.

Given the rapid and significant alteration of fatty acid synthesis and high energy CoA intermediates following both FAS and MCD inhibition, we further hypothesized that inhibition of each enzyme would potentiate the cytotoxic effect of the other. The combination of individually sublethal concentrations of cerulenin (2.5 µg/ml) or MPA (10 µg/ml) led to significant cytotoxicity (50%) in MCF7 cells. The combination of cytotoxic concentrations of both compounds produced an additive effect.

In both muscle cells and lipogenic MCF7 cells, MCD inhibition increased malonyl-CoA levels, but the downstream effects of increased malonyl-CoA levels were drastically different. In muscle cells, increased malonyl-CoA levels inhibited fatty acid oxidation. In MCF7 cells, however, the rise in malonyl-CoA levels failed to inhibit fatty acid oxidation, but enhanced fatty acid synthesis. Responding to the increase in acetyl-CoA and malonyl-CoA levels, fatty acid synthesis increased by 20% following MCD inhibition. However, unlike muscle cells, fatty acid oxidation was not reduced by MCD siRNA in MCF7 cells (data not shown). Thus, cancer cells do not have the same stringent regulation between malonyl-CoA levels and fatty acid oxidation regulation. The muscle isoform of CPT-1 with its low K_i for malonyl-CoA affords tight regulation of fatty acid

oxidation, whereas the liver form of CPT-1 has a 100-fold higher K_i for malonyl-CoA. As MCF7 cells express both ACC isoforms, this could account for the increased fatty acid synthesis without reduced fatty acid oxidation in MCF7 cells (Witters *et al.*, 1994b).

As the increase in malonyl-CoA levels paralleled cytotoxicity in MCF7 cells, it suggested that the increased malonyl-CoA levels participate in the mechanism of action of both MCD and FAS inhibition. This hypothesis leads to a number of possible mechanisms of action which may not be mutually exclusive including: (1) reduced fatty acid oxidation from malonyl-CoA inhibition of CPT-1 (perhaps depending upon CPT-1 isoform expression), (2) increased fatty acid synthesis from increased malonyl-CoA levels depleting ATP and NADPH, and (3) malonyl-CoA inhibition of succinyl-CoA dehydrogenase (complex II of the electron transport chain) promoting apoptosis (Albayrak *et al.*, 2003).

In cancer cells, elevated malonyl-CoA levels failed to inhibit fatty acid oxidation, thus this mode of action is less likely to contribute to the mechanism of action. However, MCD inhibition, through increasing malonyl-CoA and acetyl-CoA levels directly, modestly increased fatty acid synthesis. As FAS inhibition is cytotoxic to cancer cells, increasing fatty acid synthesis might be seen as advantageous to cancer cell growth. However, MCD increased fatty acid synthesis through bypassing the physiological pace-setting enzyme for fatty acid synthesis, ACC. ACC is the step which rapidly regulates fatty acid synthesis through the following mechanisms: (1) cytoplasmic citrate, the source of carbon for fatty acid synthesis, is a feed-forward allosteric activator of ACC, (2) AMPK phosphorylates and inhibits ACC during energy depletion and (3) long-chain acyl-CoA's provide pathway end-product inhibition of ACC. Thus, any effect of MCD inhibition on fatty acid synthesis occurs independently of the complex feed-forward and end-product inhibition of ACC, which is based on the cellular energy status (McGarry and Brown, 1997). A rapid, dysregulated increase in fatty acid synthesis likely constitutes a significant cellular stress. Fatty acid synthesis is an energy intensive anabolic pathway consuming 7 ATP and 14 NADPH per molecule of fatty acid synthesized (Wakil, 1989). Thus, accelerated fatty acid synthesis could potentially usurp energy as ATP from other anabolic pathways and reduce NADPH availability for reductive synthesis of macromolecules, such as DNA and RNA, contributing ultimately to cell injury or death.

