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Identification of potential caloric restriction mimetics by microarray profiling

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Dhahbi, Joseph M., Patricia L. Mote, Gregory M. Fahy, and Stephen R. Spindler. Identification of potential caloric restriction mimetics by microarray profiling. *Physiol Genomics* 23: 343–350, 2005. First published September 27, 2005; doi:10.1152/physiolgenomics.00069.2005.—To facilitate the development of assays for the discovery of pharmaceuticals capable of mimicking the effects of caloric restriction (CR) on life- and healthspan (CR mimetics), we evaluated the effectiveness of glucoregulatory and putative cancer chemopreventatives in reproducing the hepatic gene expression profile produced by long-term CR (LTCR), using Affymetrix microarrays. We have shown that CR initiated late in life begins to extend lifespan, reduce cancer as a cause of death, and reproduce approximately three-quarters of the genomic effects of LTCR in 8 wk (CR8). Eight weeks of metformin treatment was superior to CR8 at reproducing LTCR-like gene expression changes, maintaining a superior number of such changes over a broad range of statistical stringencies, and producing more Gene Ontology terms overlapping those produced by LTCR. Consistent with these results, metformin has been shown to reduce cancer incidence in mice and humans. Phenformin, a chemical cousin of metformin, extends lifespan and reduces tumor incidence in mice. Taken together, these results indicate that gene expression biomarkers can be used to identify promising candidate CR mimetics.

drug discovery; gene expression biomarkers; metformin; life span; longevity therapeutics

A MAJOR GOAL OF pharmaceutical research has been to reduce morbidity and delay mortality in the elderly. However, there are presently no authentic longevity pharmaceuticals. The reason none have been found is that no assay has existed for rapidly identifying such drugs. It is unlikely that human trials of potentially lifespan-extending pharmaceuticals will ever be conducted using death as the endpoint. Nevertheless, it appears likely that such pharmaceuticals could be developed or may already exist. Most therapeutics for human diseases were discovered using surrogate assays, with little or no knowledge of the molecular mechanisms of the disease.

We know that lifespan can be extended in experimental mammals by at least two interventions, and they are additive in their effects. Caloric restriction (CR) is a nutritional intervention capable of consistently and dramatically extending lifespan and reducing the incidence and severity of most age-related diseases, including cancer, whether it is initiated in young or older mice (10, 35). In addition, genetic ablation of growth hormone (GH) or its receptor or insulin-like growth factor I (IGF-I) or its receptor produces a dwarf phenotype and extends the lifespan of rodents (5). Such mutations are thought to slow aging because they postpone the age-related development of neoplastic diseases, immune system decline, and

collagen cross-linking (5). CR and the Ames dwarf mutation act together to additively change gene expression and increase the lifespan of mice (6). Other long-lived mouse mutants have been identified, suggesting that additional, potentially drug-gable genetic pathways exist for ameliorating age-related diseases and extending lifespan (31, 32).

The development of assays useful in the discovery of drugs capable of extending lifespan has proven problematic. Lifespan studies conducted in “short-lived” mammals such as mice might appear useful. However, a vigorous mouse strain lives ~40 mo. In addition, large groups of animals are required for such studies, making the assays too costly and time consuming to be practical. Use of enfeebled rodent strains introduces confounds into such studies and has not been helpful in identifying authentic longevity medicaments.

A number of lines of evidence, including nonhuman primate and human studies, suggest that CR-like processes may be involved in determining human longevity (39, 52). Thus the molecular mechanisms responsible for life- and healthspan extension in animals may also have applications in human health. Many gerontologists have assumed that a CR mimetic drug would need to be administered over an animal’s lifetime to resist incremental, age-related changes in physiology and gene expression. However, we recently showed that CR initiated in older mice begins within just 8 wk to extend lifespan and shift the expression of hepatic and heart genes toward the “slow-aging profile” associated with long-term CR (LTCR; Refs. 10, 11, 44). CR initially reduced deaths from tumors by 3.1-fold (10). Approximately 75% of these tumors are liver tumors (44). For these reasons, we focused the studies reported here on liver gene expression.

The data reviewed above suggest that relatively brief treatments with a CR mimetic drug should be capable of shifting the rate of aging and gene expression toward that of LTCR. This introduces the possibility that potential CR mimetics can be rapidly screened using microarray profiling as a surrogate for their effects on lifespan and health. To test this hypothesis, we evaluated the effectiveness of five potential CR mimetics at reproducing the gene expression profiles of CR in mouse liver, using Affymetrix microarrays. Because of the linkage between insulin, IGF-I, and the rate of aging, we tested the effects of the glucoregulatory compounds metformin (MET), glipizide (GLIP), GLIP plus MET (GM), and rosiglitazone (ROS) for their ability to reproduce the effects of LTCR on hepatic gene expression. We also tested soy isoflavone extract (SOY) for its LTCR-like gene expression effects, because it is often touted as a potentially lifespan-extending chemopreventative that is thought to work through mechanisms unrelated to insulin-IGF-I signaling. These data indicate that 8 wk of treatment with MET was even better at reproducing the gene expression effects of LTCR than 8 wk of CR (CR8). These results are consistent with published studies, and together they identify MET as a potential CR mimetic.

