Experimental Gerontology 46 (2011) 148–154

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Review

Glycolytic inhibition as a strategy for developing calorie restriction mimetics

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ARTICLE INFO

Article history:
Received 8 October 2010
Received in revised form 6 December 2010
Accepted 7 December 2010
Available online 15 December 2010

Section Editor: Kurt Borg

Keywords:
Calorie restriction
Diet restriction
Insulin
mTOR
Sirtuin
Glucose
Hexokinase
Resveratrol
Metformin

ABSTRACT

Calorie restriction (CR) remains the most robust environmental intervention for altering aging processes and increasing healthspan and lifespan. Emerging from progress made in many nonhuman models, current research has expanded to formal, controlled human studies of CR. Since long-term CR requires a major commitment of will power and long-term negative consequences remain to be determined, the concept of a calorie restriction mimetic (CRM) has become a new area of investigation within gerontology. We have proposed that a CRM is a compound that mimics metabolic, hormonal, and physiological effects of CR, activates stress response pathways observed in CR and enhances stress protection, produces CR-like effects on longevity, reduces age-related disease, and maintains more youthful function, all without significantly reducing food intake. Over 12 years ago, we introduced the concept of glycolytic inhibition as a strategy for developing mimetics of CR. We have argued that inhibiting energy utilization as far upstream as possible might offer a broader range of CR-like effects as opposed to targeting a singular molecular target downstream. As the first candidate CRM, 2-deoxyglucose, a known anti-glycolytic, provided a remarkable phenotype of CR, but turned out to produce cardiotoxicity in rats. Since the introduction of 2DG as a candidate CRM, many different targets for development have now been proposed at more downstream sites, including insulin receptor sensitizers, sirtuin activators, and inhibitors of mTOR. This review discusses these various strategies to assess their current status and future potential for this emerging research field.

Given the robust nature of the calorie restriction (CR) paradigm for attenuating aging processes and increasing lifespan and healthspan in a wide range of species, recent research has emerged to develop CR mimetics (CRM) (Baur, 2010; Chen and Guarente, 2007; Ingram et al., 2006; Mattson et al., 2001; Roth et al., 2005). The motivation for this effort is driven by the recognition that long-term CR as an intervention for human aging could generate several problems. First, there is the challenge of compliance to long-term dieting in which caloric intake might be reduced to 30–40% below baseline levels. Second, there are the unpleasant side effects, such as reported reductions in body temperature and libido. Third, the health disadvantages that long-term CR could bring, such as possible low bone mineral density or slow wound healing, have yet to be fully determined.

In a paper published in 1998, we first proposed the concept of a CRM and offered a candidate molecule, 2-deoxyglucose (Lane et al., 1998). Since that time, the concept of CRM has widened in its application along with the number of candidate interventions (Baur, 2010; Chen and Guarente, 2007; Ingram et al., 2006; Mattson et al., 2001; Roth et al., 2005). The research area remains a relatively small one as a recent search on PubMed yielded less than 45 citations for the term "calorie/caloric restriction mimetic" or "diet/dietary restriction mimetic." In addition to the increasing numbers of papers being published on this topic, there are an increasing number of companies that now list an interest in CRM on their websites.

In its broader meaning, CRM can apply to any intervention evoking similar benefits on aging, health, and lifespan to those of CR. These might include antioxidants, hormones, metal chelators, and appetite suppressants. Within the more narrow context that we first proposed (Lane et al., 1998), we still characterize CRM as follows (Ingram et al., 2006): (1) mimics the metabolic, hormonal, and physiological effects of CR; (2) activates stress response pathways observed in CR and enhances stress protection; (3) produces CR-like effects on longevity, reduces age-related disease, and maintains more youthful function; and (4) does not significantly reduce food intake, at least over the short-term. The latter criteria has taken on a slight modification, as we have realized that if a candidate CRM significantly alters body composition, that causes body weight/fat reductions, then it is highly likely that it might also effect reduced food intake over the long-term.

