# Protective and adaptive responses by antioxidant flavonoids

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ISBN: 978-94-6295-106-8

Cover design: Chris Lemmens ~ Kloroform
Layout: Kristien Lemmens
Printed by: Uitgeverij BOXPress, Proefschriftmaken.nl

The research presented in this dissertation was conducted at NUTRIM School for Nutrition, Toxicology and Metabolism of Maastricht University which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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Financial support for printing of this thesis was kindly provided by Greiner Bio-one (Alphen a/d Rijn).

# Protective and adaptive responses by antioxidant flavonoids

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. dr. L.L.G. Soete,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op woensdag 15 april 2015 om 12.00 uur

door

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geboren te Oupeye (België) op 14 februari 1986

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Chapter 1

# General introduction

**Oxidative stress**

Living organisms continuously produce reactive species such as free radicals. Free radicals are molecules with one or more unpaired electrons in their outer orbital, which makes them very unstable and reactive towards other molecules to obtain a paired electron. Besides free radicals also reactive species that contain a paired electron are formed, e.g. peroxynitrite. All these reactive molecules are generally divided in reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Table 1) (Kohen and Nyska, 2002).

**Table 1.** Reactive oxygen species (ROS) and reactive nitrogen species (RNS)

| | Radicals | Nonradicals |
|---|---|---|
| ROS | Superoxide, $O_2^{\bullet-}$<br>Hydroxyl, $OH^{\bullet}$<br>Peroxyl, $RO_2^{\bullet}$<br>Alkoxyl, $RO^{\bullet}$ | Hydrogen peroxide $H_2O_2$<br>Hypochlorous acid, HOCl<br>Ozone, $O_3$<br>Singlet oxygen, $\Delta g$ |
| RNS | Nitric oxide, $NO^{\bullet}$<br>Nitrogen dioxide, $NO_2^{\bullet}$ | Nitrous acid, $HNO_2$<br>Dinitrogen tetroxide, $N_2O_4$<br>Dinitrogen trioxide, $N_2O_3$<br>Peroxynitrous acid, ONOOH<br>Nitronium cation, $NO_2^+$<br>Alkyl peroxinitrites, ROONO |

ROS can be formed endogenously. The main source of ROS is aerobic respiration. Aerobic respiration requires oxygen in order to generate ATP. The process in which ATP is produced, called oxidative phosphorylation, involves electron transport. In this process oxygen is reduced to water, but 1 to 2 % of the oxygen is partly reduced (Magder, 2006). This leads to the formation of reactive species, especially superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide ($H_2O_2$). In the presence of transition metal ions, the even more reactive hydroxyl radical ($OH^{\bullet}$) can be formed. Hydroxyl radicals react immediately with virtually any molecule it encounters. Most other radicals are less reactive and thus will react more selectively (Galli et al., 2005).

ROS are also produced by e.g. peroxisomal β-oxidation of fatty acids and the cytochrome P450 system. There are also exogenous sources of radicals including ionizing radiation, ultraviolet light, toxins and cigarette smoke (Valko et al., 2007).
ROS are involved in various physiological effects including cell to cell signaling, essential in maintaining homeostasis. In addition, ROS play a pivotal role in catalytic oxidation of some endogenous compounds and xenobiotics, maintain vascular tone and are part of the physiological host defense. Moreover, increased ROS production can induce cellular senescence and apoptosis which could protect against tumor genesis or uncontrolled proliferation (Valko et al., 2007).
An excessive production of ROS results in oxidative stress, causing damage to lipids, proteins and nucleic acids (Diplock et al., 1998). Due to this (uncontrolled) redox imbalance various pathologies can emerge. An excessive ROS production has been implicated in the pathogenesis of cancer, cardiovascular disease, chronic inflammation, neurological and neurodegenerative disorders, diabetes, ischemia/reperfusion, ageing and other age-related diseases (Alfadda and Sallam, 2012; Haenen et al., 1990; Sundar et al., 2013; Van der Vliet and Bast, 1992).

The body contains potent antioxidant defenses which balance the level of oxidants and thus preserve homeostasis and protect against damage. One important pathway in the protection against ROS is the Nuclear factor erythroid 2-related factor 2 (NRF2) pathway (Lyakhovich et al., 2006). The NRF2 pathway is an antioxidant defense system to protect against oxidative damage. In this pathway KEAP1 is the inhibitor of the transcription factor NRF2. KEAP1 functions as an in vivo sensor for ROS and electrophiles, which causes NRF2 to dissociate from the complex and translocate to the nucleus (Itoh et al., 1997; Lo et al., 2006). Consequently NRF2 activates the transcription of numerous detoxifying and antioxidant genes, mediated by antioxidant response elements (ARE). The defense system regulates the level of detoxifying enzymes as well as non-enzymatic antioxidants (Figure 1).

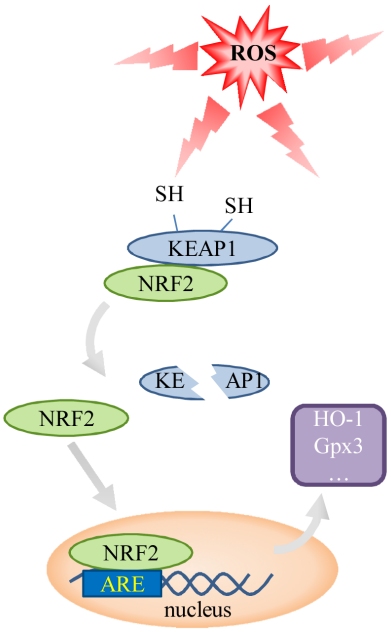


**Figure 1.** Overview of the induction of the adaptive response of the cell by ROS. ROS attack KEAP1 and activate the NRF2 pathway which stimulates the transcription of numerous detoxifying and antioxidant genes, mediated by antioxidant response elements (ARE)

Important detoxifying enzymes are superoxide dismutases (SOD), catalases, glutathione peroxidases (Gpx) and heme oxygenase 1 (HO-1) (Diplock et al., 1998; Surh et al., 2009). SOD catalyze the conversion of superoxide into oxygen and hydrogen peroxide. Three forms of SOD exist. SOD1 and SOD3 contain copper and zinc, while SOD2 contains manganese. SOD1 is located in the cytoplasm, SOD2 in the mitochondria and SOD3 is extracellular (Zelko et al., 2002).

Catalases catalyze the conversion of hydrogen peroxide to water and oxygen. The enzymes are localized in peroxisomes of the cell (Chaudiere and Ferrari-Iliou, 1999; Zamocky and Koller, 1999).

Gpx catalyze the reduction of lipid hydroperoxides to alcohol and hydrogen peroxide into water. Different forms exist; Gpx1-Gpx8 which have different substrate specificity. Gpx contain selenium and use glutathione (GSH) as cofactor (Chaudiere and Ferrari-Iliou, 1999; Flohe, 1978; Yamamoto and Takahashi, 1993).

HO-1 catalyzes the degradation of the potentially toxic heme to biliverdin, iron and carbon monoxide (Gozzelino et al., 2010).

The most important non-enzymatic antioxidants are GSH, ascorbate and uric acid.

GSH is a cysteine-containing tripeptide which is synthesized in cells and therefore not required in the diet although cysteine is an essential amino acid (Meister, 1991). The antioxidant properties of GSH come from the thiol-group in its cysteine moiety, which has reducing as well as nucleophilic properties (Zhang and Forman, 2012).

Ascorbate is an essential cellular constituent. It must be obtained from the diet because it cannot be synthesized by humans (Englard and Seifter, 1986; Pauling, 1970). Ascorbate is an electron donor and once oxidized, converted into dehydroascorbate. Ascorbate can be regenerated from dehydroascorbate by the enzyme dehydroascorbate reductase (Chaudiere and Ferrari-Iliou, 1999; Meister, 1992).

Uric acid is a product of the metabolic breakdown of purine nucleotides. Uric acid is oxidized to alantoine when it scavenges radicals (Becker, 1993; Glantzounis et al., 2005).

**Flavonoids**

Flavonoids are polyphenolic compounds ubiquitously present in fruits, vegetables and plant-derived foodstuffs. More than 5000 naturally occurring flavonoids have been identified (Nichenametla et al., 2006; Ren et al., 2003). The major dietary intake of flavonoids in Western Europe on average is 100-1000 mg per day (Hertog et al., 1993; Kozlowska and Szostak-Wegierek, 2014).

Flavonoids are divided in classes according to their chemical structure. Flavonoids have a common basic structure; a carbon skeleton, which consists of two phenyl rings (A and B) and mostly a heterocyclic ring (C) (Figure 2). The major classes are flavonols, flavones, flavanone, flavanols, anthocyanidins, dihydroflavonols and chalcones (Scalbert and Williamson, 2000).

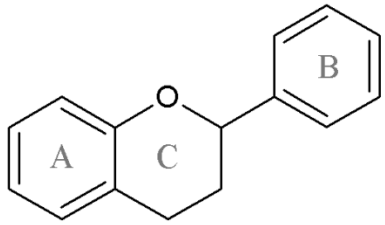


**Figure 2.** Basic structure of flavonoids

Several epidemiological studies showed that a relatively high intake of flavonoids is associated with a reduced risk of various chronic diseases, including cardiovascular diseases e.g. coronary heart diseases, lung diseases such as COPD and asthma, liver disorders (e.g. NAFLD) and certain types of cancers (Queen and Tollefsbol, 2010; Tabak et al., 2001; van de Wier et al., 2014; Visioli and Davalos, 2011). In various tumor cell lines flavonoids showed anti-proliferative and cytotoxic effects (Choi et al., 2001; Naasani et al., 2003; Wang et al., 1999). However, some studies failed to show this or even reported an adverse association (Hirvonen et al., 2001; Knekt et al., 2000; Knekt et al., 2002; Rimm et al., 1996). Further research is needed to substantiate the protective role of flavonoids in the etiology of chronic diseases.
The protective effect of flavonoids is due to their effect on a molecular level. Flavonoids display a multitude of effects including, antioxidant, antibacterial, antiviral, anti-inflammatory, vasodilatory activity (Boots et al., 2008; Cushnie and Lamb, 2005; Hu et al., 1994; Middleton et al., 2000; Nichenametla et al., 2006; Park et al., 2007; van Acker et al., 2000; Xie et al., 2014). Although it has been studied for several decades, the molecular mechanism of flavonoids involved in the protection against various pathologies is still enigmatic.

Besides naturally occurring flavonoids semisynthetic flavonoids were developed to improve their antioxidant activity, metabolic stability or water solubility. An example of such a flavonoid is 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER), a compound derived from the naturally occurring flavonoid rutin. MonoHER is a constituent of Venoruton®, a registered drug that is used in the treatment for chronic venous insufficiency. The drug contains several hydroxyethylrutosides of which

monoHER appeared to be the most powerful antioxidant (van Acker et al., 1993). The antioxidant activity was also reflected in its effective protection against doxorubicin-induced cardiotoxicity in mice (van Acker et al., 1995).

**Molecular mechanism**

Initially, the beneficial effect of antioxidants was ascribed to their direct antioxidant effect i.e. their ability to scavenge radicals (Halliwell, 1996). During the scavenging, antioxidants donate an electron or a hydrogen atom to the radical involved. In this way the radical is neutralized and the noxious potential of the radical is eliminated. However, in their protective effect, antioxidants are converted into oxidation products that take over part of the reactivity of the radical that is scavenged (Bast and Haenen, 2002). Due to this lower reactivity the oxidized antioxidant is less noxious, and thus more selective compared to that of the radical. Hence, the reactivity of oxidized antioxidants is more selective than that of the initial reactive radical (Boots et al., 2002).

When antioxidant flavonoids exert their scavenging activity, oxidation products - called quinones - are formed. To protect against the reactivity of the quinones, cells have an antioxidant network. Two well-known antioxidants in this network are ascorbate and GSH (Meister, 1994). It should be noted that they neutralize the reactivity of the quinones in a different way. Ascorbate reduces the quinones back to their parent flavonoid. In contrast, GSH forms GSH-flavonoid adducts (Boots et al., 2003). The GSH-flavonoid adduct is also reactive towards thiol containing proteins. Moreover, GSH is consumed. The way in which quinones channel their reactivity into the antioxidant network differs between flavonoids (Boots et al., 2003; Moalin et al., 2012).

**Aim and outline of the thesis**

In this thesis we further elaborate on the molecular mechanism of the antioxidant effect of flavonoids. As described in chapter 1, the molecular mechanism behind the protective effect of flavonoids is not clear yet.

The contribution of the radical scavenging activity to the antioxidant effect is questioned. Therefore, in **chapter 2** the direct antioxidant activity of the flavonoid monoHER in the protection against intracellular oxidative stress was evaluated at several levels, its site specific scavenging effect, its position in the antioxidant network and its location in the vascular system.

After scavenging, flavonoids are oxidized and become reactive themselves. In **chapter 3** the channeling of the reactivity of the structurally related oxidized flavonoids, quercetin and tamarixetin, towards the thiol containing proteins, CK or into the antioxidant network, specifically towards ascorbate, is determined.

When flavonoids scavenge ROS, the oxidative damage is prevented. The protection against ROS can backfire because the activation of the innate NRF2 pathway might be prevented as well. As a consequence, no protective adaptation is elicited because antioxidant administration prevents the endogenous defensive shield to be raised. The effect of monoHER on the cellular adaptive response is studied in **chapter 4**.

In **chapter 5** the effect of monoHER on oxidative stress and the adaptive response during the onset of NAFLD was determined in an animal model. The effect was evaluated on three levels, namely the direct damage by ROS, the NRF2-induced gene expression and the adaptive response on the cellular redox status.

In mice, monoHER protects against doxorubicin-induced cardiotoxicity. The contribution of the major metabolite, 4'-O-methylmonoHER to the antioxidant activity of monoHER was investigated in **chapter 6**. The metabolite was synthesized and its antioxidant activity was evaluated and compared to that of monoHER. Besides the potency to scavenge radicals, the channeling of the reactivity of the oxidized product towards proteins was studied.

**Chapter 7** puts the findings of the present study in a further perspective. The antioxidant, anti-inflammatory and other activities and their contribution to the beneficial health effects of the nutraceutical monoHER is discussed.

Finally, the most important results and future perspectives are discussed in **chapter 8**.

## References

Alfadda, A.A., Sallam, R.M., 2012. Reactive oxygen species in health and disease. Journal of Biomedicine & Biotechnology 2012, 936486.

Bast, A., Haenen, G.R., 2002. The toxicity of antioxidants and their metabolites. Environmental Toxicology and Pharmacology 11, 251-258.

Becker, B.F., 1993. Towards the physiological function of uric acid. Free Radical Biology & Medicine 14, 615-631.

Boots, A.W., Haenen, G.R., den Hartog, G.J., Bast, A., 2002. Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. Biochimica et Biophysica Acta 1583, 279-284.

Boots, A.W., Kubben, N., Haenen, G.R., Bast, A., 2003. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. Biochemical and Biophysical Research Communications 308, 560-565.

Boots, A.W., Wilms, L.C., Swennen, E.L., Kleinjans, J.C., Bast, A., Haenen, G.R., 2008. In vitro and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. Nutrition 24, 703-710.

Chaudiere, J., Ferrari-Iliou, R., 1999. Intracellular antioxidants: from chemical to biochemical mechanisms. Food and Chemical Toxicology 37, 949-962.

Choi, J.A., Kim, J.Y., Lee, J.Y., Kang, C.M., Kwon, H.J., Yoo, Y.D., Kim, T.W., Lee, Y.S., Lee, S.J., 2001. Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. International Journal of Oncology 19, 837-844.

Cushnie, T.P., Lamb, A.J., 2005. Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents 26, 343-356.

Diplock, A.T., Charleux, J.L., Crozier-Willi, G., Kok, F.J., Rice-Evans, C., Roberfroid, M., Stahl, W., Vina-Ribes, J., 1998. Functional food science and defence against reactive oxidative species. The British Journal of Nutrition 80 Suppl 1, S77-112.

Englard, S., Seifter, S., 1986. The biochemical functions of ascorbic acid. Annual Review of Nutrition 6, 365-406.

Flohe, L., 1978. Glutathione peroxidase: fact and fiction. Ciba Foundation symposium, 95-122.

Galli, F., Piroddi, M., Annetti, C., Aisa, C., Floridi, E., Floridi, A., 2005. Oxidative stress and reactive oxygen species. Contributions to Nephrology 149, 240-260.

Glantzounis, G.K., Tsimoyiannis, E.C., Kappas, A.M., Galaris, D.A., 2005. Uric acid and oxidative stress. Current Pharmaceutical Design 11, 4145-4151.

Gozzelino, R., Jeney, V., Soares, M.P., 2010. Mechanisms of cell protection by heme oxygenase-1. Annual Review of Pharmacology and Toxicology 50, 323-354.

Haenen, G.R., Veerman, M., Bast, A., 1990. Reduction of beta-adrenoceptor function by oxidative stress in the heart. Free Radical Biology & Medicine 9, 279-288.

Halliwell, B., 1996. Antioxidants in human health and disease. Annual Review of Nutritrion 16, 33-50.

Hertog, M.G., Feskens, E.J., Hollman, P.C., Katan, M.B., Kromhout, D., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342, 1007-1011.

Hirvonen, T., Virtamo, J., Korhonen, P., Albanes, D., Pietinen, P., 2001. Flavonol and flavone intake and the risk of cancer in male smokers (Finland). Cancer Causes & Control : CCC 12, 789-796.

Hu, C.Q., Chen, K., Shi, Q., Kilkuskie, R.E., Cheng, Y.C., Lee, K.H., 1994. Anti-AIDS agents, 10. Acacetin-7-O-beta-D-galactopyranoside, an anti-HIV principle from

Chrysanthemum morifolium and a structure-activity correlation with some related flavonoids. Journal of Natural Products 57, 42-51.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., Nabeshima, Y., 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochemical and Biophysical Research Communications 236, 313-322.

Knekt, P., Isotupa, S., Rissanen, H., Heliovaara, M., Jarvinen, R., Hakkinen, S., Aromaa, A., Reunanen, A., 2000. Quercetin intake and the incidence of cerebrovascular disease. European Journal of Clinical Nutrition 54, 415-417.

Knekt, P., Kumpulainen, J., Jarvinen, R., Rissanen, H., Heliovaara, M., Reunanen, A., Hakulinen, T., Aromaa, A., 2002. Flavonoid intake and risk of chronic diseases. The American Journal of Clinical Nutrition 76, 560-568.

Kohen, R., Nyska, A., 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicologic Pathology 30, 620-650.

Kozlowska, A., Szostak-Wegierek, D., 2014. Flavonoids--food sources and health benefits. Roczniki Panstwowego Zakladu Higieny 65, 79-85.

Lo, S.C., Li, X., Henzl, M.T., Beamer, L.J., Hannink, M., 2006. Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. The EMBO Journal 25, 3605-3617.

Lyakhovich, V.V., Vavilin, V.A., Zenkov, N.K., Menshchikova, E.B., 2006. Active defense under oxidative stress. The antioxidant responsive element. Biochemistry. Biokhimiia 71, 962-974.

Magder, S., 2006. Reactive oxygen species: toxic molecules or spark of life? Critical care 10, 208.

Meister, A., 1991. Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. Pharmacology & Therapeutics 51, 155-194.

Meister, A., 1992. On the antioxidant effects of ascorbic acid and glutathione. Biochemical Pharmacology 44, 1905-1915.

Meister, A., 1994. Glutathione, ascorbate, and cellular protection. Cancer Research 54, 1969s-1975s.

Middleton, E., Jr., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacological Reviews 52, 673-751.

Moalin, M., van Strijdonck, G.P., Bast, A., Haenen, G.R., 2012. Competition between ascorbate and glutathione for the oxidized form of methylated quercetin metabolites and analogues: tamarixetin, 4'-O-methylquercetin, has the lowest thiol reactivity. Journal of Agricultural and Food Chemistry 60, 9292-9297.

Naasani, I., Oh-Hashi, F., Oh-Hara, T., Feng, W.Y., Johnston, J., Chan, K., Tsuruo, T., 2003. Blocking telomerase by dietary polyphenols is a major mechanism for limiting the growth of human cancer cells in vitro and in vivo. Cancer Research 63, 824-830.

Nichenametla, S.N., Taruscio, T.G., Barney, D.L., Exon, J.H., 2006. A review of the effects and mechanisms of polyphenolics in cancer. Critical Reviews in Food Science and Nutrition 46, 161-183.

Park, H.H., Lee, S., Oh, J.M., Lee, M.S., Yoon, K.H., Park, B.H., Kim, J.W., Song, H., Kim, S.H., 2007. Anti-inflammatory activity of fisetin in human mast cells (HMC-1). Pharmacological research 55, 31-37.

Pauling, L., 1970. Evolution and the need for ascorbic acid. Proceedings of the National Academy of Sciences of the United States of America 67, 1643-1648.

Queen, B.L., Tollefsbol, T.O., 2010. Polyphenols and aging. Current Aging Science 3, 34-42.

Ren, W., Qiao, Z., Wang, H., Zhu, L., Zhang, L., 2003. Flavonoids: promising anticancer agents. Medicinal Research Reviews 23, 519-534.
Rimm, E.B., Katan, M.B., Ascherio, A., Stampfer, M.J., Willett, W.C., 1996. Relation between intake of flavonoids and risk for coronary heart disease in male health professionals. Annuals of Internal Medicine 125, 384-389.
Scalbert, A., Williamson, G., 2000. Dietary intake and bioavailability of polyphenols. The Journal of Nutrition 130, 2073S-2085S.
Sundar, I.K., Yao, H., Rahman, I., 2013. Oxidative stress and chromatin remodeling in chronic obstructive pulmonary disease and smoking-related diseases. Antioxidants & Redox Signaling 18, 1956-1971.
Surh, Y.J., Kundu, J.K., Li, M.H., Na, H.K., Cha, Y.N., 2009. Role of Nrf2-mediated heme oxygenase-1 upregulation in adaptive survival response to nitrosative stress. Archives of Pharmacal Research 32, 1163-1176.
Tabak, C., Arts, I.C., Smit, H.A., Heederik, D., Kromhout, D., 2001. Chronic obstructive pulmonary disease and intake of catechins, flavonols, and flavones: the MORGEN Study. American Journal of Respiratory and Critical Care Medicine 164, 61-64.
Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology 39, 44-84.
van Acker, F.A., Schouten, O., Haenen, G.R., van der Vijgh, W.J., Bast, A., 2000. Flavonoids can replace alpha-tocopherol as an antioxidant. FEBS letters 473, 145-148.
van Acker, S.A., Kramer, K., Grimbergen, J.A., van den Berg, D.J., van der Vijgh, W.J., Bast, A., 1995. Monohydroxyethylrutoside as protector against chronic doxorubicin-induced cardiotoxicity. British Journal of Pharmacology 115, 1260-1264.
van Acker, S.A., Towart, R., Husken, B.C., De Jong, J., van der Vijgh, W.J., Bast, A., 1993. The protective effect of Venoruton and its main constituents on acute doxorubicin-induced cardiotoxicity. Phlebology Supplements. 1, 31-32.
van de Wier, B., Koek, G.H., Bast, A., Haenen, G.R., 2014. The potential of flavonoids in the treatment of non-alcoholic fatty liver disease. Critical Reviews in Food Science and Nutrition., in press.
Van der Vliet, A., Bast, A., 1992. Effect of oxidative stress on receptors and signal transmission. Chemico-Biological Iinteractions 85, 95-116.
Visioli, F., Davalos, A., 2011. Polyphenols and cardiovascular disease: a critical summary of the evidence. Mini Reviews in Medicinal Chemistry 11, 1186-1190.
Wang, I.K., Lin-Shiau, S.Y., Lin, J.K., 1999. Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. European Journal of Cancer 35, 1517-1525.
Xie, Y., Yang, W., Chen, X., Ren, L., 2014. Antibacterial Activities of Flavonoids: Structure-Activity Relationship and Mechanism. Current Medicinal Chemistry.
Yamamoto, Y., Takahashi, K., 1993. Glutathione peroxidase isolated from plasma reduces phospholipid hydroperoxides. Archives of Biochemistry and Biophysics 305, 541-545.
Zamocky, M., Koller, F., 1999. Understanding the structure and function of catalases: clues from molecular evolution and in vitro mutagenesis. Progress in Biophysics and Molecular Biology 72, 19-66.
Zelko, I.N., Mariani, T.J., Folz, R.J., 2002. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radical Biology & Medicine 33, 337-349.
Zhang, H., Forman, H.J., 2012. Glutathione synthesis and its role in redox signaling. Seminars in Cell & Developmental Biology 23, 722-728.

Chapter 2

# The flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside is able to protect endothelial cells by a direct antioxidant effect

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Toxicology in Vitro. 2014; 28(4):538-43

**Abstract**

The flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) is an effective protector against doxorubicin induced toxicity which has been related to the antioxidant activity of monoHER. The present study examines the potential relevance of the direct scavenging activity of the flavonoid.

The potency of the direct antioxidant effect was confirmed by its instantaneous protection against intracellular oxidative stress in human umbilical vein endothelial cells at therapeutically achievable concentrations ($EC_{50}$ = 60 nM) underpinning the involvement of a direct scavenging activity. This direct effect of monoHER is substantiated by (i) its site specific scavenging effect, i.e. on a molecular level monoHER is positioned at the location of radical formation, (ii) its position in the antioxidant network, i.e. on a biochemical level oxidized monoHER quickly reacts with ascorbate or glutathione, (iii) its location in the vascular system, i.e. on a cellular level monoHER is localized in the endothelial and smooth muscle cells in the vascular wall.

It is concluded that the flavonoid monoHER can display a physiologically important direct antioxidant effect.

## Introduction

7-Mono-O-(β-hydroxyethyl)-rutoside (monoHER) belongs to the group of the flavonoids. Flavonoids are a group of naturally occurring polyphenolic compounds that are present in vegetables, fruits and plant-derived beverages such as tea and wine (Nichenametla et al., 2006; Ren et al., 2003). The most studied biochemical activity of this group of compounds is their ability to protect against oxidative stress. A high flavonoid intake correlates with a reduced risk of developing e.g. cardiovascular diseases, which has been linked to the antioxidant activity of the flavonoids (Diplock et al., 1998; Middleton et al., 1994). New flavonoids have been designed to optimize the health effects. MonoHER is a semi-synthetic flavonoid and is the most active component of the registered drug Venoruton®. Venoruton® protects the vascular endothelium and mitigates inflammation (Bast et al., 2007). MonoHER is also a potent protector against doxorubicin-induced cardiotoxicity. This has also been associated with its excellent antioxidant properties (Haenen et al., 1993; van Acker et al., 1993).

The relevance of a direct antioxidant effect is debated (Gordon, 2012; Hollman et al., 2011; Hu, 2011; Stevenson and Hurst, 2007). In the present study, the direct antioxidant activity of the flavonoid monoHER in the protection against intracellular oxidative stress was evaluated at several levels. To this end a relatively stable reactive oxygen species (ROS), namely $H_2O_2$, was used to induce a reproducible oxidative stress. $H_2O_2$ is formed in cells in various ways, e.g. in the oxidative phosphorylation that leads to the formation of superoxide radicals that dismutate to $H_2O_2$. $H_2O_2$ readily enters the cell through aquaporin channels (Miller et al., 2010). Intracellular, the relatively unreactive $H_2O_2$ can be converted into the extremely reactive OH-radical, that induces radical damage itself and also amplifies ROS production through e.g. enhanced intracellular iron uptake, and activation of radical production by mitochondria, NAD(P)H oxidase, xanthine oxidase and uncoupled eNOS (Cai, 2005; Li et al., 2010).