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Table 1. Drug dosages and animal weights

Treatment Group	Drug Dosage, mg*	Start Weight, g†	End Weight, g‡
CON		44.7 ± 4.4 ^{a§}	43.8 ± 2.9 ^{a§}
LTCR		22.8 ± 0.6 ^b	25.5 ± 1.1 ^c
CR8		49.1 ± 1.5 ^a	38.1 ± 1.4 ^b
MET	2,100	47.8 ± 1.4 ^a	44.1 ± 1.8 ^a
GLIP	1,050	45.5 ± 1.7 ^a	43.1 ± 0.9 ^a
GM	525 GLIP 1,050 MET	46.5 ± 2.7 ^a	44.9 ± 2.2 ^a
ROS	80	48.0 ± 1.0 ^a	47.2 ± 1.6 ^a
SOY	0.25%	48.2 ± 2.9 ^a	46.1 ± 2.0 ^a

CON, control; CR, caloric restriction; LTCR, long-term CR; CR8, CR for 8 wk; MET, metformin; GLIP, glipizide; GM, GLIP plus MET; ROS, rosiglitazone; SOY, soy isoflavone extract. *Per kg diet; SOY was percent by weight of the diet. †Mean weight ± SD at the start of treatments. ‡Mean weight ± SD after 2 mo of treatment. §Analyzed by 1-way ANOVA. The significance of difference between any 2 groups was tested by Tukey's pairwise comparisons. Within a column, values with the same superscript letter are not significantly different.

MATERIALS AND METHODS

Mice and diets. One-month-old male mice of the long-lived B6C3F1 strain were purchased from Harlan (Indianapolis, IN) and maintained as described after weaning (15). Mice were housed in groups of four per cage and fed a nonpurified diet, PMI Nutrition International Product no. 5001 (Purina Mills, Richmond, IN). At 5 mo of age, the mice were individually housed and randomly assigned to one of two groups, control (CON) or LTCR. CON mice were fed 93 kcal/wk of a defined control diet (AIN-93M, diet no. F05312, BIO-SERV). LTCR mice were fed 52.2 kcal/wk of a defined CR diet (AIN-93M 40% Restricted, diet no. F05314, BIO-SERV). The LTCR mice consumed ~40% fewer calories than the CON group. The CR diet was enriched so that the CR mice consumed approximately the same amount of protein, vitamins, and minerals per gram of body weight as the CON mice. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Riverside.

Experimental design. At 20 mo of age, cohorts of mice in the CON group were randomly assigned to one of seven experimental groups. A CON group continued to be fed 93 kcal/wk of control diet for 8 wk.

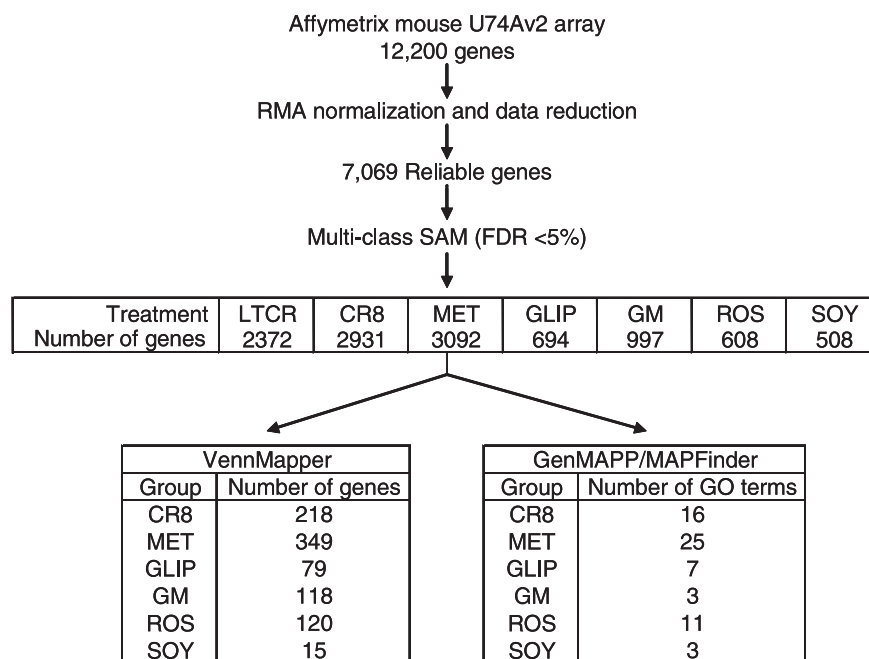
A CR8 group was fed 77 kcal/wk of CR diet for 2 wk, followed by 52.2 kcal/wk of CR diet for 6 wk. The remaining dietary groups were fed the control diets containing MET (Sigma, St. Louis, MO), GLIP (Sigma), GLIP plus MET (GM), ROS, (Avandia, SmithKline Beecham), or SOY (NOVASOY 400, Life Extension Foundation) for 8 wk at the dosages indicated in Table 1. The drugs were mixed with powdered control diet and cold-pressed into 1-g pellets (BIO-SERV). At 0900, all mice were fed 2/7 of the weekly allotment of food on Monday and Wednesday and 3/7 on Friday. The mice had free access to acidified tap water. They were fasted for 48 h and killed by cervical dislocation at 22–28 mo of age. No signs of pathology were detected in the animals used for the studies reported ($n = 4/\text{group}$). Organs were removed rapidly, flash frozen, and stored in liquid nitrogen. The weights of the mice were monitored biweekly. Mouse weights are given in Table 1.