As we have outlined previously, the development of CRM can be approached from many different angles with many potentially effective targets. Some of the more recent attempts claiming to be CRM have included manan oligosaccharide (Smith et al., 2010b), alpha-lipoic acid (Merry et al., 2008), thiazolidinediones (Wei et al., 2010), resveratrol (Baur, 2010; Baur et al., 2006; Pearson et al., 2008;

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doi:10.1016/j.exger.2010.12.001

Wood et al., 2004) and rapamycin (Harrison et al., 2009). To organize discussion of strategies taken in the development of CRM, we previously proposed the useful concept of upstream and downstream targeting (Ingram et al., 2006).

1. Sirtuins

The best example of a downstream target would be represented as the great flurry of activity surrounding research in manipulation of sirtuins as a strategy for developing CRM (Baur, 2010; Baur et al., 2006; Chen and Guarente, 2007; Howitz et al., 2003; Kaeberlein, 2010; Kume et al., 2010; Milne et al., 2007; Pearson et al., 2008; Wood et al., 2004). One company that has taken the lead in developing this strategy is Sirtris Pharmaceutical, who lists on the home page of their website the following statement, "Our drug candidates are designed to mimic certain beneficial health effects of calorie restriction, without requiring a change in eating habits by activation of sirtuins, a recently discovered class of enzymes that control the aging process."

The lead compound for the research led by Sirtris is the plant polyphenol, resveratrol, which was identified through a compound screen to be an activator of SIRT1 in mammals and its invertebrate homolog, SIRT2 (Howitz et al., 2003). A wide variety of studies has been conducted to evaluate the potential of this compound to increase healthspan and lifespan to duplicate effects of CR (Baur, 2010). In invertebrate studies including yeast (Howitz et al., 2003), nematodes (Wood et al., 2004), and drosophila (Wood et al., 2004), addition of resveratrol to the diet significantly increased median and maximum lifespan. Given other findings that knock-out of SIRT2 signaling blocked the positive effects of CR on lifespan in invertebrate models (Chen and Guarente, 2007; Wood et al., 2004), these findings were hailed as major evidence of the singular importance of signaling in this pathway for mediating the beneficial effects of CR (Chen and Guarente, 2007; Sinclair, 2005; Wood et al., 2004). When these intervention studies were expanded to mammalian models, the success was more limited. In one study of fish, resveratrol treatment was successful in increasing lifespan (Valenzano et al., 2006). In studies of mice on a normal diet, however, a resveratrol supplemented diet initiated at middle-age (12 months) did not increase mean or maximum lifespan (Baur et al., 2006; Pearson et al., 2008). There was clear evidence of the beneficial effects of the compound on healthspan, as mice on the resveratrol supplemented diet showed less cardiac pathology, greater bone health, reduced cataract incidence, and improved motor performance compared to mice on a control diet (Baur, 2010). Moreover, mice fed a high fat diet supplemented with resveratrol did exhibit improved survival compared to mice on the same diet without resveratrol (Baur et al., 2006). These resveratrol fed mice also showed improved healthspan measured by several indices, as well as a gene transcriptional profile that more closely resembled that of CR mice than control fed mice (Pearson et al., 2008).

The major thrust of development by Sirtris was to produce and characterize synthetic compounds that were direct activators of SIRT1. Several candidate compounds have been identified and become the subject of clinical investigations with the clinical application being diabetes (Feige et al., 2008; Lavu et al., 2008; Milne et al., 2007; Smith et al., 2009). Because aging is not an entity recognized by the US FDA, pharmaceutical development for CRM must seek other appropriate targets. It stands to reason, given the robust effects of CR on the glucoregulation, as demonstrated in several nonhuman primate studies (Gresl et al., 2001; Hansen et al., 1999; Lane et al., 1999), that diabetes would be a logical target for drug development.

Beyond these efforts to develop synthetic SIRT1 activators, research describing the beneficial effects of resveratrol continues to expand at a rapid pace. Resveratrol treatment in rodent models has documented protection against a great variety of insults, including ischemic stroke (Sakata et al., 2010; Yousuf et al., 2009), heart failure (Yang et al., 2010), seizures (Gupta et al., 2002; Wu et al., 2009), Parkinson's disease (Chao et al., 2008; Khan et al., 2010), and Alzheimer's disease (Karuppagounder et al., 2009).