As there was a modest increase in fatty acid synthesis following MCD inhibition, which can contribute to metabolic instability, increased malonyl-CoA levels can also directly dysregulate energy metabolism through disruption of Krebs cycle and electron transport activity. Clinical and biochemical studies of MCD deficiency, a rare inborn error of metabolism, provide another potential mechanism of action of MCD inhibition based on the pathological accumulation of malonyl-CoA (FitzPatrick *et al.*, 1999; Sacksteder *et al.*, 1999). Patients harboring an inactivating mutation of MCD exhibit elevated cellular levels of malonyl-CoA

(FitzPatrick *et al.*, 1999), resulting in high serum and urine levels of malonate, derived from the hydrolysis of malonyl-CoA to free malonate (Riley *et al.*, 1991). Malonate is a dicarboxylic acid that is a potent inhibitor of succinyl-CoA dehydrogenase, a key component of complex II of the electron transport chain and Krebs cycle (Koeppen and Riley, 1987). In addition to its function as a member of the electron transport chain, a component of complex II, CybL, has been identified as a sensor for apoptosis induction for a wide variety of commonly used chemotherapeutic agents, such as doxorubicin, paclitaxel, etoposide, menadione and cisplatin (Albayrak *et al.*, 2003). Thus, the rapid increase in malonyl-CoA after MCD inhibition could be sensed by complex II as a proapoptotic signal by the cancer cell. Preliminary studies from our laboratory have shown a transient reduction in succinyl-CoA dehydrogenase activity following pharmacological FAS inhibition in MCF7 cells (data not shown) supporting the hypothesis that elevated malonyl-CoA levels in human cancer cells may reduce complex II activity. Although the complete loss of MCD activity through inactivating mutations is not compatible with life (FitzPatrick *et al.*, 1999), pharmacological modulation of MCD activity *in vivo* has successfully augmented cardiac function during ischemia without evidence of overt toxicity (Dyck *et al.*, 2004). These *in vivo* studies provide evidence that pharmacological MCD inhibition does not induce generalized toxicity, but can be modulated to provide a therapeutic index for treatment.

As interest in cancer metabolism grows, the exploration continues to yield potential new targets and strategies for cancer therapy (Deberardinis *et al.*, 2008). As an outgrowth of investigating the mechanism of action of FAS inhibition in cancer, MCD has emerged as another potential target for cancer therapy, particularly in combination with FAS inhibitors, providing another inroad into the metabolic therapy of human cancer.

Materials and methods

Cell lines, antibodies and chemicals

MCF7 human breast cancer cells and MRC-5 human fibroblasts were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Rabbit polyclonal anti-MCD antibodies were obtained as a gift from Dr Steven Gould, Department of Biological Chemistry, Johns Hopkins University School of Medicine. Cerulenin and bromopyruvic acid were purchased from Sigma (St Louis, MO, USA). The small-molecule MCD inhibitor, MPA, was synthesized as described (Cheng *et al.*, 2006) at FASgen Inc. (Baltimore, MD, USA).

MCD siRNA preparation and transfection

To construct an siRNA for MCD, we designed a series of siRNA's with 3' overhang uridine dimers (dUdU) spanning the sequence of human MCD using the Ambion's Silencer siRNA Construction Kit (Ambion, Austin, TX, USA) (Sacksteder

et al., 1999). Based on quantitative real-time (RT)-PCR for MCD expression following transfection, we chose the sequence GGUGUACUUCUUUUCUCAUU (located 683 nucleotides downstream of the first nucleotide of the start codon of human MCD). Transfections of MCF-7 and MRC-5 cells with the MCD or control siRNA were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as recommended. Transfection efficiency was assessed under fluorescence microscopy with Cy3 labeled siRNA using the Ambion's Silencer siRNA Labeling Kit. The transfection efficiency was >80%.