Drug dosages. The dosages used for the drugs were chosen to produce a maximal, but not toxic, effect (Table 1). The MET dosage, 187 mg·kg body wt⁻¹·day⁻¹, is in the range used experimentally in chemically and genetically diabetic mice to control blood glucose levels (60–240 mg·kg body wt⁻¹·day⁻¹; Ref. 33). The GLIP dose (93 mg·kg body wt⁻¹·day⁻¹) was in the range used experimentally to control blood glucose levels in diabetic animals (100 mg·kg body wt⁻¹·day⁻¹; Ref. 34). The ROS dose (7 mg·kg body wt⁻¹·day⁻¹) was consistent with that used in other mouse studies (43). The SOY dosage is reported to prevent or reduce the growth of transplanted tumor cells and inhibit the development of chemical- or radiation-induced tumors in mice (36).

Measurement of specific mRNA levels. Liver total RNA was isolated as described from pathology-free mice ($n = 4/\text{group}$; Ref. 12). mRNA levels were measured using Affymetrix mouse U74Av2 arrays according to standard Affymetrix protocols (Affymetrix).

Probe set expression measurement and normalization. After hybridization and scanning, raw image files were converted to probe set data (*.CEL files) using Microarray Suite (MAS 5.0). Probe set data from all 32 arrays were simultaneously analyzed with the robust multichip average (RMA) method to generate normalized expression measures for each probe set (Fig. 1; Ref. 28). The data were further filtered to exclude probe data sets that were "Absent" across all 32 arrays according to the MAS 5.0 detection algorithm (1, 53).

Fig. 1. Data analysis and nos. of found genes. Affymetrix data were normalized and filtered using Microarray Suite (MAS 5.0) and robust multichip average (RMA), resulting in reliable signals from 7,069 genes. The normalized, filtered data were subjected to multi-class SAM analysis. The no. of differentially expressed genes in each group is shown. The differentially expressed genes were merged into a list of 4,155 genes. These genes were subjected to Venn Mapper analysis to identify the long-term caloric restriction (LTCR)-responsive genes significantly affected by each treatment. The genes were also subjected to GenMAPP/MAPFinder analyses to identify Gene Ontology (GO) terms common to the treatments and LTCR. CR8, 8 wk of caloric restriction; MET, metformin; GLIP, glipizide; GM, GLIP plus MET; ROS, rosiglitazone; SOY, soy isoflavone extract.



Data analyses. Normalized and filtered data from all groups were analyzed using the significance analysis of microarray (SAM) algorithm (Fig. 1; Ref. 51). Genes that were differentially expressed between two or more groups were identified using multiclass SAM analysis. We set the significance cutoff at a median false discovery rate (FDR) of $<5.0\%$. To determine the specific effects of each treatment on gene expression, each treatment group was separately compared with the CON group using a $|1.2|$ -fold change cutoff (Fig. 1).

Comparison of gene expression changes induced by LTCR and the drug treatments. To identify gene expression profiles common to drug treatments and LTCR, we used Venn Mapper, which can determine the number of statistically significant changes in gene expression between pairs of treatment groups (42). Venn Mapper reports the number and identity of up- and downregulated genes in the overlap and a table of z -values, where a z -value of 1.96 indicates a P value of 0.05 and a z -value of 2.58 indicates a P value of 0.01. The table of z -values was imported into TreeView for visualization of the z -profile (Fig. 2; Ref. 18).