Interestingly, in contrast to the many positive reports on the health benefits of resveratrol, several negative reports have also been published describing failure to replicate prolongevity effects of resveratrol in invertebrate models (Bass et al., 2007; Zou et al., 2009) or demonstrating increased longevity independent of effects on SIRT1/2 (Aljada et al., 2010; Das et al., 2010). Even several of the effects of resveratrol on gene expression stimulated by CR in mice could not be replicated (Barger et al., 2008). Moreover, a recent study questioned the validity of the original assay used to identify resveratrol as a SIRT2/1 activator (Pacholec et al., 2010). A technical artifact involving the flurophore was identified (Pacholec et al., 2010). The implication was that resveratrol did not directly activate SIRT1, nor did the synthetic compounds identified by the same assay activate the gene (Schmidt, 2010). Moreover, these authors reported that one of the Sirtris synthetic compounds, SIRT1720, also did not activate SIRT1 *in vitro* after taking into account the possible assay compound nor did it appear to have any beneficial health effects *in vivo* when given to an ob/ob mouse model (Pacholec et al., 2010). These issues are now subject to intense counter-arguments by the authors of the original resveratrol studies, and it remains to be seen what verdict will emerge (Dai et al., 2010; Schmidt, 2010).

2. mTOR signaling

As another major example of downstream targeting to create a candidate CRM, mTOR has taken center stage recently (Blagosklonny, 2010; Kapahi et al., 2010; Stanfel et al., 2009). Interest in this target emerged out of the debate over the centrality of SIRT1 in mediating the anti-aging effects of CR as well as the growing interest in autophagy (Blagosklonny, 2010; Hands et al., 2009; Kapahi et al., 2010; Salminen and Kaarniranta, 2009; Stanfel et al., 2009). mTOR is a serine/threonine protein kinase purported to be involved in regulating cell survival, cell growth, cell proliferation, cell motility, cell protein synthesis and transcription (Hay and Sonenberg, 2004; Tokunaga et al., 2004) and autophagy (Hands et al., 2009; Salminen and Kaarniranta, 2009). As a mediator of CR effects, it is well positioned to sense cellular nutrient and energy levels and redox status (Tokunaga et al., 2004). In effect, mTOR can integrate input from more upstream pathways, including insulin, IGF-1, and mitogens (Hay and Sonenberg, 2004). Reduced mTOR signaling has been reported in CR studies (Blagosklonny, 2010; Tzatsos and Kandror, 2006). Moreover, genetic downregulation of mTOR has been shown to increase lifespan in a number of model systems, including yeast, worms, and flies (Blagosklonny, 2010; Sarbassov and Sabatini, 2005; Tzatsos and Kandror, 2006; Yang et al., 2006). The most exciting findings reported recently are that pharmacological inhibition of mTOR signaling by dietary supplementation with rapamycin could increase mean and maximum lifespan in mice begun on treatment during middle age (Harrison et al., 2009) combined with a recent report of increased longevity in a cancer-prone mouse strain (Anisimov et al., 2010b). Rapamycin has a long history of interest in treatment of cancers (Ciuffreda et al., 2010; Sparks and Guertin, 2010). Many clinical trials are currently advancing, applying an analog of rapamycin, temsirolimus, in treating a variety of different tumors (Ciuffreda et al., 2010; Sparks and Guertin, 2010; Wang et al., 2005). Additionally, considerable research activity has been generated in evaluating the use of rapamycin as a treatment for a number of neurodegenerative disorders (Swiech et al., 2008; Tatar et al., 2003; Zemke et al., 2007).