The direct antioxidant activity of monoHER was quantified in a radical scavenging assay, and further evaluated on a cellular level. Since Venoruton®, with monoHER

as principle component, efficiently protects the vascular endothelium, endothelial cells (i.e. HUVEC's) were selected for the cellular experiments.

It was concluded that due to its high antioxidant potential, its interplay with other antioxidants in the antioxidant network and its presence at the site of action, the flavonoid is able to directly protect against oxidative stress.

## Materials and methods

### Chemicals

7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) was kindly provided by Novartis Consumer Health (Nyon, Switzerland). 2-Deoxy-D-ribose, ferric chloride hexahydrate ($FeCl_3.6H_2O$), ascorbic acid were purchased from Sigma (St. Louis, USA). $H_2O_2$ was obtained from Riedel-de Haën (Seelze, Germany). 2-thiobarbituric acid (TBA) and EDTA were purchased from Merck (Darmstadt, Germany). 2',7'-dichlorofluorescein-diacetate (DCFH-DA), horseradish peroxidase (HRP), reduced nicotinamide adenine dinucleotide (NADH), 2,4-dinitrophenylhydrazine (DNPH) and reduced glutathione (GSH) were acquired from Sigma (St. Louis, USA). Trichloroacetic acid (TCA) was obtained from Serva (Heidelberg, Germany). Ethanol was purchased from VWR prolabo (Fonteney sous Bois, France). Human Umbilical Vein Endothelial Cells (HUVECs) cell line-1730 was obtained from ATCC (Manassas, USA). Hank's balanced salt solution (HBSS) was obtained from Invitrogen (Breda, The Netherlands).

### Cell culture

HUVECs were cultured in T75 coated culture flasks (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) in Ham's F12-K medium (Invitrogen, Breda, The Netherlands) supplemented with 10 % (v/v) fetal calf serum (FCS; Invitrogen, Breda, The Netherlands), 0.05 mg/ml endothelial cell growth supplement (ECGS; BD Science, Franklin Lakes, USA), 1 % pen/strep (Gibco, Bleiswijk, The Netherlands) and 0.01 mg/ml heparin (LEO Pharma, Ballerup, Denmark) in a humidified atmosphere containing 5 % $CO_2$ and 95 % air at 37 °C. The maximal passage used was 20 in the measurement of intracellular oxidative stress.

**Measurement of intracellular oxidative stress**

The intracellular levels of oxidative stress were quantified by the fluorescence of DCF. The probe used has broad specificity; it detects $H_2O_2$, hydroxyl radicals, superoxide and other reactive species.

Cells (4 X $10^4$ per well) were plated out in a 96-wells plate (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) 24 h before the experiment. The cells were then incubated with 20 µM DCFH-DA in 5 % $CO_2$ and 95 % air at 37 °C for 45 min. After the medium with excess of DCFH-DA was removed, the cells were rinsed once with HBSS and subsequently incubated with 200 µM $H_2O_2$ and 0-1 µM monoHER or 1 mM ethanol in serum-free medium. The fluorescence (Ex 485 nm, Em 538 nm) was determined on a microplate reader (Spectramax microplate reader, Molecular devices, Sunnyvale, USA) as a measure of oxidative damage.

The $EC_{50}$, the concentration of monoHER which reduces intracellular oxidative stress by 50 % after 45 min, was determined by plotting the logarithm of the concentration of monoHER against the protection (in %) at time point 45 min. The $EC_{50}$ was estimated by linear interpolation between the concentration 0.01 µM and 0.1 µM.

Measurement of DCF in the medium revealed that in none of the incubations DCF leakage occurred (data now shown).

The reaction of monoHER or ethanol with $H_2O_2$ was examined by HPLC and spectrophotometrically (data not shown). Both compounds did not react with $H_2O_2$.

**Cell viability**

HUVECs (4 X $10^4$ per well) were plated out in a 96-wells plate (Greiner Bio-one, Alphen a/d Rijn, The Netherlands). The next day, medium was removed and cells were washed once with HBSS and exposed to serum-free medium containing test compounds for 45 min. After exposure, 20 µl of medium was transferred to a new 96 well plate to measure lactate dehydrogenase (LDH) activity. Fifty µl of 10 mg/ml NADH in 0.75 mM sodium pyruvate was added to the medium and incubated for 30 minutes at 37 ºC. To determine the remaining amount of pyruvate, 50 µl 0.2 mg/ml

DNPH was added to the wells and incubated at room temperature in the dark for 20 minutes. After incubation with DNPH, 50 µl 4 M NaOH was added and the mixture was incubated for 5 minutes to develop the color. Absorbance was measured at 540 nm using a Spectramax plate reader (Molecular Devices, Sunnyvale, CA, USA) and viability was calculated relative to unexposed cells (100 % viability) with 0.1 % (v/v) triton X-100 as a positive control.

**Hydroxyl radical scavenging competition assay**

In the assay, the scavenger, monoHER or ethanol, is in competition with deoxyribose for a reaction with hydroxyl radicals. The hydroxyl radicals are formed in the reaction between $Fe^{2+}$ and $H_2O_2$. Iron is kept in the reduced form by ascorbate acid.

The reaction mixtures contained, unless stated otherwise, 0-0.4 mM monoHER or 0-0.4 mM ethanol, 2.8 mM deoxyribose, 100 µM ascorbic acid and 2.8 mM $H_2O_2$ in 20 mM $KH_2PO_4/K_2HPO_4$ buffer pH 7.4. Hydroxyl radical formation was started by adding 200 µl of a solution containing 20 µM $FeCl_3$ and 0-100 µM EDTA in deoxygenated water. The mixture was incubated at 37 °C, and after 1 h 2 ml of a solution, containing 0.5 % TBA and 1.4 % TCA was added. Subsequently, the mixture was heated at 100 °C for 20 min. The test tubes were cooled to room temperature. The absorbance (A) of the reaction mixture was measured at 532 nm. By plotting the reciprocal of the relative absorbance against the concentration of the scavenger, the apparent reaction rate constant ($k_s$) of the reaction between the scavenger and the hydroxyl radicals was calculated according to (Halliwell et al., 1987).

**Reaction of oxidized monoHER with GSH and/or ascorbate**

MonoHER (50 µM) was oxidized by 1.6 nM HRP and 33 µM $H_2O_2$ in a 145 mM phosphate buffer pH 7.4, at 37 °C. The concentration of ascorbate and GSH was 40 µM when present. The reaction was monitored spectrophotometrically (monoHER consumption at 355 nm, ascorbate consumption at 270 nm) and by high-

performance liquid chromatography (HPLC) as previously described by Jacobs et al., 2010 (Jacobs et al., 2010). The absorption spectra were recorded on a Varian Carry 50 spectrophotometer (Mulgrave, Australia) from 220 to 500 nm with a scan speed of 960 nm/min, using quartz cuvettes. HPLC analysis was performed on a HP 1100 series HPLC system (Agilent Technologies, Palo Alto, USA) using a Supelcosil LC 318 column (5 µm, 25 cm x 4.6 mm) (Supelco, Bellefonte, USA). The mobile phase consisted of water with linear gradients of acetonitrile, containing 0.1 % (v/v) TFA. MonoHER was detected with a diode array detector at 355 nm (absorption maximum of monoHER).

**Localization of monoHER in carotid arteries by two photon laser scanning microscopy**

The animal protocol was approved by the Ethics committee for Animal Experiments of Maastricht University (Maastricht, The Netherlands). C57BL/6J mice were used to isolate the carotid artery. The arteries were incubated with HBSS and afterwards with 200 µM monoHER for 30 min at 37 °C. The arteries were rinsed with HBSS to remove the unbound monoHER.

The carotid arteries were imaged using TPLSM (TCS SP5 CFS AOBS with a Coherent: Chameleon Ultra II. ir. 140 fs pulsed laser system >300 KW, for 2 photon excitation at 800 nm, Emission filter: 510 - 580 nm) (Leica Microsystems, Wetzlar, Germany). A HCX APO L 20.0 x 1.00 water objective (WD = 2.0 mm) was used. A nucleus specific staining with SYTO 41 was carried out. Images were obtained using LAS-AF SP5 software. No further image processing was carried out.

**Statistics**

Three independent experiments were performed. Data are expressed as mean ± SD or a typical example is shown. Statistical analysis was performed using Student's t-test. P values $\leq 0.05$ were considered statistically significant.

## Results

### Protection against intracellular oxidative stress

$H_2O_2$ added to HUVECs in a concentration of 200 µM, induced intracellular oxidative stress (Figure 1). Immediately after addition, monoHER protected HUVECs against the intracellular oxidative stress. The $EC_{50}$ after 45 min was 60 nM. Ethanol in a concentration up to 1 mM did not protect against intracellular oxidative stress. In none of the incubations, significant LDH leakage was observed (data not shown).

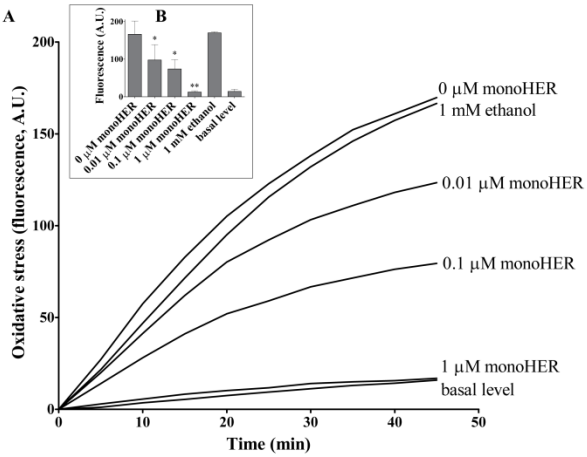


**Figure 1.** The effect of ethanol and monoHER on the intracellular oxidative stress induced by 200 µM $H_2O_2$ in human umbilical vein endothelial cells, except for the basal level, which was without $H_2O_2$. Intracellular oxidative stress is measured using DCF fluorescence as described in the materials and methods section. A typical example is shown. In the insert (B) the oxidative stress after 45 min is shown. Three independent experiments were performed in quadruplicate and data are shown as mean ± SD (significantly lower than 0 µM monoHER *P<0.05,**P<0.001).

### Hydroxyl radical scavenging/direct antioxidant activity

The competition experiment with deoxyribose showed that monoHER is a very potent antioxidant (Figure 2). The apparent second order reaction rate constant ($k_s$)

of the reaction of monoHER with the hydroxyl radicals, was 130 X $10^8$ $M^{-1}s^{-1}$. The reference compound ethanol proved to be less potent than monoHER (Figure 2). The $k_s$ of ethanol obtained was 25 X $10^8$ $M^{-1}s^{-1}$, which is similar to previously reported values (Halliwell et al., 1987).

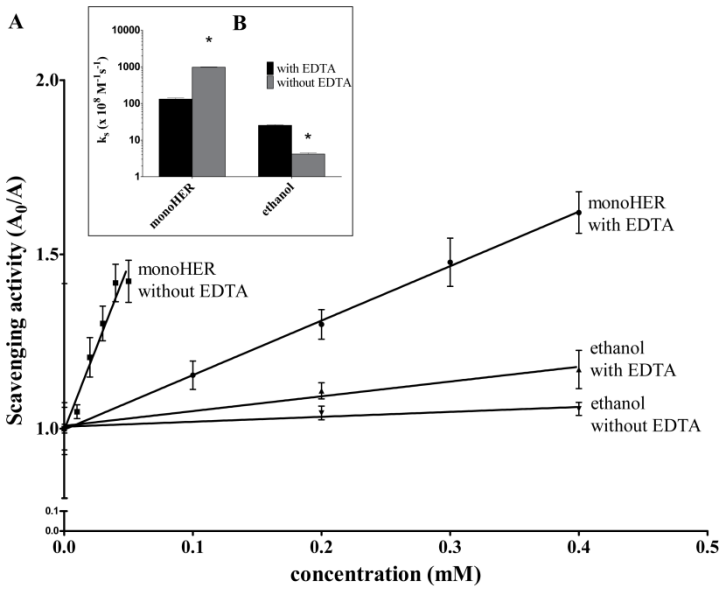


**Figure 2.** Determination of the hydroxyl radical scavenging activity of monoHER and ethanol using a competition assay with deoxyribose. The deoxyribose concentration is 2.8 mM. The concentration of the scavenger is plotted on the x-axis. To determine the scavenging activity of monoHER and ethanol, the breakdown products formed in the reaction of deoxyribose with OH-radicals were measured spectrophotometrically. MonoHER and ethanol compete with deoxyribose for the OH-radicals and the presence of these compounds reduces the concentration of deoxyribose breakdown product. The absorbance obtained in the presence of monoHER or ethanol (A) was related to the absorbance obtained without these compounds ($A_0$) which is a measure of the scavenging activity. Three independent experiments were performed and data are shown as mean ± SD. The apparent reaction rate ($k_s$) of the reaction of monoHER and ethanol with hydroxyl radicals with or without ETDA is shown in the insert (B). Data are shown as mean ± SD (n=3). *Significant difference between $k_s$ with EDTA and $k_s$ without EDTA $P<0.001$.

Omitting the chelator EDTA from the reaction mixture, increased the observed potency of monoHER (Figure 2); the apparent $k_s$ of monoHER was 980 X $10^8$ $M^{-1}s^{-1}$. The results (Figure 2) also show that the apparent $k_s$ of ethanol in the absence of EDTA (4 X $10^8$ $M^{-1}s^{-1}$) was 245 times lower than that of monoHER and six times lower than that of ethanol in presence of EDTA.

**Reactivity of oxidized monoHER with the endogenous antioxidants GSH or ascorbate**

MonoHER oxidized in the presence of GSH generates a GSH-monoHER adduct. In the presence of ascorbate, no net consumption of monoHER is observed, while ascorbate is consumed (Figure 3). The fast ascorbate consumption is due to the instantaneous reduction of oxidized monoHER to monoHER. A competition experiment demonstrated that oxidized monoHER reacted 2.5 times as fast with ascorbate (3.0 ± 0.3 µM/min) as with GSH (1.3 ± 0.1 µM/min).

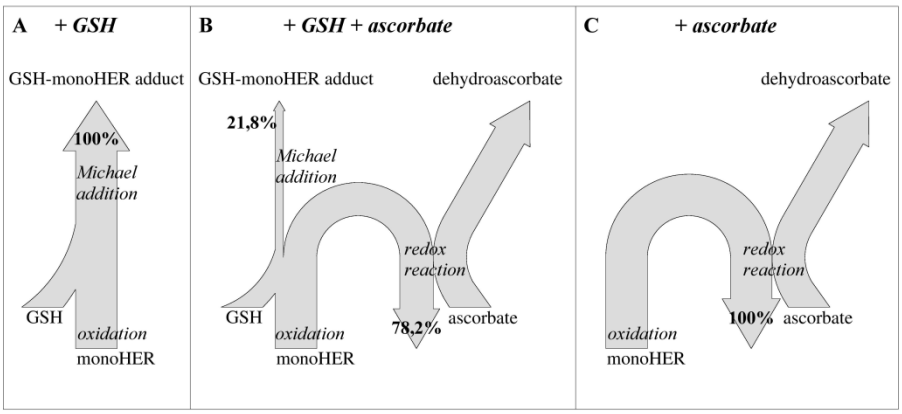


**Figure 3.** Location of monoHER in the antioxidant network. In the presence of 40 µM GSH (A), the combination of 40 µM GSH and 40 µM ascorbate (B) or 40 µM ascorbate (C), 50 µM monoHER was oxidized. The relative importance of the reactions is depicted by the width of the arrow and given by the percentage.

**Localization of monoHER in the carotid artery**

Incubation of the carotid arteries with monoHER and subsequent TPLSM analysis showed that monoHER was retained in the wall of the artery (Figure 4A and 4B). The arteries were also stained with SYTO 41 (Figure 4C). The fluorescence of this nucleus specific stain overlaps with the fluorescence of monoHER (Figure 4D).

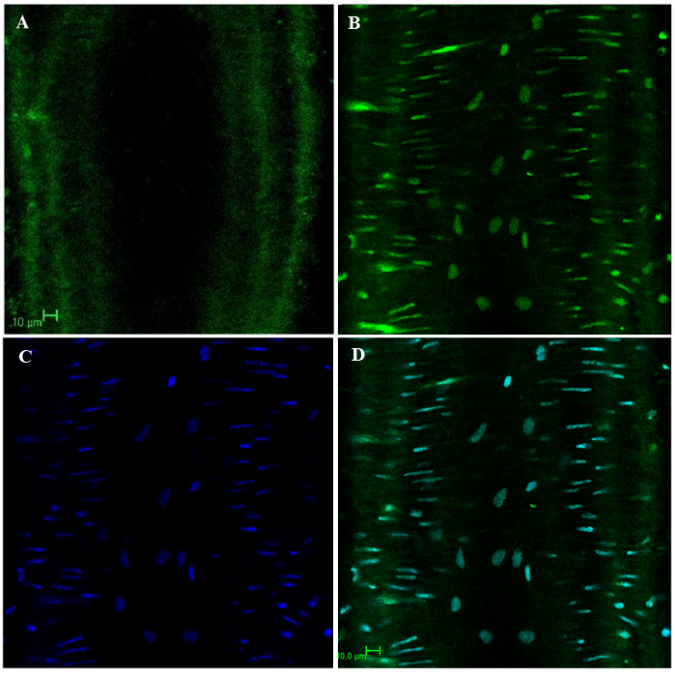


**Figure 4.** Localization of monoHER in the carotid artery. Two Photon Laser Scanning Microscopy imaging of longitudinal cross-sections of an intact carotid artery incubated with HBSS (A), 200 µM monoHER (B), a nuclei staining dye, SYTO 41 (C) and both monoHER and SYTO 41 (D). A typical example is shown. The elastic lamina autofluoresce in the same wavelength range as monoHER, i.e. emission 510-580 nm, and are therefore visible. The fluorescence obtained after addition of SYTO 41, overlaps with the fluorescence of monoHER. This indicates that monoHER is located in the nuclei of the endothelial cells (round spots) and smooth muscle cells (elongated spots).

**Discussion**

MonoHER is a semi-synthetic flavonoid which was found to display a very potent antioxidant activity (Haenen et al., 1993; van Acker et al., 1993). This flavonoid proves to have several beneficial effects on the cardiovascular system. As the main constituent of the registered drug Venoruton®, monoHER improves the function of the endothelial layer of the vessels in the treatment of chronic venous insufficiency (Cesarone et al., 2006). MonoHER was also found to effectively protect against doxorubicin-induced cardiotoxicity in mice (van Acker et al., 2000; van Acker et al., 1995).

In the present study the potential contribution of the direct antioxidant effect of monoHER against oxidative stress was elucidated by evaluating the effect at different levels i.e. the molecular, supramolecular, and cellular level.

*Protection against intracellular oxidative stress*

Initially, the beneficial effect of antioxidants was ascribed to their direct antioxidant effect i.e. their ability to scavenge radicals (Halliwell, 1996). This could directly neutralize the noxious potential of radicals. The plausibility of a physiological relevant effect of radical scavenging of antioxidants is seriously questioned because of the high concentration of antioxidant that is needed for this type of protection, a concentration that is impossible to be reached *in vivo* (Gordon, 2012; Hollman et al., 2011; Hu, 2011; Stevenson and Hurst, 2007).

In line with this hypothesis, we found that the well known OH-radical scavenger ethanol was not able to protect against intracellular oxidative stress in HUVECs, even at relatively high concentrations. Apparently, radical scavenging on its own is not sufficient to offer effective protection.

In the case of monoHER, we found that the flavonoid does protect cells against oxidative stress, even at a low concentration (the $EC_{50}$ was 60 nM). The probe used to detect oxidative stress reacts with a broad array of reactive species, suggesting a full protection by monoHER. A study in human volunteers demonstrated that monoHER can reach plasma concentrations up to 360 µM after i.v. administration

(Willems et al., 2006). This points out that monoHER can provide an effective protection at therapeutically achievable concentrations.

The *in vivo* effectiveness was demonstrated by the protection of monoHER against doxorubicin- induced cardiotoxicity in mice. MonoHER administered 30 min before doxorubicin could prevent oxidative damage to the heart (Bruynzeel et al., 2006). In this effect the antioxidant activity of metabolites of monoHER has to be taken into account (Jacobs et al., 2011a; Jacobs et al., 2011b). We have also identified that monoHER immediately offered protection in HUVECs. The fact that the protection by monoHER is instantaneous, precludes the involvement of e.g. the induction of antioxidant enzymes because such an indirect mechanism requires several hours to be effective due to the time needed for transcription factor activation, DNA transcription, mRNA production, protein synthesis and protein processing. That the effective protection was observed immediately after the addition of monoHER, confirms that a direct antioxidant activity of monoHER is involved in the protection of the HUVECs.

*Scavenging activity*

To induce oxidative stress in the cells, HUVECs were exposed to $H_2O_2$. $H_2O_2$ is relatively unreactive and can readily diffuse over membranes. By the use of $H_2O_2$, that in the concentration used did not induce LDH leakage, we were able to impose a reproducible and relatively mild oxidative stress upon the cells. $H_2O_2$ can produce intracellular oxidative stress by reacting with iron to generate the extremely reactive OH-radicals in a Fenton reaction (Wardman and Candeias, 1996). The extreme reactivity of radicals fueled the debate on the relevance of radical scavenging antioxidants, because it would be impossible for antioxidants to neutralize the radical before it attacks vital cellular compounds (Bast and Haenen, 2013). Remarkably, in the present study we found that monoHER did protect against the intracellular stress directly after addition. This also indicates that metabolites are not likely to be involved, and that this instantaneous effect is primarilly attributable to monoHER itself.

In addition, our results of the hydroxyl radical scavenging competition assay show that monoHER scavenges OH-radicals at an extremely high apparent rate in our test system - $k_s = 980 \text{ X } 10^8 \text{ M}^{-1}\text{s}^{-1}$ - which is even quicker than the diffusion rate (~ $100 \text{ X } 10^8 \text{ M}^{-1}\text{s}^{-1}$). This can be explained by site-specific scavenging. Essential for this activity is that monoHER can chelate iron (van Acker et al., 1998). The result of this chelation is that monoHER is present at the site of the radical formation, i.e. the iron ion. This enables monoHER to immediately scavenge the newly formed radical. By this site-specific scavenging, monoHER is able to prevent damage to critical biomolecules such as lipids, proteins or DNA, despite the high reactivity of the radical.

*Reactivity in the antioxidant network*

Due to the site-specific scavenging, monoHER is the first molecule to react with the generated radicals. In the scavenging reaction monoHER donates an electron or a hydrogen atom to the radical involved. The radical is converted into a relatively stable non-radical, e.g. the hydroxyl radical becomes $H_2O$. MonoHER is oxidized and takes over part of the reactivity of the radical (Bast and Haenen, 2002). Fortunately, the body is endowed with an intricate network of antioxidants in which monoHER can pass over its reactivity (Bast et al., 1991; Jacobs et al., 2010; Meister, 1994). The main antioxidants in this network are GSH and ascorbate. When oxidized monoHER reacts with GSH, a GSH-monoHER adduct is formed (Jacobs et al., 2009). Although oxidized monoHER can react with GSH, it is preferentially recycled in a redox reaction with ascorbate. The oxidized ascorbate formed in this redox reaction can, on its turn, be reduced, e.g. by dehydroascorbate reductase that uses NADH as a cofactor. In this way, the reactivity of the radical is completely neutralized and the antioxidant network fully restored.

The actual mechanism of action is that the antioxidant monoHER functions as a catalyst that (i) efficiently scavenges the radical involved, (ii) subsequently safely channels the reactivity of the radical into the endogenous antioxidant network and (iii) finally is recycled and thus helps to maintain homeostasis.

*Localization in the cardiovascular system*

Neumann et al. monitored the uptake of O-(β-hydroxyethyl)-rutosides, among which monoHER at a subcellular level, in varicose long saphenous veins, after i.v. administration (Neumann et al., 1992). They found that these flavonoids were localized in the endothelial and sub-endothelial layer of the vessel. Our results are in line with this observation. Our experiments with carotid arteries showed that monoHER is retained in the wall of these arteries with TPLSM analysis.
The fluorescence was localized in the nucleus of the cell. This finding was in line with the observation of Mukai et al. (Mukai et al., 2009). They found that flavonols added to cultured Hepa-1c1c7 cells also accumulated in the nucleus of these cells. A favorable localization in the cardiovascular system supports the protective potential of monoHER.

*Location, location, location…*

The fundamental prerequisite for a bioactive compound to have a biologic effect is that the compound has to closely interact with its biological targets, a concept coined by Ehrlich in 1913 as 'Corpora non agunt nisi fixata' (Ehrlich, 1913). At first sight, radical scavengers do not seem to meet this criterion, which fuels the debate on their biological activity. However, the present study shows that the concept of Ehrlich does apply for monoHER. The interactions needed for a biological effect are favorable on (i) the molecular level; monoHER is at the right location for radical scavenging, (ii) the supramolecular level; monoHER is located at a pivotal position in the antioxidant network and (iii) the cellular level; monoHER is located in the endothelial and smooth muscle cells in the vascular wall.

## References


Bast, A., Haenen, G.R., 2002. The toxicity of antioxidants and their metabolites. Environmental Toxicology and Pharmacology 11, 251-258.

Bast, A., Haenen, G.R., 2013. Ten misconceptions about antioxidants. Trends in Pharmacological Sciences 34, 430-436.

Bast, A., Haenen, G.R., Doelman, C.J., 1991. Oxidants and antioxidants: state of the art. American Journal of Medicine 91, 2S-13S.

Bast, A., Kaiserova, H., den Hartog, G.J., Haenen, G.R., van der Vijgh, W.J., 2007. Protectors against doxorubicin-induced cardiotoxicity: flavonoids. Cell Biology and Toxicology 23, 39-47.

Bruynzeel, A.M., Mul, P.P., Berkhof, J., Bast, A., Niessen, H.W., van der Vijgh, W.J., 2006. The influence of the time interval between monoHER and doxorubicin administration on the protection against doxorubicin-induced cardiotoxicity in mice. Cancer Chemotherapy Pharmacology 58, 699-702.

Cai, H., 2005. Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences. Cardiovascular Research 68, 26-36.

Cesarone, M.R., Belcaro, G., Pellegrini, L., Ledda, A., Vinciguerra, G., Ricci, A., Gizzi, G., Ippolito, E., Fano, F., Dugall, M., Acerbi, G., Cacchio, M., Di Renzo, A., Hosoi, M., Stuard, S., Corsi, M., 2006. Circulating endothelial cells in venous blood as a marker of endothelial damage in chronic venous insufficiency: improvement with venoruton. Journal of Cardiovascular Pharmacology and Therapeutics 11, 93-98.

Diplock, A.T., Charleux, J.L., Crozier-Willi, G., Kok, F.J., Rice-Evans, C., Roberfroid, M., Stahl, W., Vina-Ribes, J., 1998. Functional food science and defence against reactive oxidative species. British Journal of Nutrition 80 Suppl 1, S77-112.

Ehrlich, P., 1913. Address in Pathology, On Chemotherapy: Delivered before the Seventeenth International Congress of Medicine. British Medical Journal 2, 353-359.

Gordon, M.H., 2012. Significance of dietary antioxidants for health. International Journal of Molecular Sciences 13, 173-179.

Haenen, G.R., Jansen, F.P., Bast, A., 1993. The antioxidant properties of five O-(-β-Hydroxyethyl)-Rutosides of the flavonoid mixture Venoruton. Phlebology Supplements. 1, 10-17.

