SUMMARY OF KEY RIBOCEINE™ STUDIES ADAPTED FROM ORIGINAL PUBLICATIONS

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1. RiboCeine Shown to Protect Against Oxidative Stress

Roberts, J.C.; Nagasawa, H.T.; Zera, R.T.; Fricke, R.F.; Goon, D.J. W. Prodrugs of Lcysteine as protective agents against acetaminophen-induced hepatotoxicity. 2-(polyhydroxyalky)-and 2-(Polyacetoxyalky)-Thiazolidine-4(R)-Carboxylic Acids. J. Med Chem., 1987, 30, 1891-1896.

Study Background

Oxidative stress is a metabolic condition where excess free radicals are being produced. Chronic oxidative stress has been associated with many diseases and disorders including cardiovascular diseases, neurodegenerative disorders, cataract formation, inflammatory diseases, etc. Although there is no proven relationship that oxidative stress is the cause of

these diseases, there is a significant body of evidence that oxidative stress (low glutathione levels) is prevalent in many disease states and disorders.

Basic science researchers in pursuit of determining whether certain compounds are effective in reducing oxidative stress require animal models to conduct their experiments. To mimic chronic oxidative stress in animals, an acute oxidative stress model is used to permit these experiments to be conducted in shorter time periods, thereby saving on time and cost. A widely accepted acute oxidative stress animal model is the acetaminophen over-dose model, wherein severe depletion of liver glutathione is manifested when toxic doses are administered.

Acetaminophen, a widely used pain killer, is not toxic at recommended doses, and is readily metabolized by the liver to non-toxic products, which are then excreted in the urine. However, at high or toxic doses, the normal metabolic pathways become overwhelmed, and the liver will, ironically, metabolize acetaminophen through a different pathway that produces a very reactive and toxic metabolite, thus triggering the development of acute oxidative stress. This toxic metabolite is detoxified by liver glutathione; however, once liver glutathione becomes depleted, this toxic metabolite will react with liver cells leading to liver necrosis and eventually death of the animals.

To overcome acetaminophen-induced acute oxidative stress that causes severe depletion of liver glutathione, researchers administer test compounds believed to promote varying degrees of glutathione production. The compound's effectiveness is determined by the level of protection it provides to the liver, and its ability to prevent animal death. Those compounds that exhibit the best protection to the liver and having the highest animal survival rates are deemed to be the superior compounds. The level of protection directly correlates with the compound's ability to improve liver glutathione levels.

Summary: In this study, a total of 8 sulfhydryl-protected L-cysteine compounds, in addition to N-acetylcysteine (NAC) which is not sulfhydryl-protected, were evaluated in an in vivo mouse model to determine their ability to protect against liver toxicity from a high dose of acetaminophen. RiboCeine was among the sulfhydryl-protected compounds tested. In protecting the sulfhydryl (SH) group of L-cysteine, the authors used naturally-occurring endogenous substances to avoid any possibility of toxicity by the protecting substance itself.

> **Part 1**: The experimental protocol involved the administration of a lethal dose (LD90) of acetaminophen where 90% of the mice were expected not to survive plus the test compounds. Toxicity was assessed on the basis of overall survival of the animals at 48 hours, as well as histological (cell pathology) criteria of liver cell damage assessed by an independent third party "who had no knowledge of the experimental protocols or sample identity". RiboCeine was the only compound tested where no animal death occurred at 48 hours. All other compounds tested had varying rates of animal survival between 30% to 94%. The animals without intervention had only a 17% survival rate. RiboCeine "showed the best histological profile" with all animals showing a necrosis rating of +2 or below (range 4+ to 0). All other compounds tested had animals with histological ratings of 4+.

Part 2: In the in vitro study with cultured hepatocytes (liver cells), RiboCeine at 40% of the dose of NAC raised liver cell glutathione levels 130% better than NAC. Since BSO, an inhibitor of glutathione synthesis, prevented this glutathione increase, it was concluded that the L-cysteine from RiboCeine must have been utilized in the 13 newly formed glutathione.