The hypothesized mechanism for the benefits of rapamycin is the upregulation of autophagy to remove damaged or misfolded proteins to prevent their aggregation (Tatar et al., 2003; Wang et al., 2005; Zemke et al., 2007). Most impressive are recent reports demonstrating marked attenuation of amyloid-β pathology and cognitive impairments in mouse models of Alzheimer's disease treated with

rapamycin (Caccamo et al., 2010; Spilman et al., 2010), which appear to be related to increased autophagy. However, these results stand in contrast to other recent reports that treatment with rapamycin stimulates production of amyloid-β production (Marwarha et al., 2010; Zhang et al., 2010); thus, this story is still unfolding. Nonetheless, it appears that rapamycin as well as analogs of rapamycin that act to inhibit mTOR signaling may be strong candidates for development of CRM at a downstream site.

3. Insulin signaling

Turning attention to a more upstream target than mTOR and SIRT1, the search for effective CRM can logically focus on insulin signaling (Anisimov, 2003; Bartke, 2008; Ingram et al., 2006; Tatar et al., 2003). Using invertebrate models, genetic manipulation of the daf-2 pathway in a variety of targets demonstrated that reduced signaling could increase lifespan (Anisimov, 2003; Bartke, 2008; Tatar et al., 2003). A major biomarker of reduced insulin signaling is reduced plasma levels of insulin, which is observed in CR mammals and appears to be predictive of longevity in healthy humans (Roth et al., 2002). Biguanides, which include phenformin, buformin, and metformin, have emerged as the class of compounds demonstrating great promise as CRM (Onken and Driscoll, 2010). Due to their robust ability to reduce hyperglycemia, insulin, gluconeogenesis, intestinal glucose absorption, serum lipids and somatomedin, biguanides became major anti-diabetic treatments; however, clinical use of phenformin and buformin was abandoned because of problems with lactic acidosis observed in many patients (Anisimov, 2003). In a number of rodent studies, phenformin was shown to increase lifespan and reduce cancer in mouse strains susceptible to tumors (Anisimov, 2003; Anisimov et al., 2005a,b, 2008, 2010a).

Metformin has become the most widely prescribed medication in the world for treating type 2 diabetes (Kirpichnikov et al., 2002). In many long-term studies, this compound has demonstrated ability to improve the metabolic profile of diabetes apparently through several mechanisms of action. First, hepatic gluconeogenesis is suppressed. Second, metformin enhances peripheral glucose uptake, decreases absorption of glucose from the gastrointestinal tract, and increases fatty acid oxidation (Collier et al., 2006; Kim et al., 2008). Many of these actions appear to result from the activation of adenosine monophosphate-activated protein kinase (AMPK), which produces increased expression of small heterodimer protein (SHP), which inhibits expression of hepatic gluconeogenic genes, including phosphoenolpyruvate carboxykines (PEPCK) and glucose 6-phosphatase (Collier et al., 2006; Kim et al., 2008). AMPK activation also initiates GLUT4 translocation to the plasma membrane to improve insulin independent glucose uptake. Improved insulin binding to insulin receptors results in increased peripheral glucose utilization (Bailey and Turner, 1996; Collier et al., 2006; Kim et al., 2008). With metformin in such wide use, many studies have been conducted to demonstrate that use of this medication increases survival from all-cause mortality in diabetic and cardiovascular disease patients (Eurich et al., 2005; Scarpello, 2003). Additionally, there are numerous reports of reduced incidence of age-related diseases, including cancer (Ben Sahra et al., 2010; Giovannucci et al., 2010), cardiovascular disease (Papanas and Maltezos, 2009), and chronic kidney disease (Pilmore, 2010). Thus, the broad disease effects of metformin treatment are evidence of its potential as a CRM. Dhahbi et al. (2005) provided evidence that the transcriptional profile of mice treated with metformin for 8 weeks matched closely with that produced by CR. Positive effects of metformin treatment on lifespan in rodent models have been reported by Anismov and colleagues in a series of studies of cancer-prone mouse strains (Salminen and Kaarniranta, 2009; Sarbassov and Sabatini, 2005; Scarpello, 2003; Schmidt, 2010). However, in a recent study of Fischer-344 rats, we found no significant effects of metformin (300 mg/kg) on lifespan, but this finding was produced with only one dose of the drug (Smith et al., 2010a). Using the nematode model and a variety of doses, Onken and Driscoll (Sinclair, 2005) recently demonstrated increased lifespan in metformin treated worm cultures, which was dependent upon AMPK expression. In summary, metformin remains a viable CRM for which research will continue to expand because of its widespread clinical use.