Halliwell, B., 1996. Antioxidants in human health and disease. Annual Revision of Nutrition 16, 33-50.

Halliwell, B., Gutteridge, J.M., Aruoma, O.I., 1987. The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. Analytical Biochemistry 165, 215-219.

Hollman, P.C., Cassidy, A., Comte, B., Heinonen, M., Richelle, M., Richling, E., Serafini, M., Scalbert, A., Sies, H., Vidry, S., 2011. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. Journal of Nutrition 141, 989S-1009S.

Hu, M.L., 2011. Dietary polyphenols as antioxidants and anticancer agents: more questions than answers. Chang Gung Medical Journal 34, 449-460.

Jacobs, H., Koek, G.H., Peters, R., Moalin, M., Tack, J., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2011a. Differences in Pharmacological Activities of the Antioxidant Flavonoid MonoHER in Humans and Mice Are Caused by Variations in Its Metabolic Profile. Clinical Pharmacology Therapeutics 90, 852-859.

Jacobs, H., Moalin, M., Bast, A., van der Vijgh, W.J., Haenen, G.R., 2010. An essential difference between the flavonoids monoHER and quercetin in their interplay with the endogenous antioxidant network. PLoS One 5, e13880.

Jacobs, H., Peters, R., den Hartog, G.J., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2011b. Identification of the metabolites of the antioxidant flavonoid 7-mono-O-(beta-hydroxyethyl)-rutoside in mice. Drug Metabolism and Disposition 39, 750-756.

Jacobs, H., van der Vijgh, W.J., Koek, G.H., Draaisma, G.J., Moalin, M., van Strijdonck, G.P., Bast, A., Haenen, G.R., 2009. Characterization of the glutathione conjugate of the semisynthetic flavonoid monoHER. Free Radical Biology and Medicine 46, 1567-1573.

Li, W.M., Liu, H.T., Li, X.Y., Wu, J.Y., Xu, G., Teng, Y.Z., Ding, S.T., Yu, C., 2010. The effect of tetramethylpyrazine on hydrogen peroxide-induced oxidative damage in human umbilical vein endothelial cells. Basic & Clinical Pharmacology & Toxicology 106, 45-52.

Meister, A., 1994. Glutathione, ascorbate, and cellular protection. Cancer Research 54, 1969s-1975s.

Middleton, E., Jr., Kandaswami, C., 1994. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. The flavonoids: advances in research since 1986 (Harborne, J. B., Ed.), 619-652.

Miller, E.W., Dickinson, B.C., Chang, C.J., 2010. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. Proceedings of the National Academy of Sciences of the United States of America 107, 15681-15686.

Mukai, R., Shirai, Y., Saito, N., Yoshida, K., Ashida, H., 2009. Subcellular localization of flavonol aglycone in hepatocytes visualized by confocal laser scanning fluorescence microscope. Cytotechnology 59, 177-182.

Neumann, H.A., Carlsson, K., Brom, G.H., 1992. Uptake and localisation of O-(beta-hydroxyethyl)-rutosides in the venous wall, measured by laser scanning microscopy. European Journal of Clinical Pharmacology 43, 423-426.

Nichenametla, S.N., Taruscio, T.G., Barney, D.L., Exon, J.H., 2006. A review of the effects and mechanisms of polyphenolics in cancer. Critical Review in Food Science and Nutrition 46, 161-183.

Ren, W., Qiao, Z., Wang, H., Zhu, L., Zhang, L., 2003. Flavonoids: promising anticancer agents. Med Res Rev 23, 519-534.

Stevenson, D.E., Hurst, R.D., 2007. Polyphenolic phytochemicals--just antioxidants or much more? Cellular Molecular Life Sciences 64, 2900-2916.

van Acker, F.A., van Acker, S.A., Kramer, K., Haenen, G.R., Bast, A., van der Vijgh, W.J., 2000. 7-monohydroxyethylrutoside protects against chronic doxorubicin-induced cardiotoxicity when administered only once per week. Clinical Cancer Research 6, 1337-1341.

van Acker, S.A., Kramer, K., Grimbergen, J.A., van den Berg, D.J., van der Vijgh, W.J., Bast, A., 1995. Monohydroxyethylrutoside as protector against chronic doxorubicin-induced cardiotoxicity. British Journal Pharmacology 115, 1260-1264.

van Acker, S.A., Towart, R., Husken, B.C., De Jong, J., van der Vijgh, W.J., Bast, A., 1993. The protective effect of Venoruton and its main constituents on acute doxorubicin-induced cardiotoxicity. Phlebology Supplements. 1, 31-32.

van Acker, S.A., van Balen, G.P., van den Berg, D.J., Bast, A., van der Vijgh, W.J., 1998. Influence of iron chelation on the antioxidant activity of flavonoids. Biochemical Pharmacology 56, 935-943.

Wardman, P., Candeias, L.P., 1996. Fenton chemistry: an introduction. Radiat Res 145, 523-531.

Willems, A.M., Bruynzeel, A.M., Kedde, M.A., van Groeningen, C.J., Bast, A., van der Vijgh, W.J., 2006. A phase I study of monohydroxyethylrutoside in healthy volunteers. Cancer Chemotherapy and Pharmacology 57, 678-684.

Chapter 3

# The minor structural difference between the antioxidants quercetin and 4'O-methylquercetin has a major impact on their selective thiol toxicity

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International Journal of Molecular Sciences. 2014; 15(5): 7475-7484

**Abstract**

Antioxidants act as intermediates by picking up the high unselective reactivity of radicals and transferring it to other molecules. In this process the reactivity is reduced and becomes selective. This channeling of the reactivity can cause selective toxicity. The antioxidant quercetin is known to channel the reactivity towards thiol groups. The present study compares the thiol reactivity of quercetin with that of 4'-O-methylquercetin (tamarixetin) towards creatine kinase (CK), a vital protein that contains a critical thiol moiety. Our results showed that oxidized quercetin and oxidized tamarixetin both adduct CK, which then loses its enzymatic function. Ascorbate, an important representative of the antioxidant network, is able to prevent adduction to and thus the inhibition of the enzyme by tamarixetin but not by quercetin. Apparently, tamarixetin is less thiol toxic than quercetin, because - rather than adduction to CK - tamarixetin quinone prefers to pass reactivity to the antioxidant network, *i.e.*, to ascorbate. The findings exemplify that radical scavenging flavonoids pick up the reactivity of radicals and act as a pivot in directing the way the reactivity is channeled. A mere minor structural difference of only one methyl moiety between quercetin and tamarixetin appears to have a high impact on the selective, thiol toxicity.

**Introduction**

Flavonoids are polyphenols found in numerous fruits and vegetables having excellent antioxidant properties. After their discovery, the prevailing idea was that antioxidant intake was exclusively linked to health benefits. Antioxidants were thought to protect against radicals by simply scavenging them and thus fully annihilating their reactivity (Halliwell, 1996; Willcox et al., 2004). Contemporary research acknowledges this protective effect and append that by scavenging also reactive intermediates of the antioxidant are formed, which can be toxic (Bjelakovic et al., 2014).

Flavonoids are extraordinary potent antioxidants; this places them at the top of the pecking order meaning that flavonoids are first in line to scavenge reactive oxygen species (ROS). ROS take up an electron or hydrogen atom from the antioxidant flavonoid and this reduction converts the ROS into relatively harmless species. The oxidized flavonoids formed in the scavenging reaction are less reactive than the radical scavenged, which leads to selective reactivity. Oxidized flavonoids readily and specifically adduct thiol groups. Hence, oxidized flavonoids may threat vital cellular compounds containing a critical thiol group (Boots et al., 2002; Ito et al., 1988). Apparently, a paradox is hidden in the functioning of an antioxidant flavonoid. Flavonoids protect against the reactivity of ROS but the other side of the coin is that during their protective effect, a potential thiol toxic product is formed.

The thiol containing enzyme creatine kinase (CK) is crucial for energy production in cells with a high energy turnover. Oxidative stress forms an onslaught on the energy level of the cell. Inhibition of CK aggravates the energy crisis which can finally lead to cell death (Reddy et al., 2000). This prompted us to examine thiol toxicity on CK.

The selective toxicity toward protein thiols might be circumvented when the reactivity of the oxidized flavonoid is absorbed by the antioxidant network of the cell. Ascorbate, an important representative in this network, can efficiently reduce an oxidized flavonoid and thus recycle the flavonoid. In this way the radical is neutralized by channeling its reactivity safely into the antioxidant network.

The flavonoids of interest to us are quercetin and the 4'-O-methylquercetin, tamarixetin. Quercetin and tamarixetin are naturally occurring flavonoids which are present in our diet. Tamarixetin is also a metabolite of quercetin that is formed *in vivo* (Brevik et al., 2004). The cellular uptake of tamarixetin is higher than that of quercetin (Spencer et al., 2003). This supports a potential role of tamarixetin *in vivo*. In this study the canalization of the reactivity of structurally related flavonoids, quercetin and tamarixetin, is unraveled at the level of protein reactivity because toxicity arises at this level. The preference of the oxidized flavonoids to direct their reactivity towards thiol containing proteins like CK or into the antioxidant network, specifically towards ascorbate, is determined. It is found that the minor difference in structure, *i.e.*, the 4'-O-methyl group, has a profound effect.

## Materials and Methods

### Chemicals

Quercetin was purchased from Acros Organics (Geel, Belgium). Tamarixetin was obtained from Extrasynthese (Genay, France). Hydrogen peroxide ($H_2O_2$), L-ascorbic acid, horseradish peroxidase (HRP) and creatine kinase (CK) were obtained from Sigma (St. Louis, MO, USA). The creatine kinase kit was acquired from Bioo Scientific Corporation (Austin, TX, USA). Trypsin was purchased from Promega (Madison, WI, USA). Ammonium bicarbonate (ABC), acetonitrile (ACN), α-cyano-4-hydroxycinnamic acid and trifluoroacetic acid (TFA) were acquired from Sigma (St. Louis, MO, USA).

### Creatine Kinase activity

Quercetin and tamarixetin quinones were generated *in situ* by oxidizing the flavonoids (50 µM) with 50 µM $H_2O_2$ and 0.4 nM HRP in case of quercetin and 3.2 nM HRP in case of tamarixetin to obtain an equal rate of oxidation of the flavonoid (5 µM/min), in the presence of 6.2 µM CK in a 145 mM potassium-phosphate buffer pH 7.4. The influence of ascorbate was determined by adding 50 µM to the reaction mixture. The reactions were started by adding the HRP and carried out at 37 °C for 5 min.

Enzyme activity of creatine kinase (CK) in the reaction mixtures was measured by the catalytic conversion of ADP into ATP. Because ascorbate prevented the net consumption of tamarixetin, it was checked whether ascorbate did prevent the oxidation of tamarixetin. It was found that ascorbate did not inhibit the oxidation, but immediately reduced the oxidized tamarixetin (supplemental data). Hexokinase was used to convert ATP with glucose into glucose-6-phosphate. Finally, the glucose-6-phosphate formed converts $NAD^+$ into NADH in the presence of glucose-6-phosphate dehydrogenase. The formation of NADH was measured spectrophotometrically at 340 nm during 5 min and reflects the CK activity.

**Mass spectrometry**

In the presence of 6.2 µM CK in 50 mM ABC buffer pH 7.4, quercetin and tamarixetin (50 µM) were oxidized by 50 µM $H_2O_2$ and 0.4 or 3.2 nM HRP, respectively. The influence of ascorbate was determined by adding 50 µM to the reaction mixture. The oxidation reaction was carried out at 37 °C for 5 min. CK (0.5 mg/mL) was digested by adding 1 µg trypsin during 30 minutes at 37°C. The digested samples were diluted 1:10 in 0.1% TFA. After dilution, 1 µL of the digest and 1 µL of matrix solution (2.5 mg/mL α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA) were spotted on a 348-well-format target plate and air dried. Mass spectra were measured on the MALDI-TOF mass spectrometer (4800 MALDI-TOF analyzer; Applied Biosystems, Bridgewater, NJ, USA). The instrument was operated in positive reflector mode. Acquisition mass range was 800–3500 Da.

**Statistics**

All experiments were performed at least in triplicate. Data are expressed as means ± standard error (SE).

## Results

### Quercetin quinone and tamarixetin quinone inhibit creatine kinase

Quercetin quinone and tamarixetin quinone were generated *in situ* in an incubation mixture containing CK. Quercetin quinone was found to reduce CK activity (95%). Tamarixetin quinone also attenuated the activity of CK, but the extend of this reduction was less (20%) than that in the experiment with quercetin despite the equal rate of quinone formation (Figure 1).

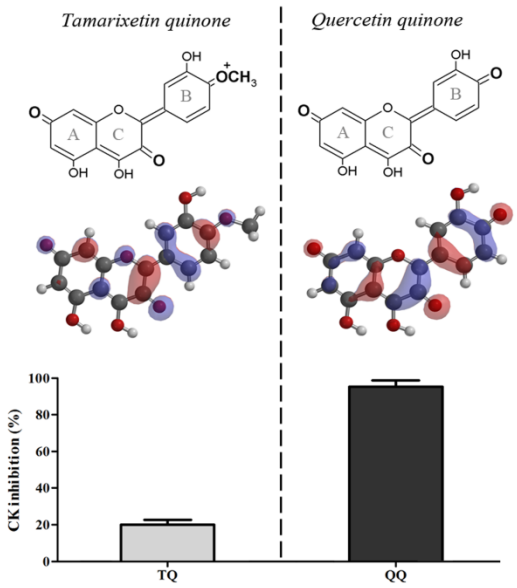


**Figure 1.** Structure and Lowest Unoccupied Molecular Orbital (LUMO) localization map of the preferred tautomer of quercetin quinone and tamarixetin quinone, and the effect of quercetin and tamarixetin oxidation on the enzyme activity of creatine kinase (CK). The carbonyl groups of quercetin quinone are positioned at maximal distance within the molecule and the LUMO is distributed over the phenolic rings, which explains why it behaves as a soft electrophile. Tamarixetin quinone has a positive charge and the LUMO is focused in the B-ring, which makes it a relatively hard electrophile. Quercetin and tamarixetin (50 µM) were oxidized by 50 µM $H_2O_2$ and 0.4 or 3.2 nM horseradish peroxidase (HRP), respectively, to obtain an equal rate of oxidation (5 µM/min). In the presence of 6.2 µM CK, the enzyme activity of CK was measured. Data are shown as mean ± SE ($n$ = 4).

Ascorbate only slightly protected against the inhibition of the activity caused by quercetin quinone (from 95% to 72%). In contrast, ascorbate completely protected the enzyme against the inactivation by the tamarixetin quinone, because the activity of CK was fully retained (Figure 2).

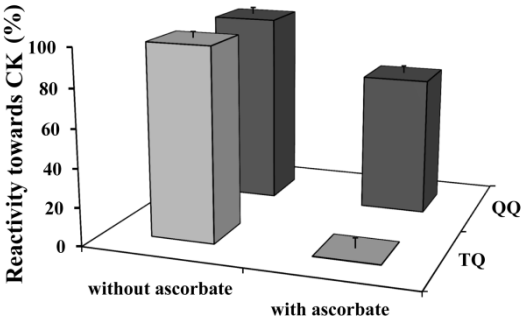


**Figure 2.** Effect of quercetin and tamarixetin oxidation on the enzyme activity of CK in presence of ascorbate. Quercetin and tamarixetin (50 µM) were oxidized by 50 µM $H_2O_2$ and HRP, at equal rate of oxidation (5 µM/min) in presence of 6.2 µM CK. The enzyme activity of CK was measured in the absence or presence of 50 µM ascorbate and expressed as percentage of the CK inhibition obtained without ascorbic acid. Data are shown as mean ± SE ($n$ = 4).

**Quercetin quinone and tamarixetin quinone adduct creatine kinase**

After reaction of CK with quercetin quinone and tamarixetin quinone and subsequent trypsin digestion, MALDI-TOF analysis showed that the amount of the native peptide fragment - GYTLPPHCSR with a mass of $m/z$ = 1130 - was reduced. Fragments with a mass of $m/z$ = 1430 or 1444 emerged after incubation with quercetin or tamarixetin, respectively (Figures 3 and 4). The increments of the mass of the fragment correspond to the molecular weight of the quinones, being 300 Da and 314 Da, respectively. Apparently, the flavonoid quinones adduct creatine kinase. The amount of adducted fragment formed was less after the reaction with tamarixetin quinone than after the reaction with quercetin quinone (Figures 3 and 4). Ascorbate effectively prevented the formation of the flavonoid-protein fragment in the case of tamarixetin quinone but not in the case of quercetin quinone (Figures 3 and 4).

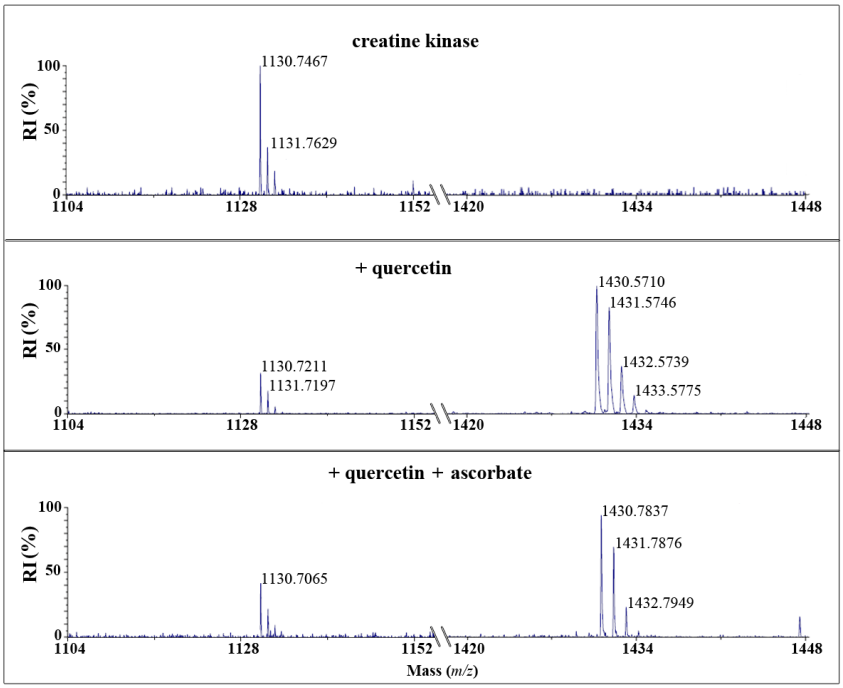


**Figure 3.** MALDI-TOF analysis of isolated creatine kinase (CK) (0.5 mg/mL) incubated with 50 µM quercetin, 0.4 nM HRP and 50 µM $H_2O_2$ with or without 50 µM ascorbate for 5 min at 37 °C. After trypsin digestion the mass spectrum of digested CK was measured. The control spectrum of CK displayed a peak at *m/z* 1130 and no peak at *m/z* 1430. The incubation with quercetin showed a peak at *m/z* 1430 which corresponds to the mass of the adduct of quercetin quinone (300 dalton) with the peptide having mass *m/z* 1130, whereas the peak at *m/z* 1130 decreased. The amino acid sequence of the peptide is GYTLPPHCSR, containing cysteine 146. The spectrum of CK incubated with quercetin in combination with ascorbate also showed a peak at *m/z* 1430. The peak at *m/z* 1130 was also present, but the relative intensity (RI) was less than the untreated CK.

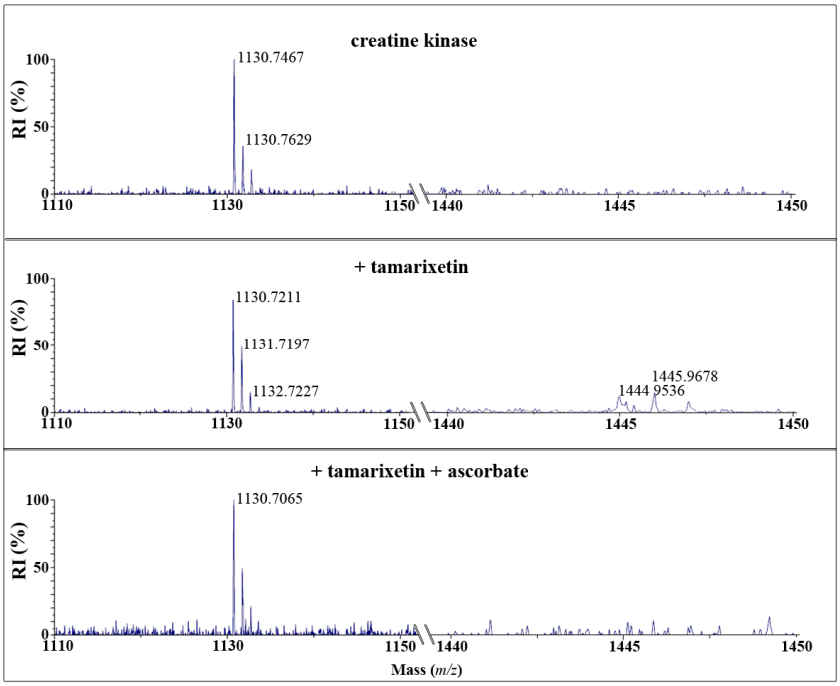


**Figure 4.** MALDI-TOF analysis of isolated creatine kinase (CK) (0.5 mg/mL) incubated with 50 µM tamarixetin, 3.2 nM HRP and 50 µM $H_2O_2$ with or without 50 µM ascorbate for 5 min at 37 °C. After trypsin digestion the mass spectrum of digested CK was measured. The control spectrum of CK displayed a peak at m/z 1130 and no peak at m/z 1444. The incubation with tamarixetin showed a peak at m/z 1444 which corresponds to the mass of the adduct of tamarixetin quinone (314 dalton) with the peptide having mass m/z 1130, whereas the peak at m/z 1130 decreased. The amino acid sequence of the peptide is GYTLPPHCSR, containing cysteine 146. The spectrum of CK incubated with tamarixetin in combination with ascorbate did not show a decrease in the intensity of the peak at m/z 1130 and an increase at m/z 1444.

**Discussion**

Amongst antioxidants, flavonoids are at the top of the pecking order meaning that these antioxidants are first in line to react with radicals. Therefore, flavonoids can effectively protect against radical toxicity (Bast and Haenen, 2013). In this protection the flavonoids become oxidized. Due to their quinone structure, oxidized flavonoids react with nucleophilic thiol groups predominantly found in GSH and cysteine residues of proteins, resulting in selective thiol toxicity (Boots et al., 2002; Boots et al., 2007; Kalyanaraman et al., 1987; Moore et al., 1988; O'Brien, 1991). The toxicity of antioxidant flavonoids emerges when they adduct thiol groups of vital cellular proteins. Actually, it is not the flavonoid itself that displays this thiol toxicity but the oxidized form generated when the flavonoid exerts its antioxidant activity. The scavenging activity protects against the unselective reactivity of the radical, but can subsequently selectively induce toxicity to vital cellular compounds containing SH-groups. Ascorbic acid—an important antioxidant in the antioxidant network which has the potential to react with the oxidized flavonoids—might circumvent this thiol toxicity and as a bonus recycle the flavonoid (Jacobs et al., 2010).

In this study the reactivity of two structurally closely related flavonoids quercetin and its 4'-O-methylated metabolite, tamarixetin have been investigated towards the thiol containing protein, creatine kinase (CK). It was found that the quinones of quercetin and tamarixetin are indeed thiol reactive. Our mass spectrometry data confirm adduction and indicate that both quinones react with cysteine 146 of CK, which is known to be essential for enzyme function (Zhao et al., 2006). The relative intensity of the adduct between CK and tamarixetin detected by MALDI-TOF was lower than that of quercetin and CK under the same experimental conditions. In accordance to these results, the activity of CK was less attenuated by tamarixetin quinone than quercetin quinone. This shows that the tamarixetin quinone is less thiol toxic than the quercetin quinone.

Ascorbate is able to efficiently protect against the inactivation of CK by oxidized tamarixetin. Remarkably, ascorbate only slightly protected against the inactivation

of CK by the quercetin quinone. Apparently, the oxidized tamarixetin prefers to react with ascorbate instead of creatine kinase. Ascorbate is able to recycle the tamarixetin quinone to its parent flavonoid. The oxidized ascorbate formed in this redox reaction can, on its turn, be reduced, e.g. by dehydroascorbate reductase that uses NADH as cofactor. In this way the reactivity of the radical is completely neutralized, tamarixetin is recycled and the enzyme inactivation is prevented. These results show that the *O*-methylation on the 4'-O position of quercetin ablates thiol toxicity.

The difference between quercetin and tamarixetin in directing the reactivity, originates from a minor change in the chemical structure and can be explained by Pearson's HSAB (Hard and Soft Acids and Bases) concept (Pearson, 1963). Oxidized tamarixetin contains a positively charged group that has a relatively high polarity, which reflects in a high LUMO (Lowest Unoccupied Molecular Orbital) energy (21.33 kJ/mol) (Moalin et al., 2012) (Figure 1). This makes oxidized tamarixetin a 'hard' electrophile which prefers to react with ascorbate, a 'hard' nucleophile. This is reflected in a high value of the competition between ascorbate and thiols (CAT), *i.e.*, 14.5 ± 3.8 (Moalin et al., 2012). The CAT was determined by dividing the rate of reaction of the oxidized flavonoid with ascorbate by the rate of reaction of the oxidized flavonoid with the thiol GSH.

The quercetin quinone lacks this highly polar group and the carbonyl groups of oxidized quercetin are positioned at maximal distance. Therefore the LUMO of oxidized quercetin is distributed over all the phenolic rings which gives rise to a low LUMO energy (−112.14 kJ/mol) (Moalin et al., 2012). Consequently, oxidized quercetin is a relatively "soft" electrophile and will prefer to react with 'soft' nucleophiles as the thiol group of GSH (the CAT of oxidized quercetin is 0.04 ± 0.03) (Moalin et al., 2012) and as shown in the present study also with protein thiols.

The present study addresses the selective toxicity of flavonoid antioxidants. Flavonoids pick up the reactivity of radicals and act as a pivot to channel the reactivity (Figure 5). Quercetin quinone channels its reactivity selectively towards thiol toxicity. The low but selective reactivity of oxidized antioxidants that directs

the reactivity to a focal point (in this case thiols) has been implicated in the toxicity of antioxidants (Bast and Haenen, 2002). The selective reactivity of tamarixetin quinone directs the reactivity towards ascorbate in the antioxidant network. This might explain why tamarixetin is found to be less toxic than quercetin in cells (Spencer et al., 2003). Apparently, the introduction of only one methyl group in quercetin, giving tamarixetin, leads to a remarkably high reduction of the selective, thiol toxicity.

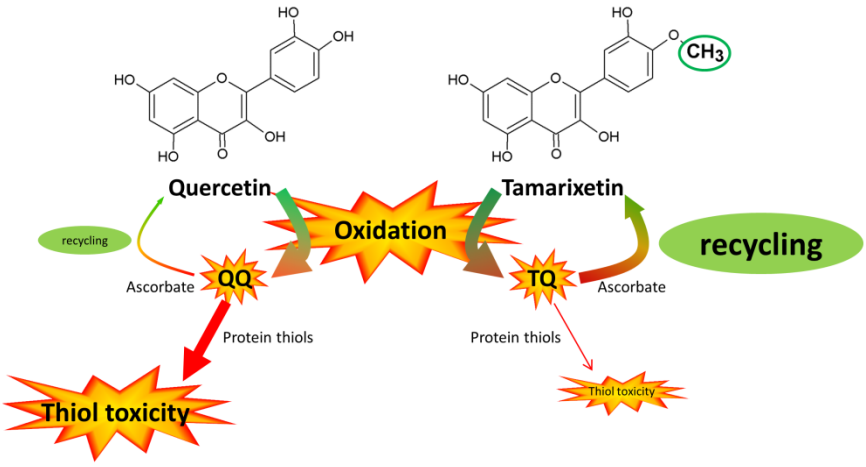


**Figure 5.** Overview of the difference in thiol toxicity between quercetin and tamarixetin. Tamarixetin and quercetin both protect against radical toxicity. The oxidized products that arise in this process (QQ and TQ) are thiol reactive. In the presence of ascorbate TQ preferentially passes its reactivity to ascorbate. An extra advantage is that ascorbate recycles TQ to the parent compound.