Real-time reverse transcription-PCR

To quantify MCD message following siRNA treatment, real-time quantitative reverse transcription-PCR was performed following RNA preparation and reverse transcription as described (Tu *et al.*, 2005). Briefly, the sequences of the sense and antisense primers used for amplification were as follows: MCD, 5'-ATTCATCAGCTTGACCCAG-3' (sense) and QJ; 5'-GGAGCTTGAGGGTCTCGTTA-3' (antisense); β -actin, 5'-GGCGGCACCACCATGTACCCT-3' (sense) and 5'-AGGGGCCGGACTCGTCATACT-3' (antisense). The β -actin gene was used as internal control in the real-time reverse transcription-PCR to normalize the results. Cycling conditions included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 95 °C denaturation for 30 s, 60 °C annealing for 30 s and 72 °C extension for 30 s. Amplification and detection were performed on an iCycler iQ Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). A negative control reaction in the absence of template was also performed for each primer pair. After completion of the cycling process, samples were subjected to a melting curve analysis to confirm the amplification specificity. For each sample, the ratio between the relative amounts of target gene (MCD) and internal control (β -actin) was calculated to compensate for variations in quantity or quality of starting mRNA as well as for differences in reverse transcriptase efficiency. The change in fluorescence of SYBR Green dye in every cycle was monitored, and the threshold cycle (C_T) above background for each reaction was calculated. The fold change in MCD relative to the β -actin internal control gene was determined by: Fold change = $2^{-\Delta(\Delta C_T)}$, where $\Delta C_T = C_{T, MCD} - C_{T, \beta\text{-actin}}$ and $\Delta(\Delta C_T) = \Delta C_{T, \text{treated}} - \Delta C_{T, \text{control}}$.

MCD activity assay, coenzyme-A and coenzyme-A derivative measurements and immunoblots

Malonyl-CoA decarboxylase activity was assayed in 5×10^5 MCF7 cells by trapping $^{14}\text{CO}_2$ from the decarboxylation of [$1,3\text{-}^{14}\text{C}$]malonyl-CoA as described (Goodwin and Taegtmeyer, 1999). The MCD inhibitor, bromopyruvic acid (Sigma), 1 mM, was used as a positive control. CoA derivatives and free CoA were measured using a modified high-pressure liquid chromatography procedure (Demoz *et al.*, 1995). After transfection at times indicated or drug treatment for 6 h, 4×10^4 MCF7 cells were homogenized in 120 μl of ice-cold 5% sulfosalicylic acid in 50 μM dithioerythritol and centrifuged at 600 g for 10 min. The supernatants were injected onto LC-18 reversed-phase column (Supelco, Bellefonte, PA, USA) and eluted with the following buffers and gradients while monitoring 254 nm: Buffer A 100 mM sodium phosphate and 75 mM sodium acetate, pH 4.6; Buffer B 70% buffer A in methanol; 0 min, 90% A; 10 min, 60% A; 17.6 min, 10% A at 1.5 ml/min. Immunoblots for MCD were prepared using rabbit anti-MCD antibodies as described (Sacksteder *et al.*, 1999).

ATP, fatty acid synthesis and oxidation measurements

Adenosine triphosphate levels are measured in the linear range of detection, using the ATP Bioluminescence Kit CLS II (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer's protocol and read on a Perkin Elmer Wallac Victor² 1420 luminometer (Perkin Elmer, Waltham, MA, USA). Fatty acid oxidation was measured by quantifying acid soluble products from [¹⁴C]palmitate oxidation in MCF7 cells (Watkins *et al.*, 1991). Fatty acid synthase was measured by [¹⁴C]acetate incorporation into lipids (Pizer *et al.*, 2000).

TUNEL, clonogenic and XTT assays

For clonogenicity studies, siRNA or compounds were added for times indicated. After 1 week of growth, clones were stained with crystal violet (0.5% in 25% methanol) and counted. 3'-TUNEL analysis was performed on MCF-7 cells that were plated and treated with MCD siRNA at 10 nM. Following fixation, cells are labeled with terminal deoxynucleotidyl transferase enzyme (200 U/ml; Sigma) along with Biotin-16-DUTP (1:100; Roche Diagnostics) as described (Zhou *et al.*, 2003). For cell counts three rows of four × 40 fields spaced evenly over the area of the well were counted for each well. Values are reported as the mean ± s.e.m. number of

BrdU labeled nuclei/total nuclei per × 40 field. Cell counts were performed on two-wells per treatment in duplicate experiments. XTT cytotoxicity assays were performed as described (Zhou *et al.*, 2007).