4. Glycolytic inhibition

We first proposed glycolytic inhibition as a logical upstream target for developing CRM (Lane et al., 1998). The main contention was that reducing cellular energy processing would stimulate the cell to induce responses similar to that induced by actual CR. Other upstream targets can be identified, such as blocking glucose absorption in the intestine or glucose transport into the cell; however, we felt that by manipulating an intracellular target, we might be most effective in triggering a robust cellular response. Fig. 1 presents the glycolytic pathway where several points of intervention can be observed, specifically inhibition of the enzymes involved in the steps in conversion of glucose to ATP. These enzymes are hexokinase, phosphoglucose isomerase, and phosphofructokinase. We proposed to target the second step in this pathway, phosphoglucose isomerase, and selected 2-deoxy-D-glucose (2DG) as the candidate CRM. 2DG was a well established glycolytic inhibitor used extensively in biochemical assays for this purpose. Regarding *in vivo* effects of 2DG, several features indicated its potential as a CRM. Specifically, injections of 2DG had been shown to inhibit tumor growth (Smith et al., 2010a), induce torpor (Smith et al., 2010b) and increase circulating levels of glucocorticoids (Dark et al., 1994).

In our initial evaluation of 2DG, we fed young male Fischer-344 (F344) rats diets supplemented (by weight) with 0.2%, 0.4%, or 0.6% 2DG to approximate doses of 100–150, 250–300, or 400–450 mg kg, respectively. Within the first few weeks, we observed that the high dose was toxic, resulting in body weight loss and a few deaths; thus, we began feeding this group the 0.6% diet every other week, and this regimen appeared to be well tolerated. Toxicity would be predicted at some high dose of 2DG because of insufficient cellular energy production. This would be consistent with the U-shaped effects of

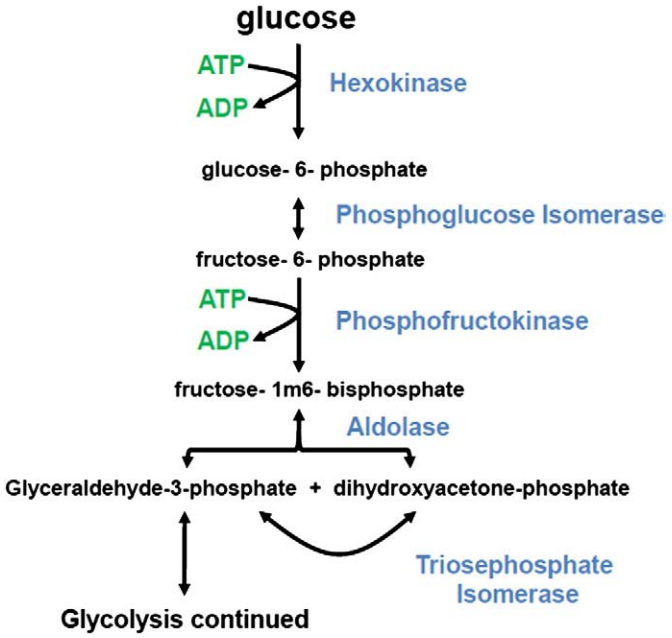

Fig. 1. Glycolysis pathway.

CR on survival. In all respects, the 2DG diets produced a physiological phenotype characteristic of CR but without significant effects on food intake. Plasma insulin and body temperature were reduced in rats on the 0.4% and 0.6% concentration 2DG diets. One concern regarding glycolytic inhibition was the production of hyperglycemia; however, rather than an increase in plasma glucose levels, we saw no significant effects on glucose with any of the diets. Thus, the major observations from this initial study were that a 2DG supplemented diet could affect two major biomarkers of CR, specifically to reduce insulin and body temperature. These two physiological markers are important as they are predictive of longevity as we reported in a study of survival data in human males obtained from the Baltimore Longitudinal Study of Aging (Tatar et al., 2003).