Functional comparison of gene expression using Gene Ontology terms. Sequential application of GenMAPP and MAPPFinder was used (9, 17). The Affymetrix probe set IDs, fold changes, and P values from the multiclass SAM and t -test analyses were exported to GenMAPP. The overlapping gene data for each treatment group were color coded and imported into MAPPFinder for global ontology analysis. The criteria for overlapping genes were defined as [(LTCR fold change ≥ 1.2 AND LTCR P value <0.05) AND (Compound fold change ≥ 1.2 AND Compound P value <0.05)] for upregulated transcripts and [(LTCR fold change ≤ -1.2 AND LTCR P value <0.05) AND (Compound fold change ≤ -1.2 AND Compound P value <0.05)] for downregulated transcripts. MAPPFinder linked the expression data of the genes meeting the criterion to the Gene Ontology (GO) hierarchy and assigned a z -score and a P value to each GO biological process, cellular component, and molecular function term. A positive z -score indicates that there are more genes meeting the criterion in a GO term than would be expected by random chance. The P value indicates a significant over- or underrepresentation of genes meeting the criterion for a GO term. The results were further filtered to include only GO terms with z -score ≥ 2 and P value <0.01 and the percentage of genes meeting the criteria ≥ 10 .

Pathway analyses. Microarray expression data were imported into PathwayAssist (<http://stratagene.com>), and all known biological relationships between the differentially expressed genes were graphically identified. PathwayAssist allows the user to query databases and direct the construction and visualization of specific biological interaction networks (BINs). ResNet, the main database used by PathwayAssist, contains molecular networks compiled from the PubMed database by natural language processing.

RESULTS

We administered MET, GLIP, GM, ROS, or SOY to mice, cold packed in their diets for a period of 8 wk. A control group received the diet alone. All these groups were isocaloric, and their weights did not vary significantly before or after the studies (Table 1). The studies also included groups that were LTCR or CR8. The ability of the drugs to reproduce the genomic effects of LTCR was assessed using the criteria described below.

Overlapping responses of CR and the candidate mimetics. We compared the changed genes identified by multiclass SAM (FDR $<5\%$; Table 2). MET actually produced more LTCR-like gene expression changes than CR8. MET produced 1,776 LTCR-like changes in gene expression, or 75% of the total LTCR-responsive genes. CR8 produced 1,675 LTCR-like changes in gene expression, or 71% of the LTCR-responsive

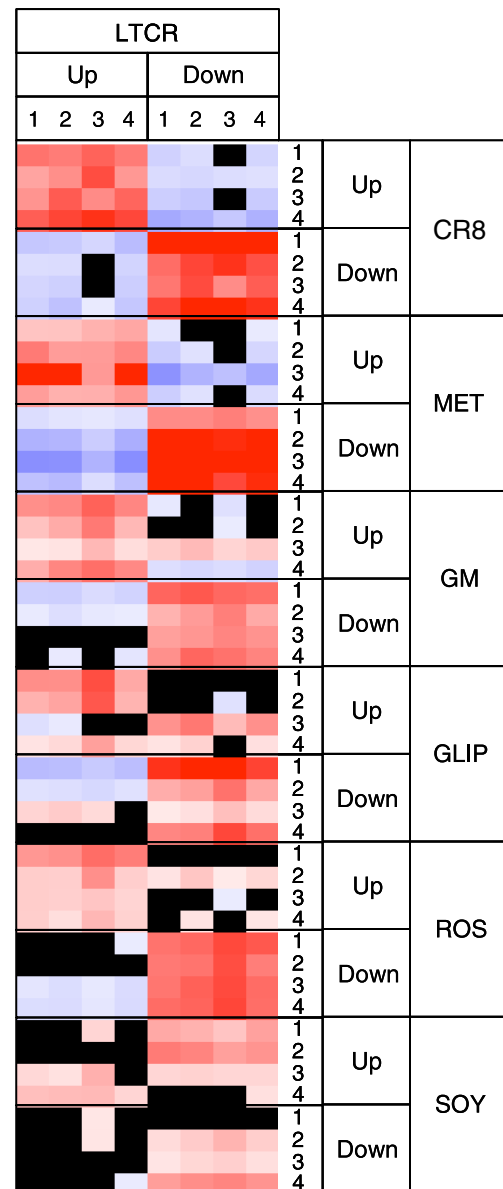


Fig. 2. Heat map of the z -values from Venn Mapper. The z -value table generated by Venn Mapper was visualized using TreeView. Each column represents up- or downregulated genes in each LTCR array. Each row represents upregulated or downregulated genes in the CR8, MET, GM, GLIP, ROS, or SOY groups. The similarity between the results found in the pairs of arrays was measured by calculating the z -value for the no. of commonly shared, differentially expressed genes. z -values are visualized by color intensity. The intensity of the color is dependent on the absolute value of the z -value. Data are shown for z -values >1.96 (P value <0.05). Red represents positive z -values (positive correlations), indicating that there are more overlapping genes than would be expected by chance alone. Blue represents negative z -values (anti-correlations), meaning there are less overlapping genes than expected by chance alone. Black indicates a z -value ≤ 1.96 (P value ≥ 0.05), indicating no similarity between the results in the 2 arrays.