## Supplemental data

Ascorbate prevented the net consumption of tamarixetin. The prevention of the oxidation of tamarixetin by ascorbate was checked.

## Materials and methods

### Spectrophotometric analysis

Tamarixetin (50 µM) was oxidized by $H_2O_2$ (50 µM) and HRP (3.2 nM) in 145 mM potassium-phosphate buffer (pH 7.4). The incubation mixtures were analyzed spectrophotometrically in presence or absence of ascorbate (50 µM). The incubation mixture of ascorbate (50 µM) with (50 µM) and HRP (3.2 nM) in 145 mM potassium-phosphate buffer (pH 7.4) was also analyzed. All spectra were recorded from 220 to 600 nm. The UV/Vis scans were started 30, 90, 150, 210, 270 sec after the addition of HRP.

## Results

### Spectrophotometric analysis of the oxidation of tamarixetin

The tamarixetin concentration decreased in the incubation mixture containing HRP/$H_2O_2$. Addition of 50 µM ascorbate to the incubation mixture shows no net consumption of tamarixetin (figure 6A and 6B). HPLC analysis of the incubation mixtures confirmed that no tamarixetin was consumed in the presence of ascorbate (data not shown). In the incubation ascorbate was consumed, evidenced in the spectrum by the decrease of the absorption at 270 nm. When tamarixetin was omitted from the incubation mixture, the ascorbate concentration did not change (figure 6C). For quercetin similar results have been reported (Moalin et al., 2012)..

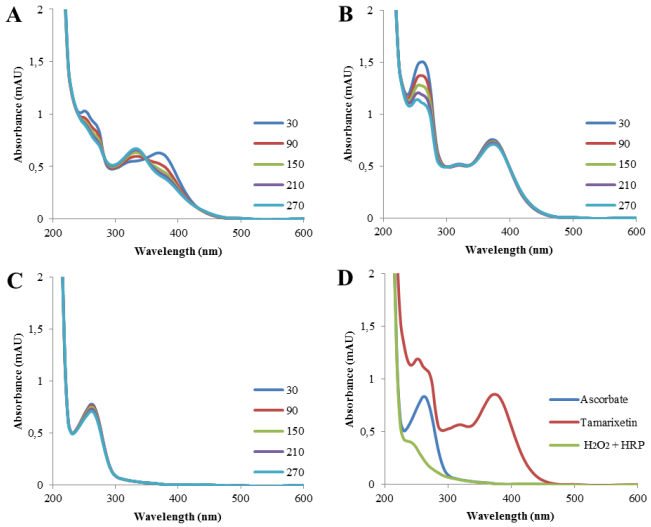


**Figure 6.** Spectrophotometrical analysis of the incubation mixture containing 50 µM tamarixetin, 50 µM $H_2O_2$ and 3.2 nM HRP (A). The same experiment was carried out in the presence of 50 µM ascorbate (B). Ascorbate was incubated with 50 µM $H_2O_2$ and 3.2 nM HRP (C). The UV/Vis scans were recorded 30, 90, 150, 210, 270 sec after addition of HRP. The spectra of 50 µM $H_2O_2$ and 3.2 nM HRP, 50 µM tamarixetin and 50 µM ascorbate are shown (D).

## Conclusion

Ascorbate did not inhibit the oxidation, but immediately reduced the oxidized tamarixetin.

## References

Bast, A., Haenen, G.R., 2002. The toxicity of antioxidants and their metabolites. Environmental Toxicology and Pharmacology 11, 251-258.

Bast, A., Haenen, G.R., 2013. Ten misconceptions about antioxidants. Trends in Pharmacological Sciences 34, 430-436.

Bjelakovic, G., Nikolova, D., Gluud, C., 2014. Antioxidant supplements and mortality. Current Opinion in Clinical Nutrition and Metabolic Care 17, 40-44.

Boots, A.W., Haenen, G.R., den Hartog, G.J., Bast, A., 2002. Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. Biochimica et Biophysica Acta 1583, 279-284.

Boots, A.W., Li, H., Schins, R.P., Duffin, R., Heemskerk, J.W., Bast, A., Haenen, G.R., 2007. The quercetin paradox. Toxicology and Applied Pharmacology 222, 89-96.

Brevik, A., Rasmussen, S.E., Drevon, C.A., Andersen, L.F., 2004. Urinary excretion of flavonoids reflects even small changes in the dietary intake of fruits and vegetables. Cancer epidemiology, biomarkers & prevention 13, 843-849.

Halliwell, B., 1996. Antioxidants in human health and disease. Annual Review of Nutrition 16, 33-50.

Ito, S., Kato, T., Fujita, K., 1988. Covalent binding of catechols to proteins through the sulphydryl group. Biochemical Pharmacology 37, 1707-1710.

Jacobs, H., Moalin, M., Bast, A., van der Vijgh, W.J., Haenen, G.R., 2010. An essential difference between the flavonoids monoHER and quercetin in their interplay with the endogenous antioxidant network. PLoS One 5, e13880.

Kalyanaraman, B., Premovic, P.I., Sealy, R.C., 1987. Semiquinone anion radicals from addition of amino acids, peptides, and proteins to quinones derived from oxidation of catechols and catecholamines. An ESR spin stabilization study. The Journal of Biological Chemistry 262, 11080-11087.

Moalin, M., van Strijdonck, G.P., Bast, A., Haenen, G.R., 2012. Competition between ascorbate and glutathione for the oxidized form of methylated quercetin metabolites and analogues: tamarixetin, 4'-O-methylquercetin, has the lowest thiol reactivity. Journal of Agricultural and Food Chemistry 60, 9292-9297.

Moore, G.A., Weis, M., Orrenius, S., O'Brien, P.J., 1988. Role of sulfhydryl groups in benzoquinone-induced Ca2+ release by rat liver mitochondria. Archives of Biochemistry and Biophysics 267, 539-550.

O'Brien, P.J., 1991. Molecular mechanisms of quinone cytotoxicity. Chemico-biological interactions 80, 1-41.

Pearson, R.G., 1963. Hard and Soft Acids and Bases. Journal of the American Chemical Society 85, 3533-3539.

Reddy, S., Jones, A.D., Cross, C.E., Wong, P.S., Van Der Vliet, A., 2000. Inactivation of creatine kinase by S-glutathionylation of the active-site cysteine residue. The Biochemical Journal 347 Pt 3, 821-827.

Spencer, J.P., Kuhnle, G.G., Williams, R.J., Rice-Evans, C., 2003. Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites. Biochemistry Journal 372, 173-181.

Willcox, J.K., Ash, S.L., Catignani, G.L., 2004. Antioxidants and prevention of chronic disease. Critical Reviews in Food Science and Nutrition 44, 275-295.

Zhao, T.J., Liu, Y., Chen, Z., Yan, Y.B., Zhou, H.M., 2006. The evolution from asparagine or threonine to cysteine in position 146 contributes to generation of a more efficient and stable form of muscle creatine kinase in higher vertebrates. The International Journal of Biochemistry & Cell Biology 38, 1614-162

Chapter 4

# The antioxidant flavonoid monoHER provides efficient protection and induces the innate NRF2 mediated adaptation in endothelial cells subjected to oxidative stress

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PharmaNutrition, 2014; 2(3): 69-74

**Abstract**

Reactive oxygen species (ROS) can react with any cellular component leading to oxidative damage. As an adaptive response to ROS, cells activate NRF2 which enforces the endogenous antioxidant defense.

Exogenous antioxidants directly neutralize the ROS and prevent the oxidative damage. This protection against ROS might backfire, because the adaptive response of the cell is prevented.

The flavonoid monoHER efficiently scavenges ROS. In this study the effect of monoHER on the oxidative stress as well as the adaptive response was investigated. It was found that monoHER protected against oxidative stress caused in HUVECs by $H_2O_2$, while monoHER did not prevent the induction of the adaptive response, including the nuclear translocation of NRF2 as well as the upregulation of *HO-1* gene expression. Remarkably, monoHER added to HUVECs which were not subjected to oxidative stress did not cause an adaptive response.

The concept that emerges is that by scavenging ROS, the oxidized monoHER formed channels the reactivity selectively to thiol groups. Oxidized monoHER adducts KEAP1, which can set the innate NRF2 machinery into motion and enforce the endogenous antioxidant defense. In this way monoHER acts as a double-edged sword in cells subjected to oxidative stress; the antioxidant offers direct protection and induces adaptation.

## Introduction

Reactive oxygen species (ROS) belong to the most aggressive molecules produced in biology and can react with any cellular constituent ranging from DNA to proteins and lipids (Bast et al., 1991). This high, non-selective reactivity forms the basis of the toxicity of ROS. Cells are protected against ROS by antioxidants (Sies, 1993). When ROS production is enhanced, cells adapt by raising their protective antioxidant shield (Chaudiere and Ferrari-Iliou, 1999). Such a cellular flexibility is far more economical and efficient than continuously wearing a suit of armor against all possible threats (Sthijns et al., 2014). The ability to adapt to ROS is triggered by activation of the innate antioxidant machinery via gene expression through the Nuclear factor erythroid 2-related factor 2 (NRF2) pathway, which results in higher cellular levels of antioxidants and antioxidant enzymes (Kobayashi and Yamamoto, 2006).

Nutrition rich in antioxidants or antioxidants administered as supplement, nutraceutical or drug are also applied in an attempt to alleviate ROS damage (Boots et al., 2008; Rietjens et al., 2007; Vetrani et al., 2013). In this respect, the semi-synthetic flavonoid antioxidant monoHER proved to be very effective. MonoHER is the most powerful antioxidant in the registered drug Venoruton® and displays excellent radical scavenging properties (Haenen et al., 1993; Lemmens et al., 2014). By scavenging, the damaging reaction of ROS with cellular compounds is prevented. In this way monoHER directly protects against cellular damage. However, the protection against ROS is expected to backfire because also the activation of the innate NRF2 pathway might be prevented. In this case, the paradoxical consequence is that no protective adaptation is elicited because antioxidant administration prevents the endogenous defensive shield to be raised.

The potentially paradoxical effect of antioxidants prompted us to investigate the effect of monoHER on the cellular adaptive response. Human Umbilical Vein Endothelial cells (HUVECs) were selected as a model since Venoruton® is used in the treatment of endothelial venous insufficiency (Cesarone et al., 2006). To induce oxidative stress, the cells were exposed to $H_2O_2$, which is a relatively stable ROS and

thus enabled us to impose a reproducible oxidative stress upon the cells. Besides, $H_2O_2$ is constantly formed by endothelial cells through various enzymatic systems, e.g. via superoxide radicals formed by NAD(P)H oxidase (NOX) which dismutate to $H_2O_2$. $H_2O_2$ easily crosses the cellular membrane through aquaporin channels (Miller et al., 2010). It can give rise to the highly toxic hydroxyl radicals.

It was confirmed that monoHER protects against oxidative stress induced by $H_2O_2$. Remarkably, monoHER did not reduce the selective adaptation against oxidative stress.

## Materials and methods

### Chemicals

7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) was kindly provided by Novartis Consumer Health (Nyon, Switzerland). Hydrogen peroxide ($H_2O_2$), 2',7'-dichlorofluorescein-diacetate (DCFH-DA), tert-Butylhydroquinone (T-BHQ), DL-Dithiothreitol (DTT), sodium fluoride (NaF), sodium molybdate ($Na_2MoO_4$), Ethylenediaminetetraacetic acid (EDTA), Protease Inhibitor Cocktail, Hepes and horseradish peroxidase (HRP) were obtained from Sigma (St. Louis, MO, USA). Lysis buffer AM1 was obtained from Active Motif (Rixensart, Belgium). Trypsin was purchased from Promega (Madison, USA). Ammonium bicarbonate (ABC), acetonitrile (ACN), α-cyano-4-hydroxycinnamic acid, 2',7'-dichlorofluorescein-diacetate (DCFH-DA) and trifluoroacetic acid (TFA) were acquired from Sigma (St. Louis, USA). HUVECs, cell line-1730 was obtained from ATCC (Manassas, USA). Hank's balanced salt solution (HBSS) was obtained from Invitrogen (Breda, The Netherlands). Kelch-like ECH-associated protein 1 (KEAP1) was purchased from Sino Biological Inc (Beijing, China).

### Cell culture

HUVECs were cultured T75 coated culture flasks (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) in Ham's F12-K medium (Invitrogen, Breda, The Netherlands) supplemented with 10 % (v/v) fetal calf serum (FCS; Invitrogen, Breda, The Netherlands), 0.05 mg/ml endothelial cell growth supplement (ECGS; BD Science, Franklin Lakes, USA), 1 % pen/strep (Gibco, Bleiswijk, The Netherlands) and 0.01 mg/ml heparin (LEO Pharma, Ballerup, Denmark) in a humidified atmosphere containing 5 % $CO_2$ and 95 % air at 37 °C. The maximal passage used was 24.

**Measurement of intracellular oxidative stress**

The intracellular levels of oxidative stress were quantified by the fluorescence of DCF.

Cells (4 X $10^4$ per well) were plated in a 96-wells plate (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) 24 h before the experiment. The cells were then incubated with 20 µM DCFH-DA in 5 % $CO_2$ and 95 % air at 37 °C for 45 min. The cells were rinsed once with HBSS, after the medium with excess of DCFH-DA was removed. Subsequently the cells were incubated with 200 µM $H_2O_2$ and 0-50 µM monoHER in serum-free medium (Lemmens et al., 2014). The fluorescence (Ex 485 nm, Em 538 nm) was determined on a microplate reader (Spectramax microplate reader, Molecular devices, Sunnyvale, USA) as a measure of oxidative damage.

For the adaptation measurements, cells (4 X $10^4$ per well) were plated in a 96-wells plate 24 h before the experiment. The cells were then incubated with serum-free medium and 200 µM $H_2O_2$ with or without 50 µM monoHER for 4 h. After a washing step with HBSS, the cells were treated with 20 µM DCFH-DA in 5 % $CO_2$ and 95 % air at 37 °C for 45 min. After the medium with excess of DCFH-DA was removed, the cells were rinsed once with HBSS and subsequently a solution containing 200 µM $H_2O_2$ was added followed by measuring the fluorescence of DCF on the microplate reader (Ex 485 nm, Em 538 nm).

The reaction of monoHER with $H_2O_2$ was examined by HPLC and spectrophotometrically (data not shown). MonoHER did not directly react with $H_2O_2$.

**Gene expression of heme oxygenase 1**

Real Time PCR (qRT-PCR) was used to measure the gene expression of heme oxygenase 1 (*HO-1*). Cells (2 X $10^5$ per well) were plated in a 6 well-plate 24h before the experiment. After washing with HBSS, the cells were exposed to 0 or 50 µM monoHER and 0-200 µM $H_2O_2$ for 3 h at 37 ˚C. After exposure cells were trypsinized. RNA was isolated with Qiazol lysis reagent (Qiagen, The Netherlands). The RNA concentration was measured using the NanoDrop System (Thermo

Scientific, Rockford, IL, USA). cDNA was prepared from 500 ng of RNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Two step qRT-PCR was performed using 12.5 µl SensiMix SYBR & Fluorescein Kit (Bioline, London, UK) with 5 µl cDNA, and 2.5 µl of 100 µM of each primer (forward: 5'-CTTCTTCACCTTCCCCAACA-3', reverse: 5'-GCTCTGGTCCTTGGTGTCAT-3') in a 25 µl reaction mixture. The cycling conditions consisted of an initial denaturation at 95 ˚C for 10 minutes, followed by 40 alternating cycles at 95 ˚C and 60 ˚C for 15 s and 45s, respectively, using the iCycler system (Bio-Rad, Hercules, CA). β-actin (forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCCGATCCACACGGAGTACT-3') was used as housekeeping gene. Fold change was calculated relative to unexposed cells.

**Mass spectrometry**

MonoHER (50 µM) was oxidized by 50 µM $H_2O_2$ and 12.8 nM HRP in presence of 0.5 mg/ml KEAP1 in 50 mM ABC buffer pH 7.4 for 15 min at 37 ˚C. KEAP1 (0.1 mg/ml) was digested by trypsin during 2 hours at 37 °C. The digested samples were diluted 1:10 in 0.1 % TFA. After dilution, 1 µl of the digest and 1 µl of matrix solution (2.5 mg/ml α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA) was spotted on a 348-well-format target plate and air dried. Mass spectra were measured on the MALDI-TOF mass spectrometer (4800 MALDI-TOF analyzer; Applied Biosystems). The instrument was operated in positive reflector mode. Acquisition mass range was 800-3500 Da.

**Nuclear extraction**

HUVECs were resuspended in hypotonic buffer (5 mM NaF, 10 µM $Na_2MoO_4$, 0.1 mM EDTA in 20 mM Hepes, pH 7.5), vortexed, and incubated on ice for 15 min. The suspension was centrifuged for 30 sec at 14000 g. The remaining nuclear pellets were resuspended in complete lysis buffer (1 µl 1 M DTT, 10 µl Protease Inhibitor Cocktail per ml Lysis Buffer AM1) and kept on ice for 30 min. The suspension was centrifuged (14,000 rpm, 10 min, 4°C), and the nuclear fraction was obtained.

**Nuclear NRF2 measurement**

HUVECs (2 X $10^6$ per well) were plated in a T75 culture flasks (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) 24h before the experiment. Cells were exposed to 50 µM monoHER with or without $H_2O_2$ for 30 min. As positive control HUVECs were incubated with 25 µM t-BHQ.

Nuclear NRF2 was determined in the nuclear extract of the cells using the TransAM NRF2 kit (Active Motif, Rixensart, Belgium). The TransAm NRF2 kit is based on ELISA. The kit contains a 96 well-plate on which oligonucleotide containing the ARE consensus binding site are immobilized. NRF2 in the nuclear extract specifically binds to this oligonucleotide. The primary antibody recognizes an epitope on NRF2 protein upon DNA binding. Addition of an HRP-conjugated secondary antibody provides a colorimetric readout quantified by spectrophotometry.

Protein concentrations in the nuclear extracts were determined using the method of Bradford (Biorad, Veenendaal, The Netherlands)

**Statistics**

All experiments were performed at least in triplicate. Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student's t-test.

## Results

### Damage by and adaptation to oxidative stress by $H_2O_2$

Two hundred µM of $H_2O_2$ induced oxidative stress in DCFH loaded HUVECs, deduced from the increase in fluorescence in time (figure 1A).
To determine adaptation to oxidative stress, cells were first pretreated with $H_2O_2$, followed by a second exposure of the pretreated cells to $H_2O_2$. The oxidative stress induced by $H_2O_2$ in the pretreated cells was significantly less than that in not-pretreated cells (figure 1A and 1C). Apparently $H_2O_2$ induces protection against a second challenge with $H_2O_2$ i.e. it induces an adaptive response to oxidative stress.

### Protection against and adaptation to oxidative stress by monoHER

MonoHER was able to fully protect against the $H_2O_2$-induced oxidative stress in the cell (Figure 1B). To determine the effect of monoHER on the adaptation to oxidative stress, cells were first pretreated with the combination of $H_2O_2$ and monoHER. This pretreatment was followed by a second exposure of the pretreated cells to only $H_2O_2$. Again, the oxidative stress induced by $H_2O_2$ in the pretreated cells was significantly less than that in not-pretreated cells. Although monoHER fully protected against oxidative stress, monoHER did not impair the adaptive response to oxidative stress (Figure 1A and 1C).
In a control experiment, cells were first pretreated with only monoHER, followed by a second exposure of the pretreated cells to $H_2O_2$. The oxidative stress induced by $H_2O_2$ in these pretreated cells was similar to that in not-pretreated cells (Figure 1A and 1C). Apparently, monoHER itself does not induce an adaptive response to oxidative stress.

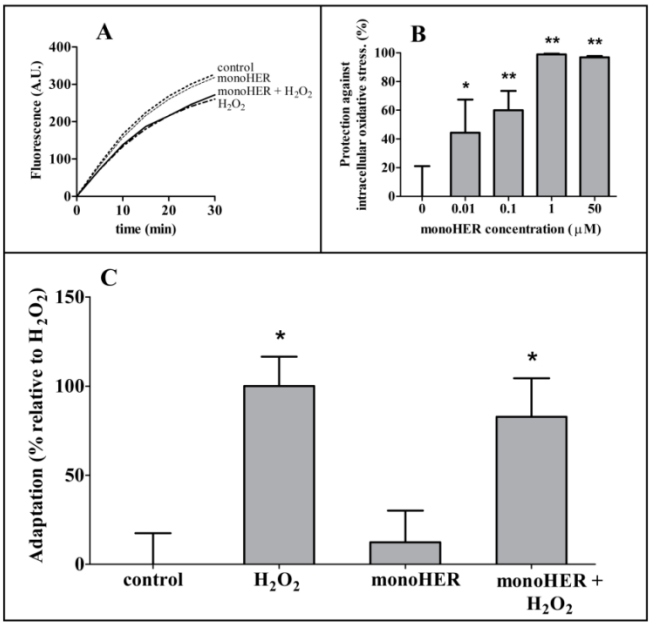


**Figure 1.** Pretreatment of HUVEC's with $H_2O_2$ and the combination of $H_2O_2$ with monoHER induces an adaptive protection against oxidative stress. Panel A shows intracellular oxidative stress in pretreated HUVEC's induced by 200 μM $H_2O_2$. The pretreatment for 4 h was with 200 μM $H_2O_2$ (curve denoted with '$H_2O_2$'); with 50 μM monoHER ('monoHER'); with the combination of 200 μM $H_2O_2$ and 50 μM monoHER ('monoHER + $H_2O_2$'); or without extra compounds ('control'). Intracellular oxidative stress was measured for 30 min using DCF fluorescence as described in the materials and methods section. Panel B shows the concentration dependent protection by monoHER against oxidative stress induced by 200 μM $H_2O_2$ in HUVEC's. Panel C depicts the adaptive response found after 30 minute incubation with 200 μM $H_2O_2$ in control HUVEC's ('control'); in HUVECs pretreated with 200 μM $H_2O_2$ ('$H_2O_2$'); in HUVECs pretreated with 50 μM monoHER ('monoHER'); in HUVECs pretreated with 200 μM $H_2O_2$ in combination with 50 μM monoHER ('monoHER + $H_2O_2$'). In panel A a typical example is shown. For panel B and C at least three independent experiments were performed in quadruplicate and data are shown as mean ± SD (*$p<0.05$, ** $p<0.01$ vs. control).

### Heme oxygenase-1 gene expression

$H_2O_2$ significantly increased the *HO-1* gene expression in HUVECs (Figure 2). MonoHER in combination with $H_2O_2$ also led to a significant increase in the *HO-1* gene expression, although this tended to be slightly less than that with $H_2O_2$ alone. MonoHER without $H_2O_2$ did not affect the *HO-1* gene expression (Figure 2).

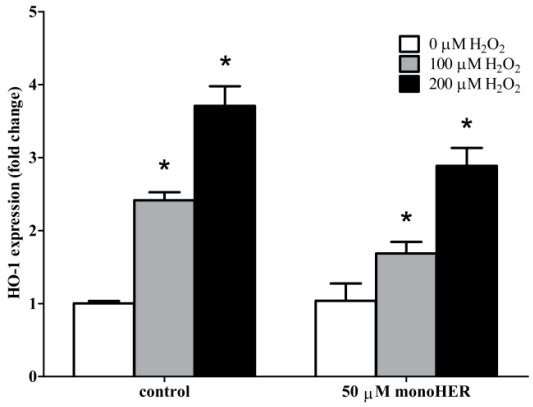


**Figure 2.** *HO-1* gene expression measured in HUVECs exposed to 0 or 50 µM monoHER and 0-200 µM $H_2O_2$ for 3 h. Bars represent fold change ($2^{-\Delta\Delta Ct}$) compared to unexposed cells. Data are shown as mean ± SD (n≥4). (*p <0.01 vs. 0 µM $H_2O_2$, 0 µM mH).

### Adduction of monoHER on KEAP1

MonoHER oxidized in the presence of KEAP1 reduced the intensity of the peak of the native peptide fragment, LNSAECYYPER with a mass of *m/z* = 1344 measured by MALDI-TOF. Instead, a new peptide fragment with a mass of *m/z* = 1996 emerged (Figure 3). The increment of the mass of the fragments corresponds to the molecular weight of the quinone of monoHER (652 Da). This indicates that the quinone formed by the oxidation of monoHER adducts KEAP1.

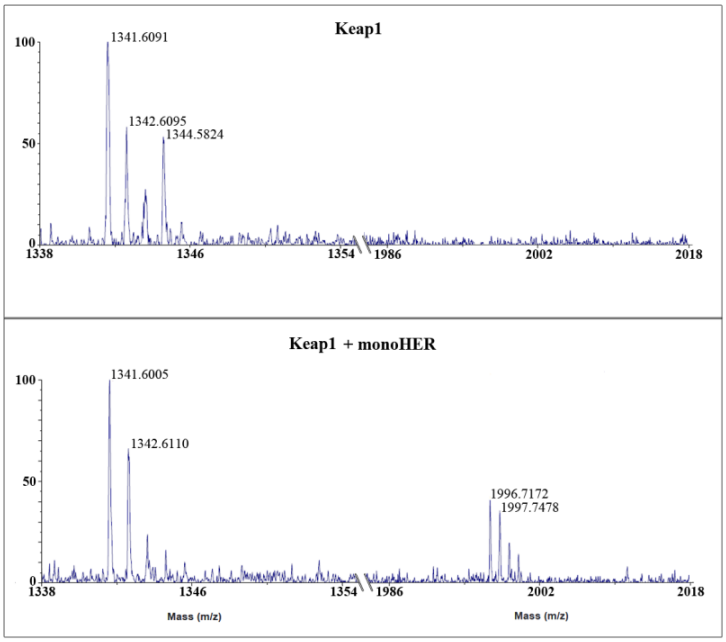


**Figure 3.** Adduction of KEAP1 by oxidized monoHER. MALDI-TOF analysis of isolated KEAP1 (0.1 mg/ml) incubated with 50 µM monoHER, 1.6 nM HRP and 50 µM $H_2O_2$, after trypsin digestion. The control spectrum of KEAP1 displayed a peak at *m/z* 1344 and no peak at *m/z* 1996. The incubation mixture with monoHER showed a peak at *m/z* 1996 which corresponds to the mass of the adduct of the monoHER quinone (652 Da) on the peptide with mass *m/z* 1344, whereas the peak at *m/z* 1344 decreased.

### NRF2 translocation

Incubation of HUVECs with 200 µM $H_2O_2$ induced translocation of NRF2 to the nucleus. Also incubation with monoHER in combination with $H_2O_2$ resulted in the translocation of NRF2 to the nucleus, whereas incubation with only monoHER did not do so. (Figure 4). The positive control, t-BHQ did induce translocation of NRF2 to the nucleus.