Since our first study of 2DG, many other studies provided additional evidence to strengthen its profile as a CRM. Several studies demonstrated 2DG protection against various *in vivo* and *in vitro* stressors similar to CR. For example, Lee et al. (1999) demonstrated that 2DG protected against glutamate excitoxicity in fetal hippocampal cells. This study also showed evidence of an up-regulation of the stress response proteins, heat shock protein-70 (HSP-70) and glutamate responsive protein-78 (GRP-78). Guo and Mattson (2000) injected rats with 2DG for 12 weeks and found that their cortical synaptosomes exhibited greater protection against iron and amyloid-peptides *in vitro*, and these synaptosome preparations also showed significantly elevated levels of HSP-70 and GRP-78 compared to control preparations. Other short-term *in vivo* studies have provided further evidence that 2DG can enhance stress protection. As examples, Yu and Mattson (1999) used a model of focal ischemia to show that 2DG treatment could attenuate cerebral damage similar to the degree observed in CR. Duan and Mattson (1999) used a mouse model of Parkinson's disease to show that 2DG treated mice, compared to controls, exhibited less dopamine depletion and faster behavioral recovery following treatment with the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). In addition, similar to *in vitro* findings, increased levels HSP-70 and GRP-78 were observed in brains of treated mice. Wan et al. (2003) reported that feeding 2DG (0.4%) to Sprague–Dawley rats for 6 months reduced serum glucose and insulin concentrations and increased ACTH and corticosterone levels, comparable to rats on a CR regimen. This study also employed telemetered measures of locomotor activity, heart rate, and blood pressure. Compared to measures in control fed groups, the latter two measures were reduced again in 2DG treated rats comparable to responses of the CR group. In a follow-up study, Wan et al. (2004) observed that 2DG-fed rats exhibited increased recovery from stress, measured as heart rate, blood pressure, and body temperature following restraint and cold water stress.

Other investigators studying the effects of 2DG on the brain indicate additional parallels to CR. For example, results have demonstrated that short-term CR (e.g. 2 weeks) increases dopamine-related locomotor responses in rats (Fuemayor and Diaz, 1984). Specifically, when challenged with injections of the dopamine agonist, amphetamine, rats exhibit hyperactivity. We have shown similar effects in rats treated with 2DG (Mamczarz et al., 2005). Specifically, we confirmed that rats on CR for 4 months exhibit an enhanced locomotor response to amphetamine and that rats fed 2DG (0.4%) also showed the same hyperactivity. Again these 2DG effects were produced without significant effects on food intake or body weight.

One of the first major hypotheses of cancer metabolism is the Warburg effect (Warburg et al., 1930), which noted that cancer cells upregulated glucose metabolism to support their propensity for rapid growth. Dependent upon cancer cell type, glycolytic enzymes are upregulated but mitochondria production is downregulated (Pedersen, 2007). This metabolic shift provides cancer cells an energetic advantage over normal cells because they can remain alive in the presence of ample oxygen or when oxygen becomes limiting. Recognition of this preferred metabolism using glucose has supported the concept that glycolytic inhibition should be an effective anti-cancer intervention, consistent with the effects of CR on cancer induction and growth (Pedersen, 2007). Prior to our proposal of 2DG as a CRM, several studies had reported beneficial effects of 2DG injections on tumor growth (Gridley et al., 1985). Several other recent studies have again confirmed the marked benefits of a 2DG supplemented feeding on tumor induction and growth. Specifically, Zhu et al. (2005) observed that 2DG markedly attenuated mammary tumor growth in female Sprague–Dawley rats produced by injection of 1-methyl-1-nitrosourea. Moreover, for this study the investigators used concentrations of 0.02% and 0.03% 2DG added to the diet after determining that the concentrations we used in our first study (Lane et al., 1998) significantly inhibited body weight growth in this rat strain, but these lower concentrations did not. Indeed, they noted that 2DG concentrations of 0.06% attenuated body weight growth. Even at the lower concentrations used, 2DG reduced serum insulin and raised serum corticosterone levels similar to CR effects, but the treatment had no significant effects on glucose, leptin, or IGF-1 values. In cultures of cancer cells (MCF-7) treated with 2DG, these investigators found upregulation in important CR-related signaling pathways, including increased levels of phosphorylated AMPK and SIRT1. Given the favorable profile of 2DG cancer treatment, some companies [Threshold Pharmaceuticals, http://www.thresholdpharm.com] attempted commercialization of this compound; however, it appears that clinical trials were terminated or were unsuccessful.