genes. Eight weeks of MET treatment also reproduced 92% of the gene expression changes produced by CR8. The other treatments affected the expression of fewer genes. These results indicate that 8 wk of treatment with CR or MET were highly effective at reproducing the gene expression profile produced by LTCR. MET has been shown to suppress tumorigenesis and/or tumor-related mortality in mice, hamsters, and

Table 2. Numerical overlap between transcriptional effects of LTCR, CR8, and each of the drug treatments

Treatment Group	Number of LTCR-Like Responses	Number of CR8-Like Responses
CR8	1,675 (71%)	
MET	1,776 (75%)	2,163 (92%)
GLIP	373 (16%)	500 (17%)
GM	479 (20%)	679 (23%)
ROS	391 (17%)	375 (13%)
SOY	254 (11%)	375 (13%)

Number of LTCR- or CR8-responsive gene expression changes produced by each treatment. Shown in parentheses are percentages of changed genes relative to LTCR or CR8.

humans, and CR8 is also effective at delaying tumor-related mortality in mice, including death from liver tumors (see DISCUSSION; Refs. 10, 44). Thus these CR8- and MET-responsive genes may have a key role in suppressing tumor-related mortality.

To further statistically quantify the overlap between LTCR and the treatments, the differentially expressed genes were analyzed using *t*-tests and Venn Mapper (Fig. 1). At a significance of $z \geq 2$, MET and CR8 again yielded the highest number of genes overlapping LTCR (349 and 218, respectively). The other treatments produced fewer such genes [ROS (120 genes), GM (118 genes), GLIP (79 genes), and SOY (15 genes)]. These results again indicate that MET reproduces the effects of LTCR even better than CR8.

To further assess the similarity of the effects of MET, CR8, and LTCR responses, the *z*-values generated by Venn Mapper were visualized as a heat map (Fig. 2). Examination of the data in this way confirmed that the co-regulated genes were upregulated and downregulated similarly by the treatments. Thus, at a high level of statistical stringency, the gene expression response to MET is highly similar to LTCR, as indicated by both the number of overlapping genes and the direction of their regulation. This overlap is significantly greater than that of any of the other drugs. On the basis of these data, MET appeared to be the most promising candidate CR mimetic.

Comparative analysis of the efficacy of the treatments at increasing fold change cutoffs. To further explore the similarity between LTCR and the treatments, we determined the number of LTCR-like changes in gene expression at increasing fold change thresholds (Fig. 3). At increasing levels of stringency, MET, followed by CR8, maintained the most overlap with LTCR. Thus, by this metric, MET induced a transcription profile that was more like LTCR than the other treatments, including CR8.

Analysis of the overlap between the treatments at increasing statistical stringency. The overlap between LTCR and the treatments was also examined by directly increasing the level of statistical stringency (Fig. 4). MET, followed distantly by CR8, maintained the highest number of overlapping genes at increasing *z*-values. At $z \geq 22$, MET treatment produced ~300 changed genes, whereas the other treatments, including CR8, produced no changes. Thus, with increasing statistical stringency, MET better duplicated LTCR than did any other treatment, including CR8.

Comparative functional analysis using GO terms. To further explore the similarities between the treatments and LTCR, we

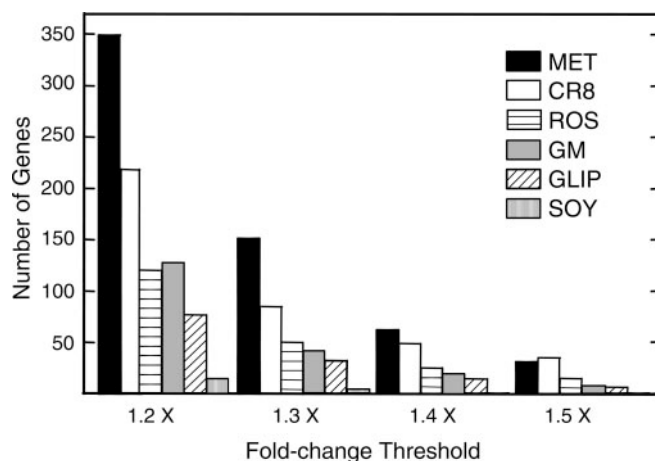


Fig. 3. The no. of overlapping genes between LTCR and the indicated treatments with increasing fold change cutoffs. Gene expression profiles induced by the different compounds were compared with the LTCR profile using Venn Mapper analysis. The comparisons were performed at a *z*-value ≥ 2 and the indicated fold change cutoffs.

used GenMAPP and MAPPFinder on the output of VennMAPPER to create an unbiased estimate of their overlapping physiological effects. The number of GO terms common to LTCR and the treatments is a quantitative measure of their functional similarity. MET produced the highest number of GO terms overlapping those of LTCR, again outstripping even CR8 (Fig. 5). These results predict substantial physiological similarity between the effects of MET and LTCR.