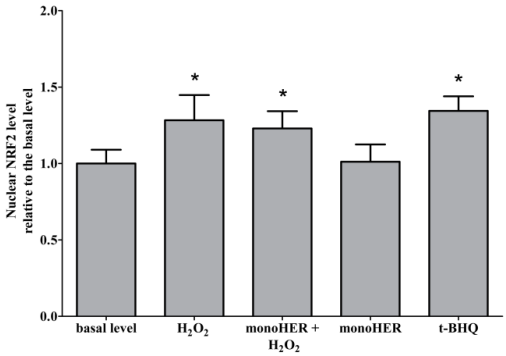


**Figure 4.** Induction of nuclear NRF2 in HUVEC's by monoHER in combination with $H_2O_2$. HUVEC's were exposed to 200 µM $H_2O_2$ or 50 µM monoHER ± 200 µM $H_2O_2$ for 30 min. t-BHQ (25 µM) was used as positive control. NRF2 was measured in the nuclear extracts. At least three independent experiments were performed and data are shown as mean ± SD (*$p<0.05$ vs. basal level).

**Discussion**

ROS possess an extreme high chemical reactivity and are therefore capable of damaging virtually any cellular component, which will eventually lead to toxicity (Bast et al., 1991). Enhanced ROS production stimulates the endogenous antioxidant defense system and enforces the innate protection against ROS toxicity (Chaudiere and Ferrari-Iliou, 1999). This cellular flexibility adapts cells to better withstand emerging oxidative stress.

Numerous exogenous antioxidants directly protect by scavenging free radicals. It is well documented that this can directly offer an excellent protection against ROS damage (Boots et al., 2008; Lemmens et al., 2014; Rietjens et al., 2007). However, the protection by this mechanism might backfire because not only the damage but also the adaptive response might be prevented. The antioxidants take away the ROS that pull the trigger for raising the cellular protective shield. In the present study, the effect of the flavonoid antioxidant monoHER on the adaptive response is therefore examined.

Oxidative stress was induced in HUVECs by $H_2O_2$. Our results show that pretreatment with $H_2O_2$ also enforces the protection of the cells against oxidative stress. This was concluded from the experiments in which cells were first pretreated with $H_2O_2$, followed by a second exposure of the pretreated cells to $H_2O_2$. The oxidative stress induced by $H_2O_2$ in the pretreated cells was significantly less than that in not-pretreated cells. Apparently, an adaptive response to oxidative stress is induced by activating the innate antioxidant defense system.

The molecular mechanism behind the adaptation involves activation of the NRF2 pathway. This is initiated by ROS-induced damage to KEAP1, the inhibitor of NRF2 (Itoh et al., 2010). The ROS damaged KEAP1 dissociates from its complex with NRF2. Upon this dissociation, NRF-2 translocates to the nucleus. NRF2 is a transcription factor which activates the expression of Antioxidant Responsive Elements (ARE), such as antioxidant and detoxifying enzymes e.g. HO-1 (Itoh et al., 1997; Itoh et al., 2004; Rushmore et al., 1991). Due to the activated NRF2 pathway, the cell upgrades its protective shield against oxidative stress. The activation of this

pathway in the HUVECs by $H_2O_2$ is evidenced by the translocation of NRF2 to the nucleus, the induced *HO-1* gene expression, and the adaptation of the cells to the second oxidative stress assault (Figure 5). This adaptation equates to the concept of hormesis: exposure to a damaging compound raises a specific protective shield (Sthijns et al., 2014).

MonoHER possesses excellent antioxidant properties. This was confirmed in the present study, as monoHER was able to fully protect HUVECs against the induced oxidative stress. MonoHER owes this activity to its strategic location (i) on a molecular level, (ii) on a supramolecular level and (iii) on a cellular level (Lemmens et al., 2014). On the cellular level monoHER is localized in the endothelial and smooth muscle cells of the vascular wall where it appears to be retained (supplemental data), and where the protective effect of the monoHER containing preparation Venoruton® is due (Neumann et al., 1992).

On the molecular level, monoHER is also exactly at the right location. The $H_2O_2$ used can produce intracellular oxidative stress by reacting with iron to generate the extremely reactive OH-radical in a Fenton reaction. In the present study we confirmed that monoHER, already at a very low concentration (0.01-1 µM), can directly protect against the oxidative stress. This efficient protection can be explained by site-specific scavenging. Essential for this activity is that monoHER can chelate iron. The result of this chelation is that monoHER is present at the site of the radical formation, i.e. the iron ion. This enables monoHER to immediately scavenge newly formed radicals. By this site-specific scavenging, monoHER is able to prevent damage to critical biomolecules such as lipids, proteins or DNA, despite the high reactivity of the radical (Lemmens et al., 2014).

Last, but not least is that monoHER is located on the supramolecular level at a pivotal position in the antioxidant network. Due to the site-specific scavenging, monoHER is the first molecule to react with the generated radicals. In the scavenging reactions, monoHER donates electrons or hydrogen atoms to the radicals involved. The radical is converted into a relatively stable non-radical, e.g. the hydroxyl radical becomes $H_2O$. MonoHER takes over part of the reactivity of the radical and is oxidized to a semi-quinone radical and monoHER quinone (Bast and

Haenen, 2002; Jacobs et al., 2009). Fortunately, the body is endowed with an intricate network of antioxidants that can react with oxidized monoHER. MonoHER quinone preferentially reacts in a redox reaction with ascorbate, and monoHER is recycled to scavenge radicals once again (Jacobs et al., 2010).

Oxidized monoHER also reacts with thiols, evidenced by the formation of a GSH-monoHER adduct after monoHER administration to human volunteers (Jacobs et al., 2009). Our mass spectrometry data confirm this thiol reactivity, since oxidized monoHER adducts the KEAP1 peptide fragment LNSAECYYPER with cysteine 489. This cysteine residue is situated in the NRF2 binding domain of the protein (Holland and Fishbein, 2010; Itoh et al., 1999). A consequence of the adduction is that the innate NRF2 machinery is set in motion and that via gene expression levels of endogenous antioxidant enzymes are increased. Indeed, the present study demonstrated that the combination of $H_2O_2$ and monoHER leads to translocation of NRF2 to the nucleus and the induction of the gene expression of *HO-1*, which cumulates in an adaptive upgrading of the protection against oxidative stress (Figure 2 and 4 and illustrated in figure 5).

Interestingly, monoHER itself did not induce an adaptive response in the HUVECs. The explanation is that monoHER itself lacks thiol reactivity. t-BHQ and acrolein, compounds that are thiol reactive themselves, were found to directly activate the NRF2 machinery (Arinze and Kawai, 2005; Kwak et al., 2003). For monoHER to do so, it first needs to be converted into a thiol reactive oxidation product. This happens when monoHER scavenges radicals. Thus, when monoHER acts as a free radical scavenger and becomes a thiol reactive quinone, it successively activates the endogenous antioxidant network. In this way the antioxidant acts as a double-edged sword; it offers direct protection as well as indirect protection. This explains why monoHER prevents the oxidative stress provoked by $H_2O_2$, and also induces the adaptive response. This dual action puts the protective effect of antioxidants like monoHER on a higher level.

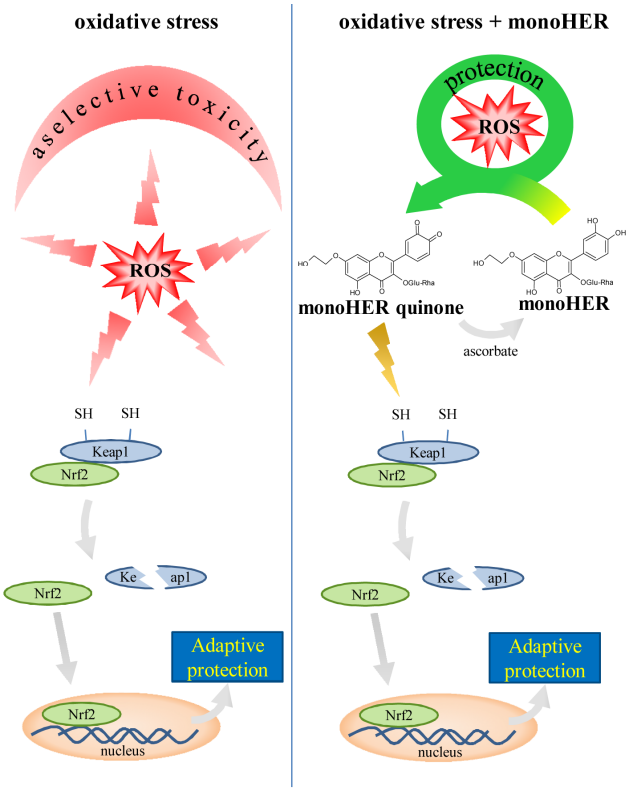


**Figure 5.** Overview of the protective effect of monoHER against ROS toxicity and the induction of the adaptive response of the cell. ROS induce toxicity and activate the NRF2 pathway which gives rise to an adaptive response in the cell. MonoHER protects by scavenging the ROS toxicity but does not prevent the adaptive response in the cells. The monoHER quinone formed during scavenging selectively adducts KEAP1 and activates the NRF2 pathway.

This molecular mechanism does not only result in a very potent antioxidant activity, it also ensures that monoHER will act selectively in a condition of oxidative stress. The unbalanced ROS production found during oxidative stress is mandatory for the formation of the thiol reactive quinone of monoHER. This will selectively activate the NRF2 pathway in cells subjected to oxidative stress. This selectivity is substantiated by the observation that monoHER itself did not induce an adaptive response in the HUVECs, while the combination of monoHER with $H_2O_2$ did.

We conclude that $H_2O_2$ causes oxidative stress in HUVECs but adapts the cells by raising the antioxidant shield to withstand new assaults. MonoHER itself does not induce adaptation under control conditions. MonoHER efficiently protects against the oxidative stress caused by $H_2O_2$ and, surprisingly, still induces the adaptive response of the cells subjected to oxidative stress. It appears that the antioxidant flavonoid monoHER provides efficient protection and induces the innate NRF2 mediated adaptation in HUVECs subjected to oxidative stress.

**Supplemental data**

The localization of monoHER in carotid arteries was studied by two photon laser scanning microscopy (TPLSM).

**Materials and methods**

The animal protocol was approved by the Ethics committee for Animal Experiments of Maastricht University (Maastricht, The Netherlands).
Two C57BL/6J mice, obtained from Maastricht University, were housed under standard conditions. The animals were allowed to adapt to the laboratory housing conditions for two weeks before the experiment was started. The animals were allowed to eat and drink water ad libitum. One mouse was injected with monoHER subcutaneously (500 mg/kg) every day during 21 days to obtain a chronic exposure. The other mouse received saline according to the same injection scheme. The mice were sacrificed 24 h after the last injection. The isolated carotid arteries were filled with HBSS before two photon laser scanning microscopy (TPLSM).

The carotid arteries were imaged using TPLSM (TCS SP5 CFS AOBS with a Coherent: Chameleon Ultra II. ir. 140 fs pulsed laser system >300 KW, for 2 photon excitation at 800 nm, Emission filter: 510 - 580 nm) (Leica Microsystems, Wetzlar, Germany). A HCX APO L 20.0 x 1.00 water objective (WD = 2.0 mm) was used. Images were obtained using LAS-AF SP5 software. No further image processing was carried out.

**Results**

Injection of monoHER in a mouse led to accumulation of monoHER in the wall of the carotid artery. The artery displayed fluorescence in the nuclei of the endothelial and smooth muscle cells (Figure 6). Similar results were obtained in carotid arteries incubated with monoHER (Lemmens et al., 2014).

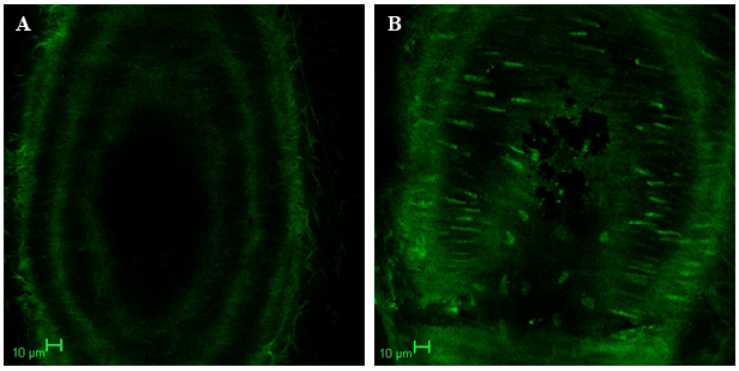


**Figure 6.** Localization of monoHER in the carotid artery. Two Photon Laser Scanning Microscopy imaging of longitudinal cross-sections of an intact carotid artery from a mouse injected with vehiculum (A) or with monoHER (B) isolated 24 h after the last injection. The elastic lamina autofluoresce in the same wavelength range as monoHER, i.e. emission 510-580 nm, and are therefore visible. The carotid artery from the mouse injected with monoHER shows fluorescence in the nuclei of the endothelial cells (round spots) and smooth muscle cells (elongated spots).

**Conclusion**

MonoHER localizes in the nuclei of the endothelial and smooth muscle cells in the carotid artery.

## References

Arinze, I.J., Kawai, Y., 2005. Transcriptional activation of the human Galphai2 gene promoter through nuclear factor-kappaB and antioxidant response elements. The Journal of Biological Chemistry 280, 9786-9795.

Bast, A., Haenen, G.R., 2002. The toxicity of antioxidants and their metabolites. Environ Toxicol Pharmacol 11, 251-258.

Bast, A., Haenen, G.R., Doelman, C.J., 1991. Oxidants and antioxidants: state of the art. The American Journal of Medicine 91, 2S-13S.

Boots, A.W., Haenen, G.R., Bast, A., 2008. Health effects of quercetin: from antioxidant to nutraceutical. European Journal of Pharmacology 585, 325-337.

Cesarone, M.R., Belcaro, G., Pellegrini, L., Ledda, A., Vinciguerra, G., Ricci, A., Gizzi, G., Ippolito, E., Fano, F., Dugall, M., Acerbi, G., Cacchio, M., Di Renzo, A., Hosoi, M., Stuard, S., Corsi, M., 2006. Circulating endothelial cells in venous blood as a marker of endothelial damage in chronic venous insufficiency: improvement with venoruton. Journal of Cardiovascular Pharmacology and Therapeutics 11, 93-98.

Chaudiere, J., Ferrari-Iliou, R., 1999. Intracellular antioxidants: from chemical to biochemical mechanisms. Food and chemical toxicology 37, 949-962.

Haenen, G.R., Jansen, F.P., Bast, A., 1993. The antioxidant properties of five O-(-β-Hydroxyethyl)-Rutosides of the flavonoid mixture Venoruton. Phlebology Supplements 1, 10-17.

Holland, R., Fishbein, J.C., 2010. Chemistry of the cysteine sensors in Kelch-like ECH-associated protein 1. Antioxidants & Redox Signaling 13, 1749-1761.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., Nabeshima, Y., 1997. An NRF2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochemical and Biophysical Rresearch Communications 236, 313-322.

Itoh, K., Mimura, J., Yamamoto, M., 2010. Discovery of the negative regulator of NRF2, Keap1: a historical overview. Antioxidants & Redox Signaling 13, 1665-1678.

Itoh, K., Tong, K.I., Yamamoto, M., 2004. Molecular mechanism activating NRF2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radical Biology & Medicine 36, 1208-1213.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D., Yamamoto, M., 1999. Keap1 represses nuclear activation of antioxidant responsive elements by NRF2 through binding to the amino-terminal Neh2 domain. Genes & Development 13, 76-86.

Jacobs, H., Moalin, M., Bast, A., van der Vijgh, W.J., Haenen, G.R., 2010. An essential difference between the flavonoids monoHER and quercetin in their interplay with the endogenous antioxidant network. PLoS One 5, e13880.

Jacobs, H., van der Vijgh, W.J., Koek, G.H., Draaisma, G.J., Moalin, M., van Strijdonck, G.P., Bast, A., Haenen, G.R., 2009. Characterization of the glutathione conjugate of the semisynthetic flavonoid monoHER. Free Radicical Biology and Medicine 46, 1567-1573.

Kobayashi, M., Yamamoto, M., 2006. NRF2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. Advances in Enzyme Regulation 46, 113-140.

Kwak, M.K., Kensler, T.W., Casero, R.A., Jr., 2003. Induction of phase 2 enzymes by serum oxidized polyamines through activation of NRF2: effect of the polyamine metabolite acrolein. Biochemical and Biophysical Research Communications 305, 662-670.

Lemmens, K.J., van de Wier, B., Vaes, N., Ghosh, M., van Zandvoort, M.A., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014. The flavonoid 7-mono-O-(beta-hydroxyethyl)-

rutoside is able to protect endothelial cells by a direct antioxidant effect. Toxicology in vitro 28, 538-543.

Miller, E.W., Dickinson, B.C., Chang, C.J., 2010. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. Proceedings of the National Academy of Sciences of the United States of America 107, 15681-15686.

Neumann, H.A., Carlsson, K., Brom, G.H., 1992. Uptake and localisation of O-(beta-hydroxyethyl)-rutosides in the venous wall, measured by laser scanning microscopy. European Journal of Clinical Pharmacology 43, 423-426.

Rietjens, S.J., Bast, A., de Vente, J., Haenen, G.R., 2007. The olive oil antioxidant hydroxytyrosol efficiently protects against the oxidative stress-induced impairment of the NObullet response of isolated rat aorta. American Journal of Physiology 292, H1931-1936.

Rushmore, T.H., Morton, M.R., Pickett, C.B., 1991. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. The Journal of Biological Chemistry 266, 11632-11639.

Sies, H., 1993. Strategies of antioxidant defense. European Journal of Biochemistry / FEBS 215, 213-219.

Sthijns, M.M., Randall, M.J., Bast, A., Haenen, G.R., 2014. Adaptation to acrolein through upregulating the protection by glutathione in human bronchial epithelial cells: The materialization of the hormesis concept. Biochemical and Biophysical Research Communications.

Vetrani, C., Costabile, G., Di Marino, L., Rivellese, A.A., 2013. Nutrition and oxidative stress: a systematic review of human studies. International Journal of Food Sciences and Nutrition 64, 312-326.

Chapter 5

# The flavonoid monoHER promotes the adaptation to oxidative stress during the onset of NAFLD

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Biochemical and Biophysical Research Communications, 2015; 456(1): 179-82

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**Abstract**

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease. An evidence-based pharmacological treatment for NAFLD is still lacking, but flavonoids have shown therapeutic potential. The present study was designed to investigate the effect of the flavonoid monoHER on the onset of NAFLD in *Ldlr*$^{-/-}$ mice on a high-fat and high-cholesterol diet. The focus was put on the effect on oxidative stress as well as the adaptive response. Wild type mice served as a control and the effect of monoHER was compared to that of a placebo.

In the *Ldlr*$^{-/-}$ group, monoHER provided only a mild protection against oxidative stress. In the placebo *Ldlr*$^{-/-}$ group an adaptive response elicited by the NRF2 antioxidant defense system was observed, evidenced by a higher *HO-1* and *Gpx3* gene expression, as well as an increased redox status, evidenced by the higher GSH/GSSG ratio. In the monoHER treated *Ldlr*$^{-/-}$ group both the adaptive response as well as the increase in redox status tended to be higher, although this did not reach significance on a group level. Unexpectedly, a strong within animal relationship was found that links a high adaptive response to a low redox status in the monoHER *Ldlr*$^{-/-}$ group. This correlation was absent in the placebo and wildtype group.

The concept that emerges is that a thiol-reactive oxidation product of monoHER, formed during oxidative stress, selectively induces the NRF2 pathway and enforces the endogenous antioxidant shield, to provide protection against NAFLD.

**Introduction**

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in Western countries affecting 20-30% of the general population, and this percentage is still on the rise. NAFLD includes a spectrum of liver disorders ranging from steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma (Berlanga et al., 2014). Up to date, no evidence-based pharmacotherapy is available for NAFLD. To find a way to prevent NAFLD, the molecular processes involved in the onset of the disease should be controlled.

One of the hallmarks in the etiology of NAFLD is oxidative stress (Koek et al., 2011; Rolo et al., 2012). During oxidative stress, the unbalanced production of reactive oxygen species (ROS) results in damage to virtually any cellular component, which explains the prominent role of ROS in NAFLD. However, ROS also destruct KEAP1, the inhibitor protein of NRF2. NRF2 serves as a master redox switch which turns on the expression of endogenous antioxidant genes e.g. *HO-1* and *Gpx3* (Itoh et al., 2004) and ultimately increases the cellular redox potential. Consequently, cells adapt to ROS through this potent feedback mechanism which enforces the protection against ROS. This fits in the current concept that health is a dynamic process characterized by the ability to adapt to challenges on a delicate homeostasis (2009).

In this concept of health, drugs are bioactive compounds that help to restore and enforce homeostasis. Numerous studies have demonstrated the beneficial effect of flavonoids - a group of antioxidant bioactives commonly found in our diet - in animal models of NAFLD (van de Wier et al., 2014). Among the flavonoids, 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) appears to be one of the most promising ones for treatment of NAFLD (van de Wier et al., 2014). This flavonoid is the main ingredient of Venoruton, a drug that has been used for several decades in the treatment of venous insufficiency. MonoHER displays excellent antioxidant activity (Lemmens et al., 2014a; Lemmens et al., 2014b), has a relatively high bioavailability and is relatively safe (van de Wier et al., 2014). Based on these favorable characteristics monoHER was selected for this study.

To evaluate its efficacy, the effect of monoHER on oxidative stress and the adaptive response during the onset of NAFLD was determined in an animal model. Previous studies have shown that *Ldlr*$^{-/-}$ mice on a high-fat and high-cholesterol diet are a suitable model to study the development of NAFLD (Bieghs et al., 2012). Wild type mice served as control and the effect of monoHER was compared to that of a placebo. The effect was evaluated on three levels, namely the direct damage by ROS, the NRF2-induced gene expression and the adaptive response on the cellular redox status.

## Material and methods

### Animals and treatment

Female C57BL/6J wildtype mice (WT mice) were obtained from Jackson laboratories (Bar Harbor, Maine, USA) and bred at the breeding facility of Maastricht University. Female LDL-receptor knockout mice on a C57BL/6J background (*Ldlr*$^{-/-}$ mice) were bred at the breeding facility of Maastricht University. Mice were maintained in a temperature- and light-controlled facility and were permitted ad libitum consumption of water and chow. At the age of 13 weeks, all mice started a high-fat and high-cholesterol diet (HFD) (D11012302) (Research Diets, New Brunswick, USA) with 0.2% cholesterol and fat derived from palm oil, which provided adequate levels of vitamins (Vitamin Mix V10001, Research Diets, New Brunswick, USA). The mice were divided in four experimental groups: WT mice treated with placebo (n=12), WT mice treated with MonoHER (n=12), *Ldlr*$^{-/-}$ mice treated with placebo (n=12) and *Ldlr*$^{-/-}$ mice treated with MonoHER (n=12). MonoHER was administered daily subcutaneously at a dosage of 500 mg/kg of body weight (25 µl per g of body weight). With this dose, monoHER provided complete protection against doxorubicin-induced cardiotoxicity in mice (van Acker et al., 1995). Daily subcutaneous injection of a physiological saline solution (25 µl per g of body weight) was used as placebo. Mice were weighed every three days and food intake was recorded for every 2 mice housed together. Diet and treatment were continued for 3 weeks, after which mice were anaesthetized with 0.05 mg/kg buprenorphine and isoflurane 0.4 l/min and sacrificed by exsanguination from the vena cava. The liver was removed and divided in different parts for further investigation. Part of the large left lobe was used for RNA isolation.

### Direct damage by ROS

To determine the direct damage by ROS, oxidative damage to poly-unsaturated fatty acids in the liver was determined by measuring malondialdehyde (MDA) formation. Liver homogenate (250 mg liver/ml) was prepared in 145 mM potassium phosphate

buffer (pH 7.4). MDA was determined by reaction of MDA with thiobarbituric acid (Haenen and Bast, 1983) and quantified by HPLC (Lepage et al., 1991).

**Cellular redox status**

The cellular redox status was assessed by quantifying the GSH/GSSG ratio. Total glutathione content and glutathione disulfide (GSSG) were measured according to the enzymatic recycling method (Julicher et al., 1988) quantifying the conversion of DTNB into TNB spectrophotometrically at 412 nm and 37 °C. The GSH/GSSG-ratio was calculated from the content of GSSG and total glutathione.

**Activation of the NRF2 antioxidant defense system**

An adaptive response by the NRF2 antioxidant defense system was evaluated by measuring *HO-1* and *Gpx3* gene expression in the liver. Liver parts that were stored in RNAlater® at -80°C were used for RNA isolation using miRNeasy Mini Kit with DNase treatment (Qiagen, Venlo, The Netherlands) according to protocol. Isolated RNA was converted into cyclic DNA (cDNA) using iScript cDNA synthesis kit (Bio-rad, Veenendaal, The Netherlands). Two step qRT-PCR was performed using 12.5 µl SensiMix SYBR & Fluorescein Kit (Bioline, London, UK) with 5 µl cDNA, and 300 nM of each primer (*HO-1*: forward: 5'-GAGCCTGAATCGAGCAGAAC-3', reverse: 5'-CCTTCAAGGCCTCAGACAAA-3', *Gpx3*: forward: 5'-CATCCTGCCTTCTGTCCCT-3', reverse: 5'-ATGGTACCACTCATACCGCC-3') in a 25 µl reaction mixture. PCR was conducted as follows: denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. Following PCR, a melting curve (60–95°C) was produced for product identification and purity check. *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) and *Ywhaz* (Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta Polypeptide) (Eurofins, Breda, The Netherlands) were used as housekeeping genes (Bruce et al., 2012). An average value of the expression of the house keeping genes *Gapdh* (forward: 5'-TTGATGGCAACAATCTCCAC-3', reverse: 5'-CGTCCCGTAGACAAAATGGT-3') and *Ywhaz* (forward: 5'-

CAGCAGATGGCTCGAGAATA-3', reverse: 5'-GAAGCATTGGGGATCAAGAA-3') was used for the calculation of the ΔCt-value. The validity was confirmed by the high within-animal correlation between the values of *Gapdh* and *Ywhaz* ($R^2$= 0.8041). Primer sequences are listed in Table 1. Data were analyzed using the MyIQ software system (BioRad, Veenendaal, The Netherlands) and were expressed as relative gene expression (fold change) using the $2^{\Delta\Delta Ct}$ method.

**Statistics**

Data were analysed with GraphPad Prism 5 (Graphpad software, CA, USA). Values presented in the study are expressed as mean ± standard deviation (SD). The relationships of the GSH/GSSG ratio with *HO-1* and *Gpx3* gene expression were evaluated using linear correlation. Differences between groups were analyzed using the Kruskal-Wallis test. Post-hoc testing was performed using the Mann-Whitney U-test with Bonferroni correction. A $P < 0.05$ was considered to be statistically significant, $P < 0.1$ and $> 0.05$ as indicating a trend.

**Results and discussion**

There were no significant differences in food intake between the groups (Supplemental data). Initial and also final body weight and liver weight did not differ between the groups. In the placebo treated *Ldlr*$^{-/-}$ mice only a trend towards increased liver/total body weight-ratio compared to placebo treated WT mice was observed, confirming that in the relatively short period of 3 weeks only the onset of NAFLD was studied.