The highest standard of proof that a compound acts as a CRM would be to demonstrate its ability to increase median and maximum lifespan. In regard to 2DG, such proof has been provided in the nematode model of aging (Schulz et al., 2007). Specifically Schulz et al. (2007) added 2DG to worm cultures and noted significant increases in lifespan that were not dependent upon SIRT2, but were dependent upon intact AMPK signaling. Moreover, this study offered a major hypothesis of how glycolytic inhibition could invoke beneficial effects similar to CR. The concept of “mitohormesis” was proposed and supported by results showing short-term production of oxidative stress that produced an adaptive response to increase stress resistance. When the worm cultures were provided antioxidant treatments, including N-acetyl-cysteine, vitamin C, or vitamin E, the prolongevity effects of 2DG were eliminated. Thus, hormesis proposes that a mild stress can improve responses to greater stressors. Over the past few years, this concept of hormesis has taken center stage regarding mechanistic hypotheses of CR (Calabrese, 2004; Mattson, 2008; Rattan, 2008), and this could apply to the actions CRM as well.

In considering the marked parallels between CR and 2DG as described above, it is clear that this compound presented a highly favorable profile as a CRM (Kang and Hwang, 2006). Unfortunately for this emerging positive picture, we conducted both short-term toxicity studies and a long-term survival study and found that the concentrations we had used (0.2–0.4%) produced cardiotoxicity in both Fischer-344 and Brown–Norway rats (Minor et al., 2010). We again confirmed that dietary supplement with 2DG matched the expected phenotype of a CRM, including reduced blood levels of glucose and insulin as well as lower body temperature. However, after 2DG treatment as short as 6 weeks, cardiotoxicity was observed in the form of vacuolarization of cardiac myocytes leading to heart failure in many rats involved in the long-term study. Additionally 2DG treated rats in the survival study had significantly increased incidence of pheochromocytoma in the adrenal medulla. While disappointing, these findings do not discourage an active search for other glycolytic inhibitors as candidate CRM.

Many targets for inhibiting glycolysis exist; therefore, many candidates could have potential efficacy as CRM. Possible targets include glucose transporters as well as other enzymatic steps in glycolysis. For example, iodoacetate acid is known to inhibit glyceraldehyde-3-phosphate. Based on preliminary *in vitro* analysis, this compound exhibited potential as a CRM regarding stress protection. Specifically, pretreatment of fetal rat hippocampal neurons with iodoacetate provided protection against several stresses, including excessive

glutamate, iron, and trophic factor withdrawal, and also produced an up-regulation of heat shock proteins, HSP-70 and HSP-90 (Guo and Mattson, 2000; Guo et al., 2001).

Regarding other candidate glycolytic inhibitors as anti-cancer treatments, currently there is great interest in 3-bromopyruvate (3BP). 3BP is a simple lactic acid analog, a brominated derivative of pyruvic acid, which acts to inhibit hexokinase II (HKII), the first step in glycolysis (Pedersen, 2007). Although no research has focused on this compound as a CRM for attenuating aging, considerable work has been accomplished in various tumor models (Pedersen, 2007). As discussed earlier, the Warburg effect, describing increased glycolytic activities of many tumors, has generated a search for effective HKII inhibitors for cancer treatment. It has been shown that many tumor lines greatly upregulate HKII activity and increase its binding to mitochondria (Pedersen, 2007). Using the VX2 rabbit model of liver cancer, Ko et al. (2001) were able to demonstrate a high level of HKII activity that was effectively lowered with an impressive reduction in growth of the tumors when treated with 3BP IP over the course of several days. Follow-up studies were even more impressive regarding tumor inhibition when the compound was delivered IV to rabbits with no evidence of pathology in other tissues. In addition, Ko et al. (2004) showed similar efficacy against AS-30D hepatoma cells in a rat model even to the point that most of the animals showed no residual signs of the cancer. 3BP was also effective against leukemia cells (Xu et al., 2005). 3BP is hypothesized to enter the tumor cells through lactic acid transporters and inhibit HKII bound to mitochondria (Pedersen, 2007).