Effects of GLIP on gene expression are dominant over those of MET. GLIP is an insulin secretagogue. The major hepatic effects of MET include insulin sensitization, reduction of gluconeogenesis, and enhancement of glucose utilization. Here, we found that administration of GLIP together with MET reduced the number of MET-responsive, LTCR-like changes in gene expression found by multiclass SAM from 3,092 to 718, a 77% reduction (Fig. 6). In contrast, MET reduced the number of LTCR-like changes in gene expression induced by GLIP by about one-half. The reduced number of MET-responsive genes may be the result of GLIP-enhanced insulin secretion, since

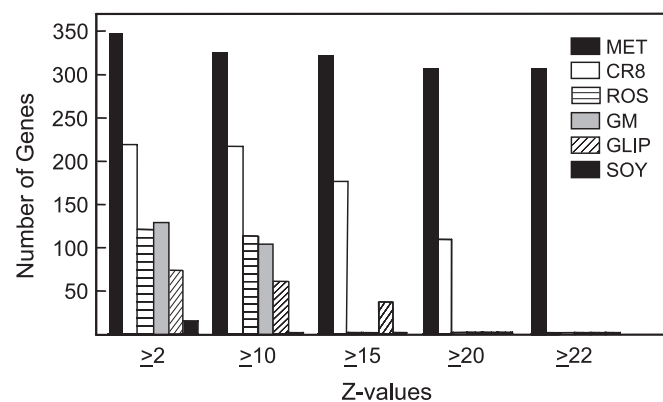


Fig. 4. The no. of overlapping genes between LTCR and the indicated treatments with increasing statistical stringency. Gene expression profiles induced by the different compounds were compared with the LTCR profile using Venn Mapper. The comparisons were performed at a fold change cutoff of 1.2 and the indicated *z*-values.

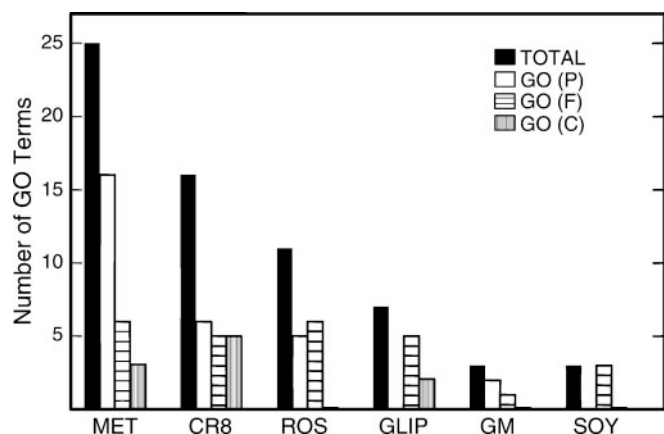


Fig. 5. Relative abundance of GO terms for genes responsive to LTCR and each drug treatment. The bars represent the total no. of GO terms and their distribution across the 3 highest-level branches in the GO tree: biological processes (P), molecular functions (F), and cellular components (C). Output from the GenMAPP/MAPFinder analyses was filtered to include only GO terms with a z -score ≥ 2 , P value < 0.01 , and the percentage of genes meeting the criterion ≥ 10 .

changes in blood glucose levels per se have few effects on hepatic gene expression (20).

Effects of the treatments on the weights of the mice. Changes in body weight cannot explain the results obtained here. There was no significant difference in the body weights of the groups (Table 1). Moreover, the drug-treated and control mice consumed the same number of calories. We inspected the cages for any signs of uneaten food before each feeding and found none. The mice assigned to the MET group weighed on average the same as the control mice. Thus the drug-induced microarray profiles result from the treatments themselves.

DISCUSSION

We investigated the hypothesis that global changes in gene expression identified using high-density microarrays can be used as surrogate biomarkers for the identification of candidate CR mimetics. We tested this hypothesis by screening a number of glucoregulatory pharmaceuticals and a putative chemopreventative for their effects on hepatic gene expression in mice. We found that 8 wk of MET treatment produced a gene expression profile that overlapped LTCR to an even greater extent than the profile produced by 8 wk of CR. We have shown previously that CR begins to enhance lifespan and reduce tumor incidence within 8 wk of its initiation (10). MET matched 75% of the gene expression changes induced by LTCR and 92% of the changes produced by CR8. Thus the analyses described here indicate that MET is a promising candidate CR mimetic.

The homology between the MET and CR profiles is consistent with the similarities in their effects on circulating insulin levels and tissue insulin sensitivity (13, 30). CR lowers blood glucose levels, thereby reducing circulating insulin levels, leading to enhanced tissue insulin sensitivity. MET enhances liver, muscle, and fat insulin sensitivity and reduces hepatic glucose output, leading to lower blood glucose and insulin levels (30). Most of the gene expression effects of CR and MET may be due to reductions in serum insulin levels, since blood glucose levels may not alter hepatic gene expression

(20). However, ROS treatment also lowered serum insulin levels by approximately one-half (unpublished results) but did not produce as many CR-like effects on gene expression. These results suggest that additional changes in physiology also may be reflected in the overlapping expression profiles of CR and MET.