Conflict of interest

Under a licensing agreement between FASgen and the Johns Hopkins University, FPK is entitled to a share of royalty received by the University on sales of products described in this article. FPK owns FASgen stock, which is subject to certain restrictions under University policy. The Johns Hopkins University, in accordance with its conflict of interest policies, is managing the terms of this arrangement.

Acknowledgements

This study was supported by a grant from the Department of Defense Breast Cancer Grant BC050452 to FPK. We thank FASgen Inc. for the generous gift of MPA.

References

- Albayrak T, Scherhammer V, Schoenfeld N, Braziulis E, Mund T, Bauer MK *et al.* (2003). The tumor suppressor cybL, a component of the respiratory chain, mediates apoptosis induction. *Mol Biol Cell* **14**: 3082–3096.
- Bandyopadhyay S, Zhan R, Wang Y, Pai SK, Hirota S, Hosobe S *et al.* (2006). Mechanism of apoptosis induced by the inhibition of fatty acid synthase in breast cancer cells. *Cancer Res* **66**: 5934–5940.
- Cheng JF, Chen M, Wallace D, Tith S, Haramura M, Liu B *et al.* (2006). Synthesis and structure-activity relationship of small-molecule malonyl coenzyme A decarboxylase inhibitors. *J Med Chem* **49**: 1517–1525.
- Deberardinis RJ, Sayed N, Ditsworth D, Thompson CB. (2008). Brick by brick: metabolism and tumor cell growth. *Curr Opin Genet Dev* **18**: 54–61.
- Demoz A, Garras A, Asiedu DK, Netteland B, Berge RK. (1995). Rapid method for the separation and detection of tissue short-chain coenzyme A esters by reversed-phase high-performance liquid chromatography. *J Chromatogr B Biomed Appl* **667**: 148–152.
- Dyck JR, Cheng JF, Stanley WC, Barr R, Chandler MP, Brown S *et al.* (2004). Malonyl coenzyme A decarboxylase inhibition protects the ischemic heart by inhibiting fatty acid oxidation and stimulating glucose oxidation. *Circ Res* **94**: e78–e84.
- Dyck JR, Lopaschuk GD. (2002). Malonyl CoA control of fatty acid oxidation in the ischemic heart. *J Mol Cell Cardiol* **34**: 1099–1109.
- Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR *et al.* (2004). Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res* **64**: 3892–3899.
- FitzPatrick DR, Hill A, Tolmie JL, Thorburn DR, Christodoulou J. (1999). The molecular basis of malonyl-CoA decarboxylase deficiency. *Am J Hum Genet* **65**: 318–326.
- Funabashi H, Kawaguchi A, Tomoda H, Omura S, Okuda S, Iwasaki S. (1989). Binding site of cerulenin in fatty acid synthetase. *J Biochem (Tokyo)* **105**: 751–755.
- Girard J, Ferre P, Foufelle F. (1997). Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annu Rev Nutr* **17**: 325–352.
- Goodwin GW, Taegtmeyer H. (1999). Regulation of fatty acid oxidation of the heart by MCD and ACC during contractile stimulation. *Am J Physiol* **277**: E772–E777.
- Koeppen AH, Riley KM. (1987). Effect of free malonate on the utilization of glutamate by rat brain mitochondria. *J Neurochem* **48**: 1509–1515.
- Kuhajda FP. (2006). Fatty acid synthase and cancer: new application of an old pathway. *Cancer Res* **66**: 5977–5980.
- Kuhajda FP, Jenner K, Wood FD, Hennigar RA, Jacobs LB, Dick JD *et al.* (1994). Fatty acid synthesis: a potential selective target for antineoplastic therapy. *Proc Natl Acad Sci USA* **91**: 6379–6383.
- McGarry JD, Brown NF. (1997). The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem* **244**: 1–14.
- Menendez JA, Mehmi I, Atlas E, Colomer R, Lupu R. (2004). Novel signaling molecules implicated in tumor-associated fatty acid synthase-dependent breast cancer cell proliferation and survival: Role of exogenous dietary fatty acids, p53-p21WAF1/CIP1, ERK1/2 MAPK, p27KIP1, BRCA1, and NF-kappaB. *Int J Oncol* **24**: 591–608.
- Pegram MD, Konecny GE, O'Callaghan C, Beryt M, Pietras R, Slamon DJ. (2004). Rational combinations of trastuzumab with chemotherapeutic drugs used in the treatment of breast cancer. *J Natl Cancer Inst* **96**: 739–749.
- Pizer ES, Thupari J, Han WF, Pinn ML, Chrest FJ, Frehywot GL *et al.* (2000). Malonyl-coenzyme-A is a potential mediator of cytotoxicity induced by fatty-acid synthase inhibition in human breast cancer cells and xenografts. *Cancer Res* **60**: 213–218.
- Riley KM, Dickson AC, Koeppen AH. (1991). The origin of free brain malonate. *Neurochem Res* **16**: 117–122.
- Sacksteder KA, Morrell JC, Wanders RJ, Matalon R, Gould SJ. (1999). MCD encodes peroxisomal and cytoplasmic forms of malonyl-CoA decarboxylase and is mutated in malonyl-CoA decarboxylase deficiency. *J Biol Chem* **274**: 24461–24468.
- Saha AK, Ruderman NB. (2003). Malonyl-CoA and AMP-activated protein kinase: an expanding partnership. *Mol Cell Biochem* **253**: 65–70.
- Shiu LY, Liang CH, Huang YS, Sheu HM, Kuo KW. (2008). Downregulation of HER2/neu receptor by solamargine enhances anticancer drug-mediated cytotoxicity in breast cancer cells with high-expressing HER2/neu. *Cell Biol Toxicol* **24**: 1–10.