Thus, the established anti-tumor effects of 3BP would qualify this compound as a candidate CRM, but there is clearly a need for further evaluation. Although many previous studies have reported little or no toxicity related to treatment with 3BP (Pedersen, 2007), a few studies have noted issues. For example, there has been one report of dose-related toxicity to the liver and gastrointestinal tract in rabbits treated with 3BP via intra-arterial delivery in doses similar to those used in previous studies (Chang et al., 2007). Second, ICV delivery of 3BP in rats can result in reduced brain metabolism, neurotransmitter function, particularly in the cholinergic system, and behavioral impairment (Froelich et al., 1995). Finally, 3BP has also been reported to have inhibitory effects on spermatozoal metabolism (Jones et al., 1996). All these effects, whether anti-tumor effects or negative effects on brain metabolism, are of course subject to dose response. We clearly understand that inhibition of energy production, or major suppression of ATP production, which could be an effective anti-tumor treatment, could be lethal for the cell and the animal. Thus, careful dose studies of 3BP are still required.

Consistent with the actions of 3BP, our latest research involving glycolytic inhibition as a strategy for developing CRM has also focused on inhibitors of HKII. We are proposing a seven carbon sugar, mannoheptulose, as a candidate CRM and have begun conducting experiments to support that proposal using *in vitro* and *in vivo* cell models (Davenport et al., 2010; Roth et al., 2009). For these experiments, we are investigating an extract of avocados that has been found to contain high concentrations of this sugar. Many other HKII inhibitors can be explored to assess their efficacy as CRM.

Rather than inhibit glycolytic enzymes, some anti-cancer drugs have focused on other steps in the glycolytic pathway. For example, several inhibitors of catalytic subunit of glucose-6-phosphatase have been proposed including Ilicicolinic acid (B), oxodiperoxo(1,10-phenanthrolin)vanadate, and tetrahydrothienylpyridine (Parker, 2001). These candidates can be explored to assess their individual efficacy in combination with other glycolytic inhibitors.

5. Summary

Many other targets exist for developing CRM beyond glycolytic inhibition. We covered a few candidates in this review, but have not mentioned several others that mimic mechanisms of CR, including, antioxidants, mitochondrial biogenerators, autophagy stimulators, and inhibitors of insulin signaling. We focused on targets for glycolytic inhibition in this review because it is our contention that such interventions would most directly mimic the metabolic actions of CR, that is, triggering cellular responses to a perceived reduction in energy production. We predict that this upstream manipulation should have a broader range of CR-like effects acting through multiple mechanisms more than would be expected from targeting a downstream target, such as sirtuin stimulation. However, we should recognize that resveratrol stimulation of sirtuin signaling did have a broad range of CR-like effects on the healthspan of mice but did not significantly affect lifespan in normally fed mice. On the other hand, acting on another downstream target of CR signaling, rapamycin, as an inhibitor of mTOR signaling, has been demonstrated to increase lifespan in older mice. Thus, the question remains open regarding strategies of upstream and downstream targeting for development of CRM. Recognizing that CR acts through multiple signaling pathways, we have also previously proposed the possibility that “cocktails” of CRM acting through multiple systems could be more effective than actions through only one pathway (Ingram et al., 2006; Roth et al., 2005). These open questions do not deny the fact that the area of CRM research is rapidly expanding to investigate many potential candidates with the potential to increase both healthspan and lifespan without the required reductions in food intake that CR imposes.

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