Health effects of MET. LTCR has long been known to reduce the incidence, progression, and growth of cancer, in part by increasing the rate of apoptosis in mitotically competent, and therefore more cancer-prone, tissues (27). Recently, we have shown that CR rapidly reduces the rate of growth (and perhaps onset) of tumors (10). Consistent with the evidence presented here, chronic MET treatment of female transgenic HER-2/neu mice increased their mean lifespan by 8% and their maximum lifespan by 13% (3). It also significantly reduced the incidence and size of mammary adenocarcinomas and increased the mean latency of the tumors. Phenformin, a chemical cousin of MET, extends lifespan of C3H mice by ~23% while reducing tumor incidence by 80% (2, 16). Further, MET treatment may be associated with reduced cancer risk in patients with type 2 diabetes (19). MET completely protects hamsters fed a high-fat diet from malignant, hyperplastic, and premalignant pancreatic lesions (40). Indirect evidence suggests that signaling through AMP-activated protein kinase may mediate both the anti-diabetic and inhibitory effects of MET on cancer cell growth and division (25). AMP-activated protein kinase may be involved in regulating the lifespan of *Caenorhabditis elegans*, *Drosophila*, and yeast (4, 24, 49). MET is also effective in the treatment of polycystic ovary syndrome (26). Finally, MET therapy has been shown to inhibit the development of metabolic syndrome in humans, which is associated with decreased risk of cardiovascular- and diabetes-associated morbidity and mortality (37). Together, these results suggest that microarray biomarkers can be used to identify promising candidate CR mimetics.

Probable function of the MET- and CR-responsive genes. The above conclusions assume that each of the transcriptional responses to CR are of approximately equal importance to its physiological effects. There are a number of lines of evidence supporting this assumption. First, like many of the genetic mutations known to extend lifespan, CR induces a substantial number of changes in gene expression (32). Most lifespan-extending mutations are in signal transduction-related genes,

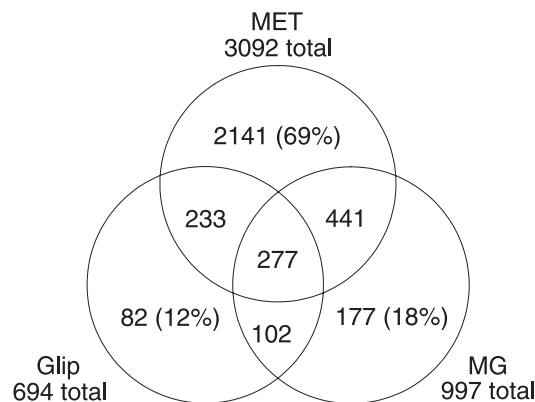


Fig. 6. Differential gene regulation by MET, GLIP, and a combination of the drugs. The numerical overlap between the genes regulated by each treatment is shown.

which regulate batteries of other genes. For example, the Ames dwarf mutation acts additively with LTCR to extend lifespan and change the expression of a substantial number of genes (50). Thus changes in the expression of significant numbers of genes appear to be linked to the lifespan and anti-cancer effects of most or all of the known lifespan interventions. Second, 8 wk of CR is sufficient to initiate its anti-cancer effects in the liver (10). Eight weeks of MET treatment reproduced 75% of the gene expression effects of LTCR and 92% of the effects of CR8 in liver. Furthermore, essentially all of these CR-induced changes were reversed by 8 wk of control feeding. Thus the gene expression effects of LTCR and CR8 are linked temporally to their biological effects. Third, the lifespan extension and anti-disease effects of CR are highly pleiotropic. For example, they clearly differ in liver and heart (10, 11, 44). In concert with these observations, the gene expression changes in liver and heart are different and numerous but easily reconciled with their tissue-specific physiological effects (10, 11, 44). Thus many of the overlapping gene expression effects of MET, LTCR, and CR8 may produce or result from similarities in their physiological effects.

GO terms. The transcriptional response of the liver to LTCR is well characterized in mice (7, 10, 14, 44, 46, 50). The number of overlapping GO terms produced by the treatments is an unbiased measure of their functional similarity. By this measure, MET and LTCR are more similar functionally than even CR8 and LTCR.