A trend was seen towards a higher average level of lipid peroxidation (MDA) in *Ldlr*$^{-/-}$ mice compared to WT mice (Figure 1A), although this difference failed to reach significance. No pronounced effect of monoHER against ROS damage was seen. This could be due to the relatively large variation in the results. An explanation for the relatively large variation is that the onset of the disease is examined. At the onset of the disease, the effects are relatively small compared to the biological variation. Moreover, the time course of the gradual progression into a clear manifestation of the disease will vary between the mice studied. The effect was only examined at a single point in time, and this also contributes to a relatively large inter-animal variation. The relatively large variation might explain that only a mild effect was found on a group level.

The general protective effect of monoHER against oxidative stress can be explained by its ability to scavenge ROS (Lemmens et al., 2014b). However, scavenging might block the activation of the innate NRF2 pathway by ROS. In this case, the paradoxical consequence is that no protective adaptation is elicited because antioxidant administration prevents the endogenous defensive shield to be raised. This has fueled the vexed dispute on the use of antioxidants in the prevention and treatment of diseases (Bast and Haenen, 2013). However, in the present study it was found that monoHER did not block the adaptive response; in contrast, a trend towards further increased levels of *HO-1* and *Gpx3* gene expression was found with monoHER (Table 1).

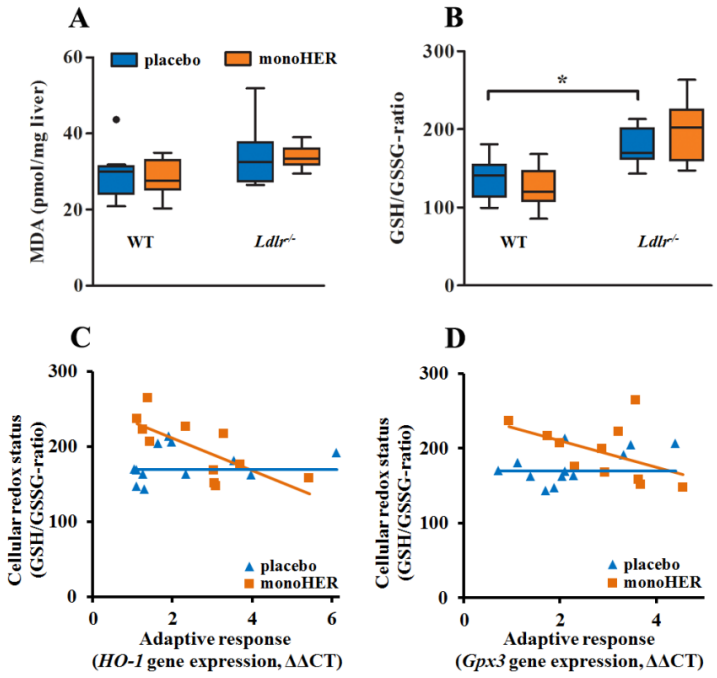


**Figure 1. Direct damage by ROS (determined as MDA formation) and cellular redox status (determined as GSH/GSSG ratio).** In panel A and B the Tukey Box-plot is shown of MDA and GSH/GSSG ratio, measured in liver tissue of WT and *Ldlr*$^{-/-}$ mice treated with placebo or monoHER**.** * $P < 0.01$. The outlier is included in the statistical evaluation. Panel C and D show the within-animal linear correlation between the cellular redox status and the NRF2-dependent adaptive response during the onset of NAFLD in the liver of *Ldlr*$^{-/-}$ mice. In the group treated with monoHER, the adaptive response, expressed as *HO-1* (C) and *Gpx3* (D) gene expression, was relatively high at a low cellular redox status, indicated by a low GSH/GSSG ratio ($P = 0.012$ and $P = 0.046$ respectively). This relationship is lacking in the placebo group.

The higher NRF2 induced gene expression in *Ldlr*$^{-/-}$ mice (Table 1) was corroborated by the adaptive response on the cellular redox status (figure 1B). In *Ldlr*$^{-/-}$ mice monoHER also tended to further increase the GSH/GSSG-ratio in *Ldlr*$^{-/-}$ mice (Figure 1B).

That monoHER does not inhibit the adaptive response, can be explained by the formation of a thiol reactive oxidation product of monoHER when it scavenges radicals. This oxidation product of monoHER selectively adducts a cysteine residue in KEAP1 and this adduction of KEAP1 induces the adaptive response (Lemmens et al., 2014a). In fact, the rather unspecific reactivity of ROS that does not only destroy KEAP1 but also vital cellular components such as other proteins and DNA, is exchanged for the much more selective reactivity of the oxidized product of monoHER towards KEAP1 (Lemmens et al., 2014a).

**Table 1. Activation of the NRF2 antioxidant defense system.** *HO-1* and *Gpx3* gene expression was measured to evaluate the activation of the NRF2 antioxidant defense system in liver tissue of WT and *Ldlr*$^{-/-}$ mice treated with placebo (p) or monoHER. Data represent fold change compared to WT + p group. Mean ± SD is shown. * $P < 0.05$ compared to WT + p group, ** $P < 0.05$ compared to WT + monoHER group

| **Gene** | **WT p** | **WT monoHER** | ***Ldlr*$^{-/-}$ p** | ***Ldlr*$^{-/-}$ monoHER** |
|---|---|---|---|---|
| ***HO-1*** | 1.00±0.40 | 0.98±0.29 | **2.27±1.48*** | **2.66±1.25**** |
| ***Gpx3*** | 1.00±0.57 | 1.43±0.51 | **2.21±1.01*** | **3.02±1.09**** |

MonoHER administration did not induce an adaptive response in the WT animals. In this respect, it should be noted that monoHER itself lacks thiol reactivity. MonoHER first needs to be converted into a thiol-reactive oxidation product to induce an adaptive response (Lemmens et al., 2014a). This conversion happens when monoHER scavenges radicals. In the control animals the generation of the thiol-reactive oxidation product will be relatively low. Therefore, no adaptive response as a result of monoHER administration is expected in the control animals.

The formation of the thiol-reactive oxidation product, exclusively during oxidative stress can be seen as a form of targeting. It results in an adaptive enforcement of the antioxidant system when this is needed. Interestingly, in the monoHER treated *Ldlr*$^{-/-}$ mice also evidence for targeting was seen. We expected that monoHER would activate NRF2 which subsequently would results in a higher cellular redox status. This was indeed found, but to our surprise in the monoHER group an improved

redox status was associated with a relatively low NRF2 activation. Apparently, monoHER administration results in a relatively potent adaptation. In the monoHER treated *Ldlr*$^{-/-}$ mice NRF2 activation was relatively high at a low GSH/GSSG ratio. This indicates that a relatively low redox status, i.e. a low GSH/GSSG ratio, tends to increase NRF2 activation in the monoHER treated *Ldlr*$^{-/-}$ group. Moreover, in the liver of animals that have a relatively high GSH/GSSG ratio, no evidence for further stimulation of NRF2 by monoHER was seen, indicating that in the animals that have adapted, no further adaptation is needed (Figure 1C and 1D). In retrospect, this relationship is more logical than the anticipated high redox status when the adaptive response is high, i.e. that in the adapted animals more adaptation is needed, which of course is not necessary. No correlation was found in the corresponding placebo group and in both control groups with WT mice probably because there was no substantiated adaptation. The higher level of adaptation and the reported correlation with NRF2 activation in monoHER treated *Ldlr*$^{-/-}$ mice was absent in the other group. This indicates that monoHER has promoted the adaptive response.

In the present *in vivo* study, only a mild protective effect of monoHER against oxidative stress was seen. As mentioned above, this may be related to the relatively low level of oxidative stress and relatively large variation in the lipid peroxidation marker (Figure 1A) because the onset of pathology was examined. An additional reason for this mild protection is that monoHER is administered once a day, whereas the half-life of monoHER is only half an hour (Abou El Hassan et al., 2003). Most of the time, monoHER will be practically absent (Jacobs et al., 2011), and for full protection by radical scavenging the compound has to be present continuously at a relatively high concentration. Scavenging of radicals at moments when concentrations of monoHER are relatively high, can explain the subtle effect of monoHER administration on oxidative stress. In addition, as mentioned above, the scavenging of radicals and the concurrent formation of the thiol-reactive oxidation product can selectively activate the innate NRF2 machinery. As demonstrated previously, a single and acute exposure to a thiol-alkylating agent can induce an adaptive response (Levonen et al., 2014; Sthijns et al., 2014; Wakabayashi et al., 2004). This would mean that in order for monoHER to induce an adaptive response,

only part of the radicals needs to be scavenged since this already leads to the formation of the thiol-alkylating oxidation product of monoHER.

Flavonoids have therapeutic potential in NAFLD which relates to their protection against oxidative stress. Our study supports a modified mechanism of action of the antioxidant flavonoid monoHER, which might also apply for other free radical scavenging antioxidants, as illustrated in Figure 2. The results show that the direct scavenging of radicals is only one aspect of protection by monoHER. The present study shows that activation of the antioxidant defense system (i.e. the activation of NRF2) by the oxidized flavonoid also plays a crucial role in the protective effect against oxidative stress (i.e. the improved GSH/GSSG ratio). This molecular mechanism does not only result in a very potent antioxidant effect, it also ensures that monoHER will act selectively in a condition of oxidative stress. In this way monoHER empowers the buffering capacity of the body to maintain homeostasis and to prevent that relatively mild but nevertheless insidious and persistent perturbations gradually progresses into a severe pathology, such as NAFLD.

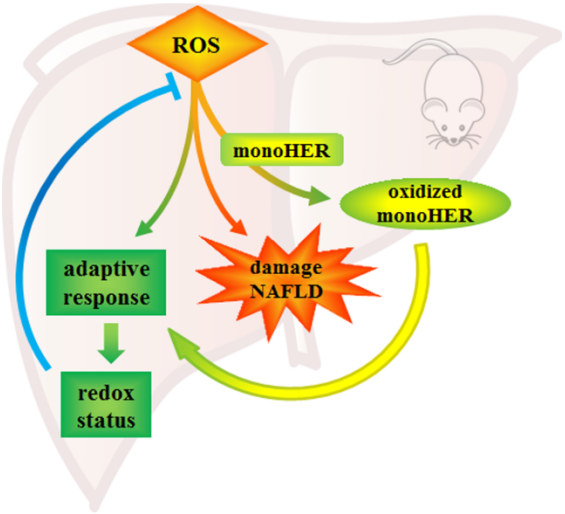


**Figure 2.** Overview of the protective effect of monoHER in the onset of NAFLD in mice. The high and therefore non selective reactivity of ROS results in damage to virtually any cellular compound. This oxidative damage is a hallmark in the etiology of NAFLD. ROS also activate

NRF2 which leads to adaptation. By scavenging the ROS, oxidation products of monoHER are formed that promote the adaptation of the cell to oxidative stress. This helps to maintain homeostasis and prevents that relatively mild perturbations gradually progress into a severe pathology such as NAFLD.

**Acknowledgements**

The authors would like to thank Leonie Jonkers (Department of Toxicology, Faculty of Health, Medicine and Life Sciences, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands) for the help with the treatment of the mice. We are also thankful to Kelly Waagmeester (Department of Toxicology, Faculty of Health, Medicine and Life Sciences, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands) for the help with the RNA isolation and measuring of the gene expression.

## Supplemental data

**Table 2. Biometric data of mice.** Initial and final body weight, body weight change, liver weight, liver/total body weight-ratio, average food intake of WT and *Ldlr*$^{-/-}$ mice treated with placebo (p) or monoHER. Mean ± SD is shown.

| | **WT p** | **WT monoHER** | ***Ldlr*$^{-/-}$ p** | ***Ldlr*$^{-/-}$ monoHER** |
|---|---|---|---|---|
| **Initial body weight (g)** | 20.8±1.3 | 20.9±1.1 | 20.9±1.1 | 21.0±1.0 |
| **Final body weight (g)** | 21.3±1.5 | 21.5±1.2 | 21.3±1.5 | 20.8±1.5 |
| **Body weight change (g)** | 0.4±0.7 | 0.6±1.1 | 0.4±1.2 | -0.1±0.8 |
| **Liver weight (g)** | 1.00±0.14 | 1.01±0.10 | 1.05±0.12 | 1.04±0.11 |
| **Liver/total body weight-ratio (%)** | 4.69±0.40 | 4.69±0.32 | 4.95±0.35 | 4.92±0.34 |
| **Average food intake (g/day/cage)** | 4.0 (0.3) | 4.1 (0.3) | 4.3 (0.3) | 4.4 (0.3) |

## References

2009. What is health? The ability to adapt. Lancet 373, 781.

Abou El Hassan, M.A., Kedde, M.A., Zwiers, U.T., Tourn, E., Haenen, G.R., Bast, A., van der Vijgh, W.J., 2003. Bioavailability and pharmacokinetics of the cardioprotecting flavonoid 7-monohydroxyethylrutoside in mice. Cancer Chemotherapy and Pharmacology 52, 371-376.

Bast, A., Haenen, G.R., 2013. Ten misconceptions about antioxidants. Trends in Pharmacological Sciences 34, 430-436.

Berlanga, A., Guiu-Jurado, E., Porras, J.A., Auguet, T., 2014. Molecular pathways in non-alcoholic fatty liver disease. Clinical and Experimental Gastroenterology 7, 221-239.

Bieghs, V., Van Gorp, P.J., Wouters, K., Hendrikx, T., Gijbels, M.J., van Bilsen, M., Bakker, J., Binder, C.J., Lutjohann, D., Staels, B., Hofker, M.H., Shiri-Sverdlov, R., 2012. LDL receptor knock-out mice are a physiological model particularly vulnerable to study the onset of inflammation in non-alcoholic fatty liver disease. PLoS One 7, e30668.

Bruce, K.D., Sihota, K.K., Byrne, C.D., Cagampang, F.R., 2012. The housekeeping gene YWHAZ remains stable in a model of developmentally primed non-alcoholic fatty liver disease. Liver International 32, 1315-1321.

Haenen, G.R.M.M., Bast, A., 1983. Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. FEBS letters 159, 24-28.

Itoh, K., Tong, K.I., Yamamoto, M., 2004. Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radical Biology and Medicine 36, 1208-1213.

Jacobs, H., Peters, R., den Hartog, G.J., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2011. Identification of the metabolites of the antioxidant flavonoid 7-mono-O-(beta-hydroxyethyl)-rutoside in mice. Drug Metabolism and Disposition 39, 750-756.

Julicher, R.H., Sterrenberg, L., Haenen, G.R., Bast, A., Noordhoek, J., 1988. The effect of chronic adriamycin treatment on heart kidney and liver tissue of male and female rat. Archives of Toxicology 61, 275-281.

Koek, G.H., Liedorp, P.R., Bast, A., 2011. The role of oxidative stress in non-alcoholic steatohepatitis. Clinica Chimica Acta 412, 1297-1305.

Lemmens, K.J., Sthijns, M.M., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014a. The antioxidant flavonoid monoHER provides efficient protection and induces the innate Nrf2 mediated adaptation in endothelial cells subjected to oxidative stress. PharmaNutrition 2, 69-74

Lemmens, K.J., van de Wier, B., Vaes, N., Ghosh, M., van Zandvoort, M.A., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014b. The flavonoid 7-mono-O-(beta-hydroxyethyl)-rutoside is able to protect endothelial cells by a direct antioxidant effect. Toxicology in Vitro 28, 538-543.

Lepage, G., Munoz, G., Champagne, J., Roy, C.C., 1991. Preparative steps necessary for the accurate measurement of malondialdehyde by high-performance liquid chromatography. Analytical Biochemistry 197, 277-283.

Levonen, A.L., Hill, B.G., Kansanen, E., Zhang, J., Darley-Usmar, V.M., 2014. Redox regulation of antioxidants, autophagy, and the response to stress: implications for electrophile therapeutics. Free Radical Biology and Medicine 71, 196-207.

Rolo, A.P., Teodoro, J.S., Palmeira, C.M., 2012. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. Free Radical Biology and Medicine 52, 59-69.

Sthijns, M.M., Randall, M.J., Bast, A., Haenen, G.R., 2014. Adaptation to acrolein through upregulating the protection by glutathione in human bronchial epithelial cells: the

materialization of the hormesis concept. Biochemical and Biophysical Research Communications 446, 1029-1034.

van Acker, S.A., Kramer, K., Grimbergen, J.A., van den Berg, D.J., van der Vijgh, W.J., Bast, A., 1995. Monohydroxyethylrutoside as protector against chronic doxorubicin-induced cardiotoxicity. British Journal of Pharmacology 115, 1260-1264.

van de Wier, B., Koek, G.H., Bast, A., Haenen, G.R., 2014. The potential of flavonoids in the treatment of non-alcoholic fatty liver disease. Critical Reviews in Food Science and Nutrition., in press.

Wakabayashi, N., Dinkova-Kostova, A.T., Holtzclaw, W.D., Kang, M.I., Kobayashi, A., Yamamoto, M., Kensler, T.W., Talalay, P., 2004. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. Proceedings of the National Academy of Sciences of the United States of America 101, 2040-2045.

Chapter 6

# The contribution of the major metabolite 4'-O-methylmonoHER to the antioxidant activity of the flavonoid monoHER

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**Abstract**

The antioxidant flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) effectively protects against doxorubicin-induced cardiotoxicity in mice. Doxorubicin is a very effective anticancer drug. The clinical use of doxorubicin is limited by severe cardiotoxicity. Free radicals play a crucial role in this toxicity.

In this study the involvement of the major metabolite of monoHER, 4'-O-methylmonoHER (methylmonoHER) in the protective effect of monoHER is studied. MethylmonoHER displayed antioxidant activity i.e. TEAC, hydroxyl and superoxide radical scavenging activity; nevertheless monoHER appeared to be superior compared to methylmonoHER. As a result of scavenging, flavonoids are oxidized and display reactivity towards thiols. Oxidized methylmonoHER, is far less thiol reactive towards creatine kinase than monoHER, which indicates that methylmonoHER is less toxic towards thiol containing enzymes. The thiol-reactivity of oxidized methylmonoHER was also negligible towards KEAP1 compared to monoHER. These results indicate that methylmonoHER hardly protects against radical damage via scavenging or via activating the NRF2 defense system. Also in HUVECs, methylmonoHER provided far less protection against oxidative stress ($EC_{50}$ > 100 µM) than monoHER which was a very potent protector ($EC_{50}$ = 80 nM). The results indicate that the contribution of methylmonoHER to the protection against doxorubicin-induced cardiotoxicity by monoHER is relatively low.

**Introduction**

Doxorubicin is an indispensable anticancer agent. However, the severe cardiotoxic effects limit the use of doxorubicin (Lipshultz et al., 2005). The formation of free radicals plays a crucial role in this toxicity (Horenstein et al., 2000; Xu et al., 2001). The heart is vulnerable because its antioxidant capacity is relatively low (Julicher et al., 1988). Therefore, antioxidants which scavenge or prevent the formation of free radicals were selected to provide protection. The proof of this concept is that the semisynthetic antioxidant flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) effectively protects against doxorubicin-induced cardiotoxicity in mice (van Acker et al., 1995).

Our knowledge on the mode of action of antioxidant flavonoids has drastically increased. For several flavonoids it is known that metabolites of these flavonoids contribute to their effect (Ruijters et al., 2013). In this respect, much attention is given to O-methylated metabolites that are more readily taken up than the parent flavonoid, have an improved metabolic stability and display biological activity (Steffen et al., 2008), as demonstrated for the 4'-O-methyl metabolite of quercetin (Spencer et al., 2003). Similarly, a contribution of the 4'-O-methyl metabolite to the overall effect of monoHER has been suggested (Jacobs et al., 2011). 4'-O-methylmonoHER (methylmonoHER) is the major metabolite of monoHER and the AUC and $C_{max}$ of methylmonoHER in mice are higher than those of monoHER (Jacobs et al., 2011). This prompted us to study the antioxidant activity of this metabolite.

In this study the 4'-O-methylated metabolite of monoHER was synthesized and its antioxidant activity was evaluated and compared to that of monoHER. Besides the potency to scavenge radicals, the channeling of the reactivity of the oxidized product towards proteins was studied.

## Material and methods

### Chemicals

7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) was kindly provided by Novartis Consumer Health (Nyon, Switzerland). 2-Deoxy-D-ribose, ferric chloride hexahydrate ($FeCl_3.6H_2O$), ascorbic acid, dimethylformamide (DMF), diethyl ether, methyl iodide, sodium bicarbonate ($NaHCO_3$), 2',7'-dichlorofluorescein-diacetate (DCFH-DA), and silica gel were purchased from Sigma (St. Louis, USA). $H_2O_2$ was obtained from Riedel-de Haën (Seelze, Germany). 2-thiobarbituric acid (TBA) and EDTA was purchased from Merck (Darmstadt, Germany). 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), ammonium bicarbonate (ABC), acetonitrile (ACN), α-cyano-4-hydroxycinnamic acid and trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO, USA). Azobisamidinepropane (ABAP) was purchased from Polysciences Inc. (Warrington, UK).

### Synthesis of 4'-O-methylmonoHER

MonoHER was methylated with an excess of methyl iodide and 22 equivalents $NaHCO_3$ dissolved in DMF. The reaction mixture was stirred at room temperature overnight.

Under these circumstances the 4'-OH is mainly methylated. The product was extracted with diethyl ether. The crude product was purified by column chromatography on silica gel using a gradient of ethanol/toluene. The purity was confirmed using HPLC analysis. MethylmonoHER was identified by $^{1}H$ and $^{13}C$ NMR (Supplemental material).

### Hydroxyl radical scavenging assay

In the assay, the scavengers, monoHER or methylmonoHER, are in competition with deoxyribose for a reaction with hydroxyl radicals. The hydroxyl radicals are formed in the reaction between $Fe^{2+}$ and $H_2O_2$. Iron is kept in the reduced form by ascorbic acid.

The reaction mixtures contained 0-0.1 mM monoHER or methylmonoHER, 2.8 mM deoxyribose, 100 µM ascorbic acid and 2.8 mM $H_2O_2$ in 20 mM $KH_2PO_4/K_2HPO_4$ buffer pH 7.4. Hydroxyl radical formation was started by adding 200 µl of a solution containing 20 µM $FeCl_3$ and 0-100 µM EDTA in deoxygenated water. The mixture was incubated at 37 °C, and after 1 h 2 ml of a solution, containing 0.5 % TBA and 1.4 % TCA was added. Subsequently, the mixture was heated at 100 °C for 20 min. The test tubes were cooled to room temperature. The absorbance (A) of the reaction mixture was measured at 532 nm. By plotting the reciprocal of the relative absorbance against the concentration of the scavenger, the apparent reaction rate constant ($k_s$) of the reaction between the scavenger and the hydroxyl radicals was calculated according to (Haenen et al., 1993).

**Superoxide radical scavenging assay**

The superoxide radical scavenging activity of monoHER and methylmonoHER was determined according to Den Hartog et al. (den Hartog et al., 2003). Superoxide radicals were generated by xanthine 0.1 mM and xanthine oxidase 10 mU/mL. The radicals were detected by using nitroblue tetrazolium (NBT; 50 µM). The $EC_{50}$, the amount of scavenger required to achieve an NBT reduction by a 50 % decrease, was determined.

**Total antioxidant capacity (TEAC) assay**

ABTS radical solution was prepared by heating (70 °C) a solution of ABTS (1.23 mg/mL) and ABAP (3.96 mg/mL) in 50 mM phosphate buffer pH 7.4 for 20 min. 50 µl of 0-20 µM monoHER or 0-50 µM methylmonoHER was added to 950 µl of the ABTS radical solution. After 5 min of incubation at 37 °C, the absorption at 734 nm was measured. The decrease in absorption after 5 min relative to blank (buffer) was related to that of Trolox. The TEAC value gives the concentration of trolox that has a similar antioxidant capacity as the sample.

**Reaction of oxidized monoHER or methylmonoHER with GSH**

To obtain an equal rate of oxidation (5 µM/min) monoHER and methylmonoHER (50 µM) were oxidized by 50 µM $H_2O_2$ and 1.6 or 128 nM HRP, respectively in a 145 mM phosphate buffer pH 7.4, at 37 °C. If present, the concentration of GSH was 50 µM. The reaction was monitored by high-performance liquid chromatography (HPLC) as previously described by Jacobs et al., 2010 (Jacobs et al., 2010). HPLC analysis was performed on a HP 1100 series HPLC system (Agilent Technologies, Palo Alto, USA) using a Supelcosil LC 318 reversed-phase column (5 µm, 25 cm x 4.6 mm) (Supelco, Bellefonte, USA). The mobile phase consisted of water with linear gradients of acetonitrile, containing 0.1 % (v/v) TFA. MonoHER and methylmonoHER were detected with a diode array detector at 355 nm.

**Creatine Kinase activity**

MonoHER and methylmonoHER quinones were generated *in situ* by oxidizing the flavonoids (50 µM) with 50 µM $H_2O_2$ and 1.6 or 128 nM HRP respectively, in the presence of 6.2 µM creatine kinase (CK) in a 145 mM potassium-phosphate buffer pH 7.4. The reactions were started by adding the HRP and carried out at 37 °C for 5 min.

Enzyme activity of CK in the reaction mixtures was measured by the catalytic conversion of ADP into ATP. Hexokinase was used to convert ATP with glucose into glucose-6-phosphate. Finally, the glucose-6-phosphate formed converts $NAD^+$ into NADH in the presence of glucose-6-phosphate dehydrogenase.

The formation of NADH was measured spectrophotometrically at 340 nm during 5 min representing the CK activity.

**Mass spectrometry**

MonoHER and methylmonoHER (50 µM) were oxidized by 50 µM $H_2O_2$ and 1.6 or128 nM HRP in the presence of 0.5 mg/ml KEAP1 or 6.2 µM CK in 50 mM ABC buffer pH 7.4 for 15 min at 37 ˚C. KEAP1 was digested by trypsin during 2 hours at

37 ° C. CK was digested for 30 min. The digested samples were diluted 1:10 in 0.1 % TFA. After dilution, 1 µl of the digest and 1 µl of matrix solution (2.5 mg/ml α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA) was spotted on a 348-well-format target plate and air-dried. Mass spectra were measured on a MALDI-TOF mass spectrometer (4800 MALDI-TOF analyzer; Applied Biosystems). The instrument was operated in positive reflector mode. Acquisition mass range was 800-3500 Da.

**Cell culture**

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured in T75 coated culture flasks (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) in Ham's F12-K medium (Invitrogen, Breda, The Netherlands) supplemented with 10 % (v/v) fetal calf serum (FCS; Invitrogen, Breda, The Netherlands), 0.05 mg/ml endothelial cell growth supplement (ECGS; BD Science, Franklin Lakes, USA), 1 % pen/strep (Gibco, Bleiswijk, The Netherlands) and 0.01 mg/ml heparin (LEO Pharma, Ballerup, Denmark) in a humidified atmosphere containing 5 % $CO_2$ and 95 % air at 37 °C. The maximal passage used was 20 in the measurement of intracellular oxidative stress.

**Measurement of intracellular oxidative stress**

The intracellular levels of oxidative stress were quantified by the fluorescence of dichlorofluorescein (DCF). The probe used has broad specificity; it detects $H_2O_2$, hydroxyl radicals, superoxide and other reactive species.

Cells (4 X $10^4$ per well) were plated out in a 96-wells plate (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) 24 h before the experiment. The cells were then incubated with 20 µM DCFH-DA in 5 % $CO_2$ and 95 % air at 37 °C for 45 min. After the medium with excess of DCFH-DA was removed, the cells were rinsed once with HBSS and subsequently incubated with 200 µM $H_2O_2$ and 0-1 µM monoHER or 0-100 µM methylmonoHER in serum-free medium. The fluorescence (Ex 485 nm, Em 538 nm) was determined on a microplate reader (Spectramax

microplate reader, Molecular devices, Sunnyvale, USA) as a measure of oxidative damage.