Conclusion: The collective in vivo and in vitro data suggested that RiboCeine was readily bioavailable, was superior to the other compounds tested, and should serve as an effective cysteine delivery system to the liver.

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RIBOCEINE WAS SHOWN TO BE 300% MORE EFFECTIVE

THAN NAC IN RAISING LIVER GLUTATHIONE LEVELS

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Table 1. Increased GSH Content of Rat Hepatocytes after Incubation with L-cysteine Prodrugs

Prodrug	conen, mM	[GSH] ± SE, nmol/ 10^6 cells	[GSH] rel to controls
none (control)		35.4 ± 0.78	1.0
GlcCys (1g)	1.0	75.2 ± 2.15	2.1
RibCys (1d)	1.0	61.2 ± 1.52	1.7
XylCys (1e)	1.0	58.3 ± 0.99	1.6
GalCys (1f)	1.0	58.0 ± 2.00	1.6
ManCys (1g)	1.0	57.8 ± 0.87	1.6
GlysCys (1a)	1.0	46.1 ± 1.10	1.3
LyxCys (1c)	1.0	45.9 ± 1.95	1.3
AraCys (1b)	1.0	42.9 ± 2.17	1.2
N-acetyl-L-cysteine (NAC)	2.5	45.8 ± 1.27	1.3

On the table above, even though 2 ½ times more NAC was used in the incubation of these hepatocytes, the glutathione levels were 30% lower than the cells that were incubated with RiboCeine (RibCys). Therefore, RiboCeine was at least 300% more effective in raising liver cell glutathione than NAC was in this liver cell model.

2. Organ Glutathione Levels after RiboCeine Roberts, J.C.; Francetic, D.J. Time course for the elevation of glutathione in numerous organs of L1210-bearing CDF1 mice given the L-cysteine prodrug, RibCys. Toxicology Letters, 1991, 59, 245-251. Study Background It is well accepted by the scientific community that glutathione levels in cells are homeostatically controlled, and dramatic elevations in glutathione levels are not expected because its biosynthesis is stringently controlled by feedback inhibition1 . Therefore, glutathione may only achieve a certain level before the biosynthetic machinery would be turned off, regardless of the level of amino acid precursors available in the cell1 . However, in the presence of a toxic substance that requires glutathione for detoxification, the levels of glutathione in the liver are continually decreasing and virtually depleted in some cases (acetaminophen overdoses). The value of these glutathione

elevation studies is not in the “absolute increase of glutathione achieved”, but rather the compound’s ability to maintain glutathione levels under oxidative stress conditions. Glutathione levels in many organs fall as the result of fasting, and therefore, compounds can be tested in fasting animals for their ability to elevate and maintain organ glutathione. Summary: In this study, RibCys (RiboCeine) was evaluated in fasting mice for its ability to improve glutathione levels in numerous organs at different time points after RiboCeine administration. After 8 to 10 hours of fasting, glutathione levels dropped approximately 43% in the liver, 41% in the bladder, 31% in the kidney, 25% in the heart and 60% in muscle. Other organs, such as the spleen, pancreas, and lung, showed no significant differences in glutathione levels between the two nutritional states. 15 Results: “Glutathione in the liver was elevated 1.5 fold compared to untreated controls at the 16 hour time point. Kidney glutathione also was maximal at 16 hours and achieved 1.6 times control values. Glutathione in muscle achieved 2.5 times the levels in control animals, while the bladder was elevated 2.1 fold and the heart 1.8 fold.” Conclusion: The authors concluded that “RibCys was able to maintain, and, in some organs, continued to elevate glutathione even though the animals were subject to continued fasting”. “The results from the present studies support the hypothesis that RibCys can serve as a reservoir for the crucial glutathione precursor, L-cysteine, and continually supply the amino acid as glutathione synthesis proceeds.” 1 Meister A, J., Glutathione metabolism and its selective modification. Biol. Chem., 1988, 273, 17205-17208.