BINs contributing to the CR-mimicking effects of MET. The co-regulation of genes by LTCR and MET implies that they utilize common transcription factors and upstream effectors. To further elucidate the functional similarities between the treatments and LTCR, we utilized PathwayAssist. Of the 349 genes co-regulated by LTCR and MET, 206 could be mapped to the ResNet database (version 3.0). These genes were used to build BINs using "Common Regulators," "Common Targets," and "Direct Interactions." The major CR- and MET-associated effector pathways identified by Common Regulators included insulin, tumor necrosis factor (TNF), fibroblast growth factor-2, epidermal growth factor, and elements of their related signal transduction pathways, including Fos, Mapk1 and -8, and P53.

Common Targets identified the major cellular processes targeted by the CR-mimicking effects of MET as apoptosis and cell survival, differentiation, cell proliferation, and focal contact. Direct Interactions indicated that the predominant genetic links between LTCR and MET are closely associated with chaperones. The chaperone network was further analyzed using the "Expand Pathway" option. It identified downregulation of the mostly endoplasmic reticulum chaperones TRA1, HSPA5, GRP58, and DNAJB11 and the related transcription factor XBP1 as major features of the CR-mimicking effects of MET. These chaperones are involved in apoptosis, proliferation, differentiation, inflammation, oxidative stress, and other cellular processes.

We previously found that CR negatively regulates endoplasmic reticulum chaperone expression in liver and several other tissues (7, 10, 45, 48, 50). Chaperone overexpression reduces apoptosis and promotes tumorigenesis, while underexpression enhances apoptosis and prevents tumor formation (29, 47). Chaperone overexpression also is associated with the acquisition of resistance to chemotherapeutics and cell-mediated im-

munity (8, 22, 23, 41, 47). Chaperones can inhibit key effectors of the apoptotic machinery including the apoptosome, the caspase activation complex, and apoptosis-inducing factors (21). For example, HSPA5 (also known as BiP and GRP78) inhibits proapoptotic signaling in part by directly blocking caspase activation (38). These overlapping effects are consistent with the anti-cancer effects of MET and CR.

Other potential regulators of apoptosis and tumorigenesis. Lifespan extension by CR and disrupted IGF-I signaling is associated with reduced serum insulin levels and increased insulin sensitivity (13). MET is an insulin-sensitizing agent with potent anti-hyperglycemic actions (30). Consistent with these properties, Common Regulators identified insulin as a major regulatory factor in both MET and LTCR action. In addition to its glucoregulatory actions and its effects on energy and lipid metabolism, insulin can also be a mitogen or a regulator of apoptosis. Signaling by insulin and the other effectors discussed above converges on the early growth response-1 (ERG1) gene, which increased expression in CR- and MET-treated mice. Signaling through mitogen-activated protein kinase-1 (MAPK1), MAPK8, FOS, and transformation-related protein 53 (TP53; also known as p53), which were also implicated by Common Regulators in the action of both MET and LTCR, also converges on ERG1. ERG1 is a zinc finger, nuclear localized, positive transcription factor that acts as a tumor suppressor. Thus positive regulation of ERG1 may mediate part of the carcinoprotective effects of CR and MET.

MAPK1 has a central role in the induction of apoptosis in response to various stressors, as does MAPK8, which is required for the cytochrome c-mediated cell death pathway (induced by TNF α and UV radiation). FOS family members are also associated with apoptotic cell death, as well as with proliferation and differentiation. Likewise, TP53, a tumor suppressor, plays an essential role in the regulation of the cell cycle and apoptosis. Regulation by TNF and TP53 converges on caspase 6 (CASP6), which was induced by both MET and LTCR. CASP6 is thought to function downstream in the caspase activation cascade.

According to Common Regulators, epidermal growth factor and nerve growth factor- β (NGFB) regulation converges on programmed cell death 4 and neoplastic transformation inhibitor (PDCD4), which was induced by both LTCR and MET. PDCD4 encodes a nuclear-localized tumor suppressor that appears to play a major role in the regulation of apoptosis. NGFB is also associated with HSPA5 regulation. The potential TNF-mediated induction of I κ B kinase- α (CHUK) is another link between LTCR, MET, and apoptosis identified by Common Regulators. CHUK phosphorylates the I κ B family, marking them for ubiquitination and degradation, which can lead to the induction of apoptosis. TANK-binding kinase 1 is another NF κ B-related gene induced by both LTCR and MET. The protein encoded by this gene is similar to the I κ B kinases. It can mediate NF κ B activation in response to growth factors by releasing it from inhibition by the I κ B proteins TANK and TRAF2. Another proapoptotic effect of LTCR and MET is downregulation of TRAF4, which mediates signal transduction by members of the TNF receptor superfamily. This protein interacts with the neurotrophin receptor, p75 (also known as NTR/NTSR1) and antagonizes NF κ B activation and NTR-induced cell death.

In conclusion, the studies described herein show that high-density microarray profiles of gene expression in the liver of LTCR mice can be used to screen compounds and identify potential CR mimetics. These results suggest that drugs capable of reproducing some or all of the life- and healthspan-extending effects of CR may be identified in this manner. They also indicate that MET is a potential CR mimetic.

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