The $EC_{50}$, the concentration of monoHER or methylmonoHER which reduces intracellular oxidative stress by 50 % after 45 min, was determined by plotting the logarithm of the concentration of monoHER or methylmonoHER against the protection (in %) at time point 45 min. The $EC_{50}$ of monoHER was estimated by linear interpolation between the concentration 0.01 µM and 0.1 µM.

Measurement of DCF in the medium revealed that in none of the incubations DCF leakage occurred (data now shown).

The reaction of monoHER or methylmonoHER with $H_2O_2$ was examined by HPLC and spectrophotometrically (data not shown). Both compounds did not react with $H_2O_2$.

**LUMO map**

The software program Spartan '06 (Wavefunction, Irvine, CA, USA) was used to generate the lowest unoccupied molecular orbital (LUMO) maps of simplified oxidized monoHER and oxidized methylmonoHER.

**Statistics**

All experiments were performed at least in triplicate. Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student's t-test.

## Results

### Radical scavenging activity

#### *Hydroxyl radical scavenging activity*

The apparent second order reaction rate constant ($k_s$) of the reaction of monoHER with the hydroxyl radicals, was 240 X $10^8$ $M^{-1}s^{-1}$ (Figure 1), which is similar to previously reported values (Lemmens et al., 2014b). The $k_s$ of methylmonoHER obtained was 110 X $10^8$ $M^{-1}s^{-1}$.

Omitting the chelator EDTA from the reaction mixture, increased the observed scavenging potency of monoHER threefold, whereas it had no effect on the scavenging potency of methylmonoHER (Figure 1).

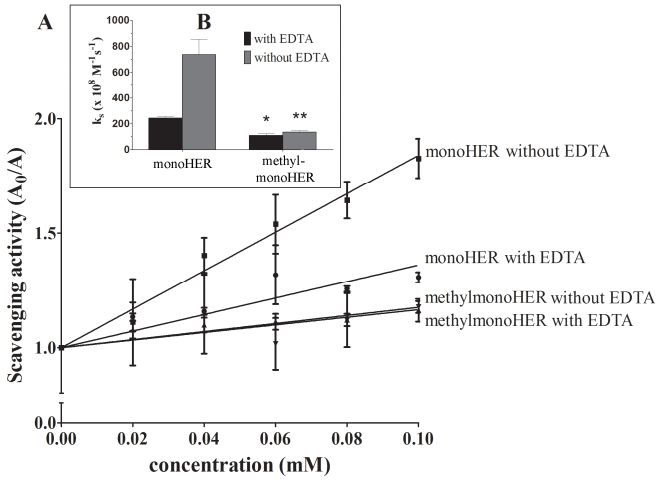


**Figure 1.** Determination of the hydroxyl radical scavenging activity of monoHER and methylmonoHER using a competition assay with deoxyribose. The deoxyribose concentration is 2.8 mM. The concentration of the scavenger is plotted on the x-axis. To determine the scavenging activity of monoHER and methylmonoHER, the breakdown products formed in the reaction of deoxyribose with OH-radicals were measured spectrophotometrically. MonoHER and methylmonoHER compete with deoxyribose for the OH-radicals and the presence of these compounds reduces the concentration of deoxyribose breakdown product. The absorbance obtained in the presence of monoHER or methylmonoHER (A) was related to

the absorbance obtained without these compounds ($A_0$) which is a measure of the scavenging activity. Three independent experiments were performed and data are shown as mean ± SD. The apparent reaction rate ($k_s$) of the reaction of monoHER and methylmonoHER with hydroxyl radicals with or without ETDA is shown in the insert (B). Data are shown as mean ± SD (n=3). * $p<0.05$, ** $p<0.01$ vs monoHER with and without EDTA, respectively.

### *Superoxide radical scavenging activity*

The $EC_{50}$ of monoHER in the superoxide radical scavenging assay was 22 µM. The $EC_{50}$ of methylmonoHER was higher than 100 µM (Figure 2). Trolox had an $EC_{50}$ of 295 µM.

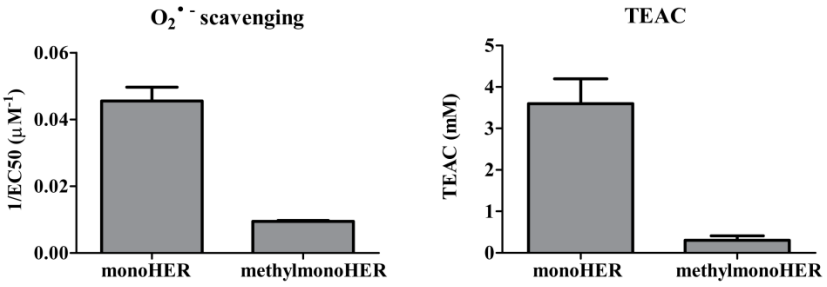


**Figure 2. Superoxide radical scavenging activity and Trolox Equivalent Antioxidant Capacity (TEAC-) values of monoHER and methylmonoHER**. Superoxide radicals were spectrophotometrically detected by NBT. The reciprocal of the $EC_{50}$, the amount of monoHER and methylmonoHER required to achieve a 50 % NBT reduction, was shown.
The TEAC-value is the decrease in absorption caused by monoHER and methylmonoHER, related to that of Trolox. Data are shown as mean ± SD (n=3).

### Antioxidant capacity (TEAC)

MonoHER had a TEAC value of 3.6 ± 0.6 mM. MethylmonoHER had a TEAC value of 0.3 ± 0.1 mM (Figure 2). Trolox is used to calibrate the assay and has a TEAC of 1.

**Reaction of the oxidized antioxidant**

***Reactivity towards GSH***

HPLC analyses show that oxidation of monoHER and methylmonoHER by HRP and $H_2O_2$ leads to the consumption of monoHER and methylmonoHER at a rate of about 5 µM/min (Figure 3A and B). In the presence of GSH a new peak eluted at an identical position of the GSH-monoHER adduct (Figure 3C), confirming previous observation (Jacobs et al., 2009). Oxidation of methylmonoHER in the presence of GSH did result in a GSH-adduct (Figure 3C).

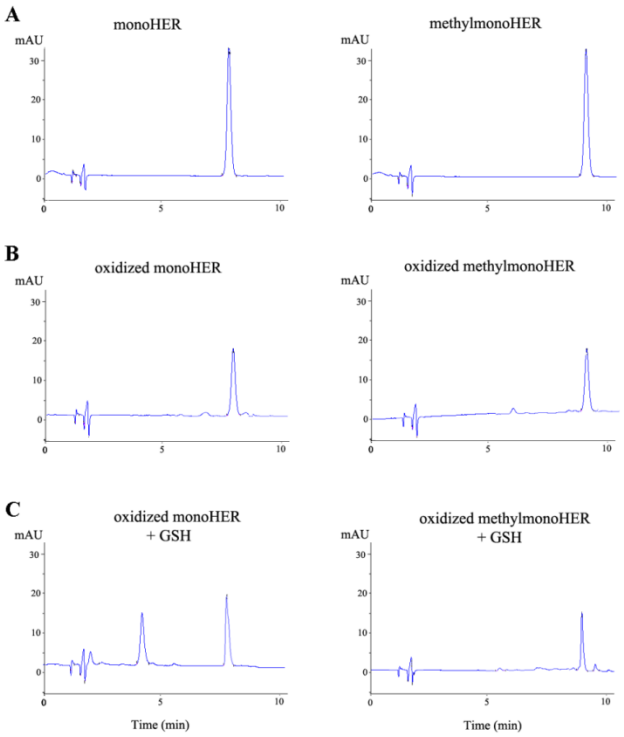


**Figure 3.** HPLC analysis of 50 µM monoHER and methylmonoHER and of the incubation mixture containing 50 µM monoHER or methylmonoHER (A), with 50 µM $H_2O_2$ and 1.6 nM HRP or 128 nM HRP, respectively (B). The incubations were also carried out in the presence of 50 µM GSH (C). The different incubation mixtures were injected on the HPLC system 5 minutes after addition of HRP. The chromatograms are detected at 355 nm. A typical example is shown. The retention times of monoHER and methylmonoHER were 7.5 min and 8.5 min, respectively.

***Reactivity towards proteins***

*Reactivity towards CK and KEAP1*

MonoHER and methylmonoHER were oxidized in the presence of CK. Oxidized monoHER was found to reduce CK activity (25%). Oxidized methylmonoHER did not change the activity of CK (Figure 4). Control experiments revealed that methylmonoHER and monoHER when not oxidized, did not inhibit CK.

MonoHER oxidized in the presence of CK reduced the intensity of the peak of the native peptide fragment, GYTLPPHCSR with a mass of m/z = 1131 measured by MALDI-TOF (peptide sequences were determined by the PeptideMass program via the ExPASy World Wide Web server). Instead, a new peptide fragment with a mass of m/z = 1783 emerged (Figure 4).

MonoHER oxidized in the presence of KEAP1 reduced the intensity of the peak of the native peptide fragment, LNSAECYYPER with a mass of m/z = 1346. Instead, a new peptide fragment with a mass of m/z = 1997 emerged (Figure 5).

The increment of the mass of the fragments corresponds to the molecular weight of the quinone of monoHER (652 Da). This indicates that the quinone formed by the oxidation of monoHER adducts CK and KEAP1.

MethylmonoHER did not show an adduct with CK or KEAP1 (Figure 4 and 5).

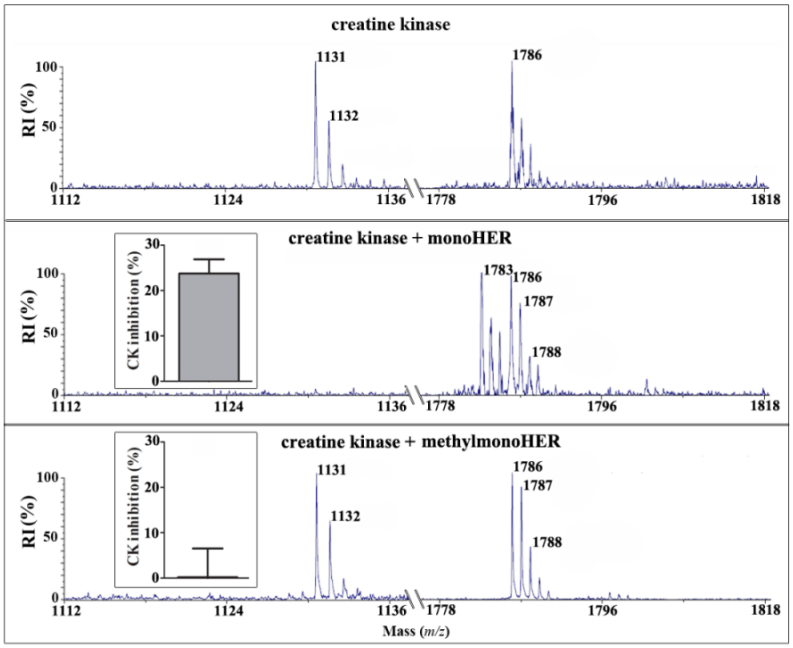


**Figure 4. Reactivity of monoHER and methylmonoHER towards CK.** MALDI-TOF analysis of CK incubated with 50 µM monoHER or methylmonoHER, 1.6 nM or 128 nM HRP and 50 µM $H_2O_2$ for 5 min at 37˚C. After trypsin digestion the mass spectrum of digested CK was measured. The control spectrum of CK displayed a peak at *m/z* 1131 and no peak at *m/z* 1783. The incubation with monoHER showed a peak at *m/z* 1783 which corresponds to the mass of the adduct of monoHER quinone (652 dalton) with the peptide having mass *m/z* 1131, whereas the peak at *m/z* 1131 decreased. The incubation with methylmonoHER did not show alterations in the peaks.

The inserts show the effect of oxidized monoHER and methylmonoHER on the enzyme activity of CK. MonoHER and methylmonoHER (50 µM) were oxidized by 50 µM $H_2O_2$ and HRP, at an equal rate of oxidation (5 µM/min) in the presence of 6.2 µM CK. The enzyme activity of CK was expressed as percentage of the control. Data are shown as mean ± SD (n = 3).

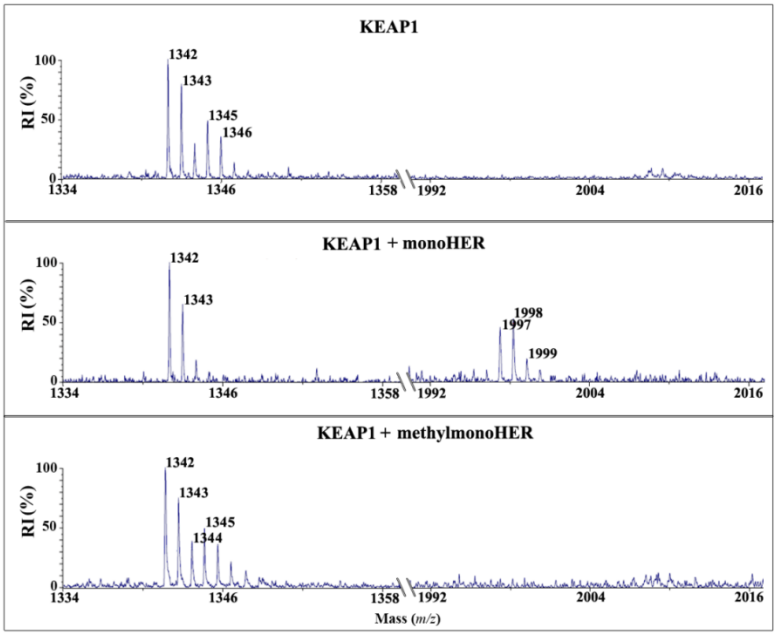


**Figure 5.** Adduction of KEAP1 by oxidized monoHER and by oxidized methylmonoHER. MALDI-TOF analysis of isolated KEAP1 (0.1 mg/ml) incubated with 50 µM monoHER or methylmonoHER, 1.6 or 128 nM HRP and 50 µM $H_2O_2$, after trypsin digestion. The control spectrum of KEAP1 displayed a peak at *m/z* 1345 and no peak at *m/z* 1997. The incubation mixture with monoHER showed a peak at *m/z* 1997 which corresponds to the mass of the adduct of the monoHER quinone (652 Da) on the peptide with mass m/z 1345, whereas the peak at m/z 1345 decreased. The incubation with methylmonoHER showed no new peaks.

### Protection against intracellular oxidative stress

$H_2O_2$ added to HUVECs in a concentration of 200 µM, induced intracellular oxidative stress (Figure 6). Immediately after addition, monoHER protected HUVECs against the intracellular oxidative stress. The $EC_{50}$ of monoHER after 45 min was 80 nM. MethylmonoHER in a concentration of 100 µM mildly protected against intracellular oxidative stress. The $EC_{50}$ of methylmonoHER after 45 min was higher than 100 µM. In none of the incubations, significant LDH leakage was observed (data not shown).

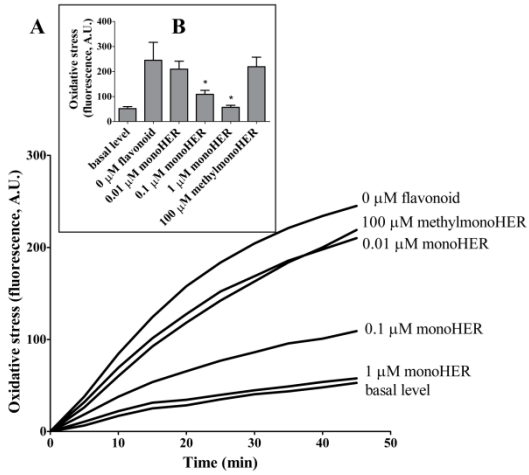


**Figure 6.** The effect of monoHER and methylmonoHER on the intracellular oxidative stress induced by 200 µM $H_2O_2$ in human umbilical vein endothelial cells. For the basal level, no $H_2O_2$ was used. Intracellular oxidative stress was measured using DCF fluorescence as described in the materials and methods section. A typical example is shown. In the insert (B) the oxidative stress after 45 min is shown. Three independent experiments were performed in triplicate and data are shown as mean ± SD (significantly lower than 0 µM flavonoid *$p<0.001$).

**Discussion**

MonoHER protects against doxorubicin-induced cardiotoxicity in mice (van Acker et al., 2000). In this protective effect, metabolites of monoHER may be involved (Jacobs et al., 2011). In the present study the primary metabolite, methylmonoHER was examined. This metabolite was synthesized, and the antioxidant activity of the metabolite was determined and compared to that of the parent compound, monoHER.

In the TEAC, superoxide and hydroxyl radical scavenging assay methylmonoHER was less potent than monoHER. It has been reported that the high hydroxyl radical scavenging activity of monoHER is due to its ability to chelate iron. This results in site-specific scavenging of radicals (Lemmens et al., 2014b). The hydroxyl radical scavenging assay shows that methylmonoHER is not able to site-specifically scavenge the hydroxyl radicals, indicating that methylmonoHER lacks the iron chelating ability of monoHER.

The difference in the scavenging activity observed between monoHER and the 4'-O-methylated metabolite can be explained by a higher electron donating effect of the aromatic 4'-OH group in monoHER than the O-methyl group in methylmonoHER. Several studies have also demonstrated that methylation of one of the OH-groups of the catechol containing antioxidants (the B ring of monoHER contains a catechol group) decreases the hydroxyl radical-, superoxide radical- and peroxynitrite scavenging activity (Rietjens et al., 2007). The importance of the 4'-OH for the intrinsic antioxidant activity has also been demonstrated for quercetin derivatives (Moalin et al., 2011).

In their reaction with radicals, the antioxidant flavonoids are oxidized. These oxidized compounds are less reactive than the radicals they scavenged. In this way the reactivity becomes more selective by converting the non-selective reactivity of radicals into a relatively selective thiol reactivity of the oxidized flavonoid. Reaction of these oxidized flavonoids with reactive thiols of essential proteins results in cellular toxicity (Boots et al., 2002; Ito et al., 1988). The reactivity of oxidized monoHER towards thiols, e.g. GSH, is well described and also takes place in vivo.

Thus, a GSH-monoHER adduct was detected in vivo in the bile of healthy volunteers who received monoHER by intravenous infusion (Jacobs et al., 2009). Our results confirm the reactivity of oxidized monoHER towards GSH and CK an enzyme containing crucial cysteine groups. CK is important for energy production in cells. Inhibition of CK aggravates the energy crisis, which can finally lead to cell death (Reddy et al., 2000). With the use of MALDI-TOF, an adduct between oxidized monoHER and the enzyme was demonstrated. In addition, the enzyme activity of CK was attenuated by oxidized monoHER. This illustrates the potential thiol toxicity of monoHER.

The other side of the coin of the thiol reactivity of oxidized monoHER is that it can also induce an adaptive response, because it can adduct reactive thiol groups on KEAP1. The adduction of KEAP1 sets the NRF2 machinery in motion, which regulates the production of endogenous antioxidant enzymes (Itoh et al., 2004). In this way the thiol reactivity of oxidized monoHER is advantageous, because it leads to the enforcement of the cellular defense system.

The thiol reactivity of oxidized methylmonoHER was also investigated. Interestingly, it was found that oxidized methylmonoHER did not inhibit CK. It also did not react with GSH or KEAP1. Apparently, the methylation of monoHER at 4’-O drastically reduces the thiol reactivity of its oxidation product.

The difference between monoHER and methylmonoHER in directing the reactivity of their oxidation products can be explained by Pearson’s HSAB concept (Pearson, 1963). The Lowest Unoccupied Molecular Orbital (LUMO) of oxidized methylmonoHER is localized only in the B-ring and oxidized methylmonoHER contains a positively charged group that has a relatively high polarity (Figure 7). This results in a high LUMO energy which makes it a hard electrophile. Hard electrophiles react with hard nucleophiles. Oxidized methylmonoHER will therefore not react with thiols because they have a soft nucleophilic character.

Oxidized monoHER is a softer electrophile than oxidized methylmonoHER. The LUMO of oxidized monoHER is distributed over the B-ring and part of the C ring which results in a relatively lower LUMO energy (Figure 7). Nevertheless, oxidized

monoHER will also prefer to pass its reactivity to hard nucleophiles but because of its relatively softer character it also has affinity for thiols.

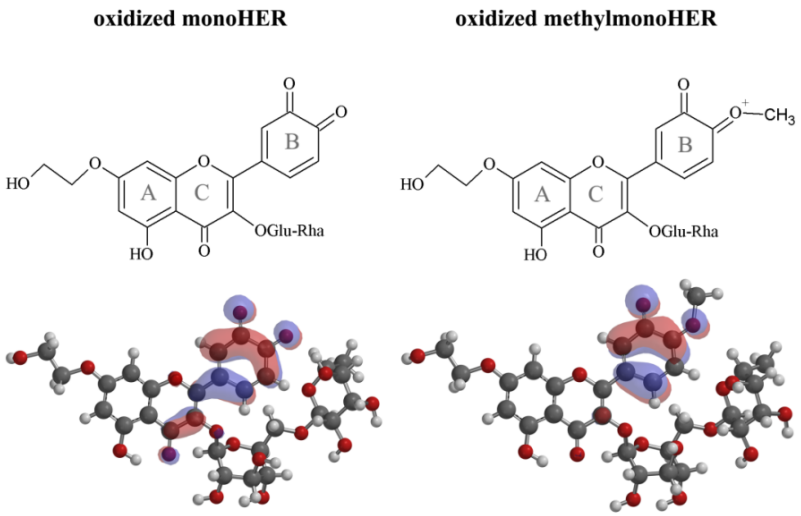


**Figure 7.** Structure and Lowest Unoccupied Molecular Orbital (LUMO) localization map of oxidized monoHER and methylmonoHER. The LUMO of oxidized monoHER is not completely distributed over the phenolic rings but only the B and part of the C rings, which explains why it behaves as a hard electrophile. The LUMO of oxidized methylmonoHER is only focused in the B-ring and oxidized methylmonoHER has a positive charge, which makes it an even harder electrophile compared to monoHER.

In the cell experiment monoHER, at very low concentrations, was able to fully protect HUVECs against oxidative stress induced by $H_2O_2$. This probably involves the site specific scavenging activity. MonoHER can also indirectly protect against oxidative stress because the thiol reactive oxidation product - which is formed during the scavenging of radicals - is able to activate the NRF2 system by reacting with KEAP1. The activation of the NRF2 antioxidant defense system will give rise to an adaptive response (Lemmens et al., 2014a).

MethylmonoHER, even at a concentration much higher than that of monoHER, could not offer direct protection in the HUVECs against oxidative stress. This is in

line with the poor scavenging activity of methylmonoHER. Due to the poor scavenging activity, hardly any oxidation product will be formed. In addition, the oxidation product of methylmonoHER does not react with KEAP1. This suggests that methylmonoHER will not boost the NRF2 antioxidant defense system.

The present study shows that the intrinsic antioxidant effect of methylmonoHER is less than that of monoHER, which means that despite of its relatively high $C_{max}$ and AUC, the contribution of methylmonoHER to the protection against doxorubicin-induced cardiotoxicity is relatively low.

## References

Boots, A.W., Haenen, G.R., den Hartog, G.J., Bast, A., 2002. Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. Biochimica et Biophysica Acta 1583, 279-284.

den Hartog, G.J., Haenen, G.R., Vegt, E., van der Vijgh, W.J., Bast, A., 2003. Superoxide dismutase: the balance between prevention and induction of oxidative damage. Chemico-Biological Interactions 145, 33-39.

Haenen, G.R., Jansen, F.P., Bast, A., 1993. The antioxidant properties of five O-(-β-Hydroxyethyl)-Rutosides of the flavonoid mixture Venoruton. Phlebology Supplements 1, 10-17.

Horenstein, M.S., Vander Heide, R.S., L'Ecuyer, T.J., 2000. Molecular basis of anthracycline-induced cardiotoxicity and its prevention. Molecular Genetics and Metabolism 71, 436-444.

Ito, S., Kato, T., Fujita, K., 1988. Covalent binding of catechols to proteins through the sulphydryl group. Biochemical Pharmacology 37, 1707-1710.

Itoh, K., Tong, K.I., Yamamoto, M., 2004. Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radical Biology and Medicine 36, 1208-1213.

Jacobs, H., Moalin, M., Bast, A., van der Vijgh, W.J., Haenen, G.R., 2010. An essential difference between the flavonoids monoHER and quercetin in their interplay with the endogenous antioxidant network. PLoS One 5, e13880.

Jacobs, H., Peters, R., den Hartog, G.J., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2011. Identification of the metabolites of the antioxidant flavonoid 7-mono-O-(beta-hydroxyethyl)-rutoside in mice. Drug Metabolism and Disposition 39, 750-756.

Jacobs, H., van der Vijgh, W.J., Koek, G.H., Draaisma, G.J., Moalin, M., van Strijdonck, G.P., Bast, A., Haenen, G.R., 2009. Characterization of the glutathione conjugate of the semisynthetic flavonoid monoHER. Free Radical Biology and Medicine 46, 1567-1573.

Julicher, R.H., Sterrenberg, L., Haenen, G.R., Bast, A., Noordhoek, J., 1988. The effect of chronic adriamycin treatment on heart kidney and liver tissue of male and female rat. Archives of Toxicology 61, 275-281.

Lemmens, K.J., Sthijns, M.M., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014a. The antioxidant flavonoid monoHER provides efficient protection and induces the innate Nrf2 mediated adaptation in endothelial cells subjected to oxidative stress. PharmaNutrition.

Lemmens, K.J., van de Wier, B., Vaes, N., Ghosh, M., van Zandvoort, M.A., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014b. The flavonoid 7-mono-O-(beta-hydroxyethyl)-rutoside is able to protect endothelial cells by a direct antioxidant effect. Toxicology in vitro 28, 538-543.

Lipshultz, S.E., Lipsitz, S.R., Sallan, S.E., Dalton, V.M., Mone, S.M., Gelber, R.D., Colan, S.D., 2005. Chronic progressive cardiac dysfunction years after doxorubicin therapy for childhood acute lymphoblastic leukemia. Journal of clinical oncology 23, 2629-2636.

Moalin, M., Strijdonck, G.P., Beckers, M., Hagemen, G., Borm, P., Bast, A., Haenen, G.R., 2011. A planar conformation and the hydroxyl groups in the B and C rings play a pivotal role in the antioxidant capacity of quercetin and quercetin derivatives. Molecules 16, 9636-9650.

Pearson, R.G., 1963. Hard and Soft Acids and Bases. Journal of American Chemical Society 85, 3533-3539.

Reddy, S., Jones, A.D., Cross, C.E., Wong, P.S., Van Der Vliet, A., 2000. Inactivation of creatine kinase by S-glutathionylation of the active-site cysteine residue. The Biochemical Journal 347 Pt 3, 821-827.

Rietjens, S.J., Bast, A., Haenen, G.R., 2007. New insights into controversies on the antioxidant potential of the olive oil antioxidant hydroxytyrosol. Journal of Agricultural and Food Chemistry 55, 7609-7614.

Ruijters, E.J., Weseler, A.R., Kicken, C., Haenen, G.R., Bast, A., 2013. The flavanol (-)-epicatechin and its metabolites protect against oxidative stress in primary endothelial cells via a direct antioxidant effect. European Journal of Pharmacology 715, 147-153.

Spencer, J.P., Kuhnle, G.G., Williams, R.J., Rice-Evans, C., 2003. Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites. Biochemical Journal 372, 173-181.