- Swinnen JV, Brusselmans K, Verhoeven G. (2006). Increased lipogenesis in cancer cells: new players, novel targets. *Curr Opin Clin Nutr Metab Care* **9**: 358–365.
- Tu Y, Thupari JN, Kim EK, Pinn ML, Moran TH, Ronnett GV *et al*. (2005). C75 alters central and peripheral gene expression to reduce food intake and increase energy expenditure. *Endocrinology* **146**: 486–493.
- Wakil S. (1989). Fatty acid synthase, a proficient multifunctional enzyme. *Biochemistry* **28**: 4523–4530.
- Warburg O, Posener K, Negelein E. (1924). Über den stoffwechsel der tumoren. *Biochem Z* **152**: 319–344.
- Watkins PA, Ferrell Jr EV, Pedersen JI, Hoefler G. (1991). Peroxisomal fatty acid beta-oxidation in HepG2 cells. *Arch Biochem Biophys* **289**: 329–336.
- Witters LA, Gao G, Kemp BE, Quistorff B. (1994a). Hepatic 5'-AMP-activated protein kinase: zonal distribution and relationship to acetyl-CoA carboxylase activity in varying nutritional states. *Arch Biochem Biophys* **308**: 413–419.
- Witters LA, Kemp BE. (1992). Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. *J Biol Chem* **267**: 2864–2867.
- Witters LA, Widmer J, King AN, Fassihi K, Kuhajda F. (1994b). Identification of human acetyl-CoA carboxylase isozymes in tissue and in breast cancer cells. *Int J Biochem* **26**: 589–594.
- Zhou W, Han WF, Landree LE, Thupari JN, Pinn ML, Bililign T *et al*. (2007). Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells. *Cancer Res* **67**: 2964–2971.
- Zhou W, Simpson PJ, McFadden JM, Townsend CA, Medghalchi SM, Vadlamudi A *et al*. (2003). Fatty acid synthase inhibition triggers apoptosis during S phase in human cancer cells. *Cancer Res* **63**: 7330–7337.