Steffen, Y., Gruber, C., Schewe, T., Sies, H., 2008. Mono-O-methylated flavanols and other flavonoids as inhibitors of endothelial NADPH oxidase. Archives of Biochemistry and Biophysics 469, 209-219.

van Acker, F.A., van Acker, S.A., Kramer, K., Haenen, G.R., Bast, A., van der Vijgh, W.J., 2000. 7-monohydroxyethylrutoside protects against chronic doxorubicin-induced cardiotoxicity when administered only once per week. Clinical Cancer Research 6, 1337-1341.

van Acker, S.A., Kramer, K., Grimbergen, J.A., van den Berg, D.J., van der Vijgh, W.J., Bast, A., 1995. Monohydroxyethylrutoside as protector against chronic doxorubicin-induced cardiotoxicity. British Journal of Pharmacology 115, 1260-1264.

Xu, M.F., Tang, P.L., Qian, Z.M., Ashraf, M., 2001. Effects by doxorubicin on the myocardium are mediated by oxygen free radicals. Life sciences 68, 889-901.

Chapter 7

# The health effects of the nutraceutical 7-mono-O-(β-hydroxyethyl)-rutoside Bridging the gap between nutrition and medicine

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* equal contribution

**Abstract**

MonoHER is a semisynthetic flavonoid, derived from the naturally occurring flavonoid rutin, and has beneficial health effects. MonoHER has shown protective effects in diseases in which oxidative stress plays an important role. Its antioxidant properties are involved in the protection. Besides its antioxidant activities monoHER also has anti-inflammatory properties which can be advantageous in the protection of disorders in which inflammation is an important factor. The multitude of physiological effects prompted us to review the mechanisms and processes behind the health effects of monoHER.

Because of the beneficial effects, monoHER could be used in the treatment of several diseases. We conclude that monoHER is a nutraceutical that forms a bridge between nutrition and drugs.

**Introduction**

In ancient cultures, there was no clear difference between nutrition and drugs. This is probably best illustrated by the adage of one of the founders of medicine, Hippocrates: "Let food be thy medicine and medicine be thy food". In the 20$^{th}$ century, the idea evolved that drugs and nutrition are separate entities. A drug became a synthetic compound that was carefully designed to effectively cure a specific disease. Nutrition was regarded as a source of energy, building blocks and essential nutrients, like vitamins, that need to be consumed in sufficient amounts to prevent deficiencies. In the last decades our knowledge on the health effect of nutrition has tremendously increased and the gap between drugs and nutrition closes down again.

The group of nutritional compounds in the spotlight, for quite some time, is the flavonoids. This group, that comprises over 5000 different compounds, is abundantly present in plant derived foodstuffs. A wealth of data indicates that the regular intake of flavonoids has a beneficial effect on chronic disorders, including cardiovascular diseases and neurodegenerative disorders and possibly even cancer. Flavonoids may display prophylactic as well as curative effects, or alleviated pathological symptoms. In this way this group of so called "nutraceuticals" illustrates the closing of the gap between nutrition and drugs.

This review focusses on the nutraceutical, 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER). MonoHER is a semisynthetic flavonoid and is a constituent of Venoruton, a registered drug that is used in the treatment of chronic venous insufficiency. Venoruton also contains other structurally related hydroxyethylrutosides (HERs), i.e. diHER, triHER and tetraHER. These HERs are derived from the flavonoid rutin by substituting its hydroxyl groups with O-β-hydroxyethyl groups. Of the HERs, monoHER appeared to be the most powerful antioxidant (Haenen et al., 1993; van Acker et al., 1993).

This review addresses the health effects of the flavonoid monoHER and its possible use as a nutraceutical.

**Chemical characterization**

MonoHER is formed by chemically modifying the naturally occurring flavonoid rutin, abundantly present in Buckwheat. In the synthesis of monoHER, a hydroxyethyl group is linked to the oxygen at the 7- position in the A ring of rutin (Figure 1). The hydroxyethyl groups were introduced to improve the water solubility, which was regarded as rate limiting factor in the biological effect of the nutritional compound.

Like most flavonoids, monoHER consists of three rings referred to as the A, B and C rings (Figure 1). The basic structure of monoHER is similar to the structure of quercetin; it contains an ortho-dihydroxy group in the B ring (catechol), and a C2-C3 double bond and 4-oxo function in the C ring. MonoHER also contains a rutinose group (glucose + rhamnose) at the 3-O position in the C ring.

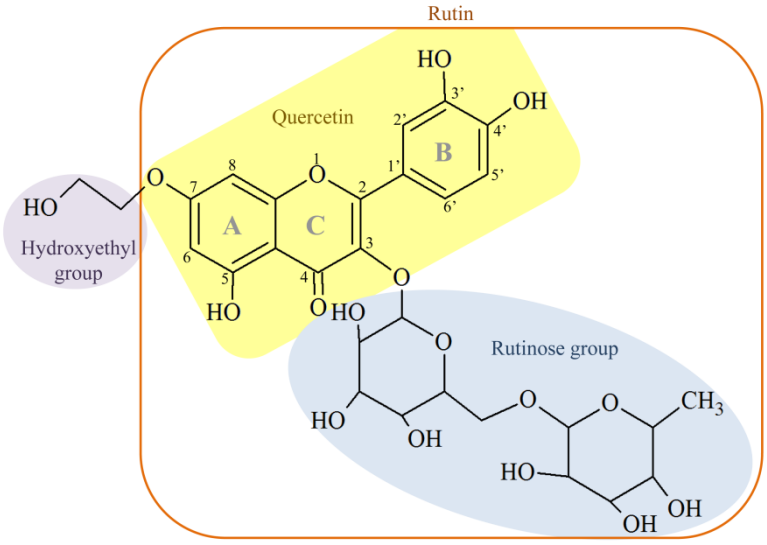


**Figure 1.** Structural formula of the nutraceutical monoHER. It consists of a nutrient (rutin) build up by quercetin and rutinose, which is chemically modified by the chemical addition of a hydroxyethyl group.

**Bioavailability and pharmacokinetics**

Before a compound can exert a systemic effect, it first has to be taken up. The bioavailability and pharmacokinetics of monoHER in mice has been studied after different routes of administration. The levels of monoHER in plasma and heart were also measured.

After oral administration to mice, no monoHER could be detected in plasma of mice, indicating that monoHER has a very poor oral bioavailability. The oral bioavailability was estimated to be less than 1 % (Abou El Hassan et al., 2003a). After intravenous (i.v.), intraperitoneal (i.p). and subcutaneous (s.c.) administration, monoHER could be detected in plasma and in the heart. After administration the maximal concentration is reached relatively quickly and after the peak the concentration decreases rapidly. MonoHER could not be detected for longer than 2 hours after i.v., i.p. or s.c. administration (Abou El Hassan et al., 2003a). After administration of labeled monoHER, radioactivity was measured in plasma for at least 6 h when radiolabeled monoHER was administered. It was suggested that the HERs of Venoruton® (including monoHER) are taken up in the vascular wall (Neumann et al., 1992). Neumann et al. found that these flavonoids were localized in the endothelial and sub-endothelial layer of varicose long saphenous veins after i.v. administration. Recently, it was found that monoHER was retained in the wall of the carotid arteries of mice after i.p. administration, more specifically in the nucleus (Lemmens et al., 2014a; Lemmens et al., 2014c).

The bioavailability after i.p. administration was 30%, after s.c. administration 40%. The pharmacokinetics of monoHER were determined (Table 1). I.p. administration of monoHER (500 mg/kg) showed a maximal concentration ($C_{max}$) of 131 µM and 35.3 µM in plasma and heart, respectively after 5-15 min. MonoHER disappeared from the plasma and heart tissue with a half-life ($t_{1/2}$) of about 30 min. The area under the curve ($AUC^{\infty}$) was 6.3 µmol min/ ml in plasma (Abou El Hassan et al., 2003a).

Toxicity studies of monoHER in several animal models revealed neither acute, nor chronic toxic effects nor teratogenic effects after oral administration of monoHER (Berte, 1974; Chesterman et al., 1973; Davies and Collins, 1973a, b, c; Hunter et al., 1973; Lueschner, 1974). To evaluate the possible side effects of monoHER and to investigate the pharmacokinetics of monoHER in men, a phase I clinical trial was performed (Willems et al., 2006). The study was performed as a single blind, randomized trial in healthy volunteers. MonoHER was administered as an i.v. infusion in 10 minutes. MonoHER was dissolved in 5% dextrose and the pH was adjusted to 8.4. Up to 1500 mg/m$^2$ (equal to a dose of approximately 3 gram per volunteer) monoHER was well-tolerated and no serious side-effects were observed. At this dose, a mean peak plasma concentration of 360 ± 69 μM and a mean $AUC^{\infty}$ of 6.8 ± 2.1 μmol min/ml, which is equal to mice administered i.p. with 500 mg/kg monoHER, were obtained (Table 2). MonoHER was still detectable in plasma after 8 hours. The data also showed that monoHER is rapidly distributed and eliminated from the plasma compartment, which corresponds with a rapid uptake in and elimination from heart tissue as found in mice. From this phase I study, it could be concluded that 1500 mg/m$^2$ of monoHER is a safe dose to be used in further clinical studies (Willems et al., 2006).

**Table 1.** Summary of the pharmacokinetic parameters of monoHER in plasma and heart tissue of mice treated with 500 mg/kg i.v., i.p. and s.c (Abou El Hassan et al., 2003a).

| | **i.v.** | | **i.p.** | | **s.c.** | |
|---|---|---|---|---|---|---|
| **pK paramaters** | Plasma | Heart | Plasma | Heart | Plasma | Heart |
| $C_{max}$ (nmol/ml) | 2 X $10^3$ | 0.4 X $10^{3*}$ | 131 | 35.3* | 229.3 | 68.4* |
| $t_{max}$(min) | 0 | 0 | 5-15 | 5-15 | 10-20 | 10 |
| $t_{1/2final}$ (min) | 11.8 | 16.2 | 28.5 | 25.7 | 23.9 | 29.3 |
| $AUC^{\infty}$ (μmol min/ml) | 24.7 | 5.6* | 6.3 | 1.6* | 8.0 | 2.0* |
| $AUC^{0\text{-}120min}$ (μmol min/ml) | 20.5 | 4.9* | 6.1 | 1.6* | 8.0 | 2.0* |
| MRT (min) | 10.9 | 12.1 | 43.6 | 43.0 | 34.1 | 41.5 |

* Expressed per gram wet tissue

**Table 2.** Summary of the pharmacokinetic parameters of monoHER in plasma of healthy volunteers treated with 1500 mg/m$^2$ i.v. (Willems et al., 2006).

| | **i.v.** |
|---|---|
| **pK paramaters** | Plasma |
| $C_{max}$ (nmol/ml) | 360 ± 69 |
| $t_{1/2\alpha}$ (min) | 5.0 ± 1.6 |
| $t_{1/2\beta}$ (min) | 27 ± 11 |
| $t_{1/2\gamma}$ (min) | 168 ± 148 |
| $AUC^{\infty}$ (µmol min/ml) | 6.8 ± 2.1 |
| MRT (min) | 33.4 ± 8.8 |

The metabolism of monoHER was investigated in mice (Jacobs et al., 2011c). MonoHER was mainly excreted via bile in feces (Barrow and Griffiths, 1971, 1974; Hackett and Griffiths, 1977), therefore the metabolism was determined in the bile fluid. Thirteen different metabolites were characterized. The observed routes of monoHER metabolism were methylation, glucuronidation, oxidation of its hydroxyethyl group, GSH conjugation, and hydrolysis of its disaccharide (Figure 2). The metabolism of monoHER was also investigated in the bile fluid of healthy volunteers (Jacobs et al., 2011b). The same metabolites were found in humans as previously in mice. However, the relative amounts of the metabolites were quite different (Figure 2). The major metabolic route in mice appeared to be methylation while in men especially glucuronidation was observed.

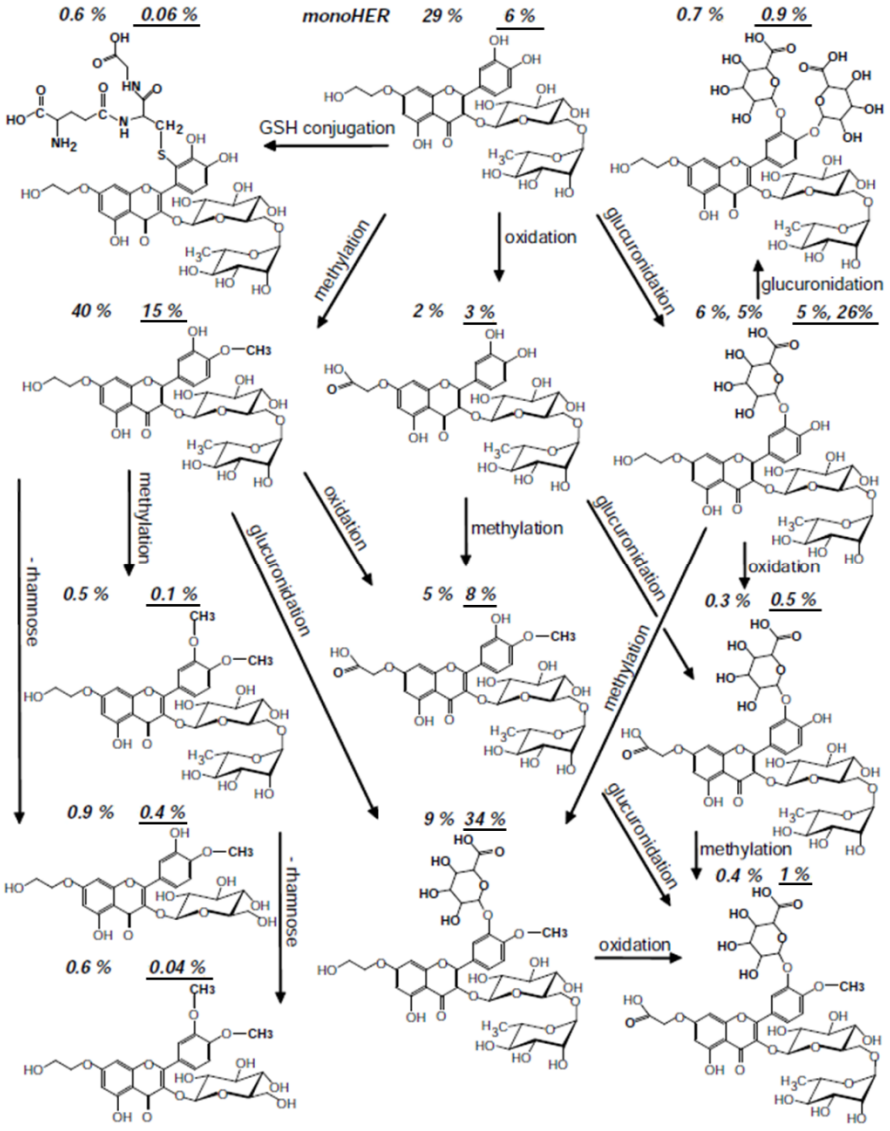


**Figure 2.** Proposed metabolism of monoHER in mice and men. The percentages which are not underlined indicate the relative amounts of each metabolite in mice (Jacobs et al., 2011c). The underlined percentages indicate the relative amounts of each metabolite in men (Jacobs et al., 2011b).

## Molecular mechanisms

### *Radical scavenging*

MonoHER is an effective radical scavenger. It scavenges a wide variety of radicals; hydroxyl, superoxide radicals (Haenen et al., 1993; Lemmens et al., 2014c). This excellent scavenging can be traced down to several structural elements. The catechol moiety in the B ring and the C2-C3 double bond and 4-oxo function in the C ring contribute to the scavenging activity of monoHER (van Acker et al., 1996). During scavenging, monoHER is oxidized by the radical, and the oxidized monoHER is relatively stable due to delocalization of the free electron over the π-system.

### *Iron chelation*

In addition to the radical scavenging, monoHER also has the ability to chelate iron. The result of this chelation is that monoHER is present at the site of the radical formation, i.e. the iron ion. This enables monoHER to immediately scavenge the newly formed radical (van Acker et al., 1998). This 'site-specific scavenging' is essential for its excellent hydroxyl radical scavenging activity. MonoHER scavenges OH-radicals at an extremely high reaction rate - $k_s = 980 \text{ X } 10^8 \text{ M}^{-1}\text{s}^{-1}$ - which is, remarkably, even quicker than the diffusion rate ($\sim 100 \text{ X } 10^8 \text{ M}^{-1}\text{s}^{-1}$).
By this site-specific scavenging, monoHER is able to prevent damage to critical biomolecules such as lipids, proteins or DNA, despite the high reactivity of the radical.

### *Adaptation*

Oxidized monoHER, which is formed after scavenging radicals, is reactive itself. However, the reactivity of the oxidized product is less reactive and thus more selective than the radical. Oxidized monoHER can channel reactivity towards thiols, evidenced by the formation of a GSH-monoHER adduct after monoHER administration to human volunteers (Jacobs et al., 2009). It has been reported that oxidized monoHER adducts KEAP1, which functions as an in vivo sensor for ROS

and electrophiles (Lemmens et al., 2014a). By reacting with KEAP1, the Nuclear factor erythroid 2-related factor 2 (NRF2) endogenous antioxidant defense system is activated. NRF2 dissociates from the KEAP1-NRF2 complex and translocates to the nucleus. NRF2 binds to antioxidant response elements (ARE) in the promoter region of genes encoding various antioxidants and phase II detoxifying enzymes e.g. heme oxygenase-1 (HO-1), glutathione peroxidases (Gpx), superoxide dismutase (SOD). MonoHER itself does not activate the NRF2 system because it lacks the thiol reactivity. In this way monoHER induces an adaptive response in cells exposed to oxidative stress (Figure 3). MonoHER empowers the buffering capacity of the body to maintain homeostasis and to prevent that relatively mild but nevertheless insidious and persistent perturbations gradually progress into a severe pathology (Lemmens et al., 2014a).

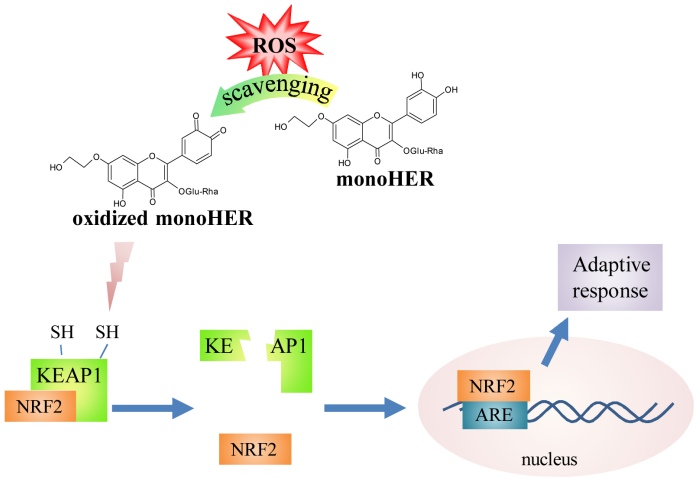


**Figure 3.** Overview of the protective effect of monoHER against ROS toxicity and the induction of the adaptive response of the cell. MonoHER protects by scavenging the ROS. The oxidized monoHER formed during scavenging selectively adducts KEAP1 and activates the NRF2 pathway (Lemmens et al, 2014a).

***Protein binding***

The thiol reactivity of oxidized monoHER can also lead to enzyme inhibition. Oxidized monoHER can react with critical thiol groups of a protein which will lead to function loss.

**Health effects of monoHER**

MonoHER has already been applied in the treatment of several diseases. Its beneficial health effects are reviewed separately. The involvement of the various molecular mechanisms and processes in the health effects is depicted in Figure 4.

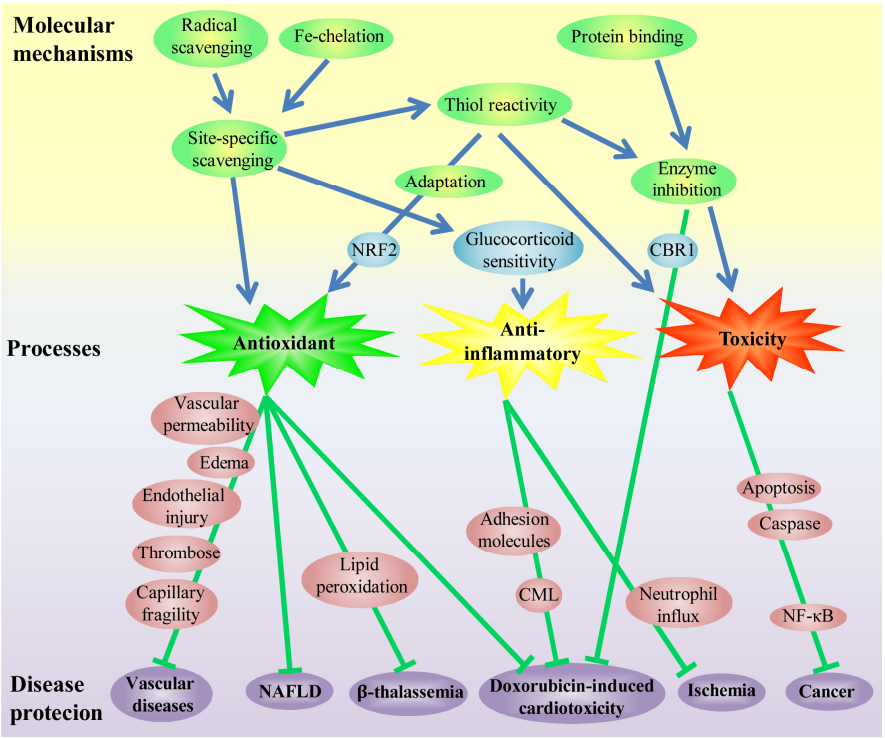


**Figure 4.** Schematic overview of the molecular mechanisms and processes involved in the beneficial health effects of monoHER. (NRF2: Nuclear factor erythroid 2-related factor 2, CBR1: carbonyl reductase 1, CML: $N^{\varepsilon}$-(carboxymethyl) lysine, NF-κB: nuclear factor-κB, NAFLD: Non-alcoholic fatty liver disease)

***Vascular diseases***

In chronic venous insufficiency oxidative stress plays an important role. Venoruton, the rutin mixture containing monoHER, is used in the treatment of chronic venous insufficiency for over several decades.
It has also been shown that monoHER has antioedematous properties in rats by inhibiting the action of bradykinine, histamine and carrageenin (Lecomte and Van Cauwenberge, 1974). Moreover, monoHER showed antithrombotic effects (Hladovec, 1977a; Mirkovitch, 1977). It prevented endothelial injury caused by nicotine and citrate (Hladovec, 1977b, 1978), it increased skin capillary resistance in a rat model (Gabor, 1981) and reduced microvascular permeability in frogs (Kendall et al., 1993).

***NAFLD***

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in Western countries. One of the hallmarks in the etiology of NAFLD is oxidative stress (Koek et al., 2011; Rolo et al., 2012). MonoHER has shown to be a potent protector against NAFLD in mice by inducing an adaptive response (Lemmens et al., 2014b). MonoHER induces the NRF2 pathway and enforces the endogenous antioxidant shield, to provide protection against NAFLD.

***β-thalassemia***

β-thalassemia is an autosomal blood disorder caused by mutations in the gene encoding β-globin, resulting in reduced or absent β-chain synthesis of hemoglobin. This leads to an excessive body iron store which will result in the formation of free radicals. Oxidative damage plays a significant role in the premature destruction of erythrocytes in thalassemias (Origa and Galanello, 2011).
As an effective radical scavenger and iron chelator, monoHER treatment of β-thalassemic mice resulted in substantial improvements in several parameters related to membrane oxidative stress and lipid peroxidation (de Franceschi et al., 2004).

***Doxorubicin-induced cardiotoxicity***

Because of its excellent iron chelating and radical scavenging properties (Lemmens et al., 2014c), monoHER was tested as a protector against doxorubicin-induced cardiotoxicity. Doxorubicin is a very effective antitumor agent, but its clinical use is limited by the occurrence of a cumulative dose-related cardiotoxicity, resulting in congestive heart failure (Bast et al., 2007; Lipshultz et al., 2005; Singal and Iliskovic, 1998). Doxorubicin-induced cardiotoxicity presumably results from free radicals, which are produced during redox-cycling of doxorubicin (Horenstein et al., 2000; Julicher et al., 1988; Xu et al., 2001). MonoHER protected almost completely (92.7%) against doxorubicin-induced cardiac damage in an isolated atrium model (van Acker et al., 1993). In mice, cardioprotection was observed when monoHER was administered as an intraperitoneal (i.p.) dose of 500 mg/kg five times/week in combination with a weekly intravenously (i.v.) dose of 4 mg/kg doxorubicin for a period of six weeks (van Acker et al., 1995). A single i.p. injection (500 mg/kg) of monoHER only once a week 1 hour before doxorubicin administration (4 mg/kg, i.v.) also gave complete protection (van Acker et al., 2000) (Figure 3).

Both *in vitro* and *in vivo*, monoHER protected against the toxic effect of doxorubicin on cardiac cells without interfering with the cytostatic effect on cancer cells (van Acker et al., 1997).

To unravel the mechanism underlying the selective protective effects of monoHER, it was investigated whether monoHER (1 mM) affects doxorubicin-induced apoptosis in neonatal rat cardiac myocytes (NeRCaMs), human umbilical vein endothelial cells (HUVECs) and the ovarian cancer cell lines A2780 and OVCAR-3. The data indicated that monoHER might act by suppressing the activation of molecular mechanisms that mediate either caspase-dependent or -independent cell death (Bruynzeel et al., 2007b). However, the concentration of monoHER needed (1 mM) indicates that the *in vitro* inhibition of the antitumor effect of doxorubicin is not relevant for the clinical application of monoHER.

The time interval between monoHER and doxorubicin did not show a significant change in protection against doxorubicin-induced cardiac damage (Bruynzeel et al.,

2006). Abou El Hassan et al. showed that the cardioprotection observed in mice is not caused by a pharmacokinetic interaction between monoHER and doxorubicin.

Some studies suggest that inflammation induced by doxorubicin plays a role in its cardiotoxic effects (Deepa and Varalakshmi, 2006; Hecker, 1990; Hou et al., 2005). To investigate whether doxorubicin could induce an inflammatory response *in vitro*, HUVECs were incubated with increasing concentrations of doxorubicin. Doxorubicin affected both the viability and proliferation capacity of endothelial cells (Abou El Hassan et al., 2003b). Doxorubicin also increased the adhesion of neutrophils, which was accompanied by the overexpression of VCAM and E-selectin. MonoHER was able to protect against these doxorubicin-induced inflammatory effects (Abou El Hassan et al., 2003b) (Figure 5).

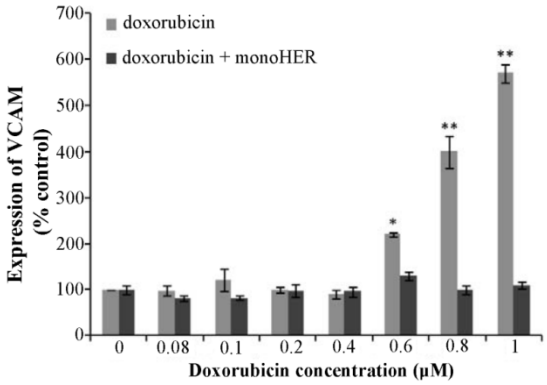


**Figure 5.** Overexpression of VCAM 25 h after incubation with doxorubicin. The combined treatment with monoHER prevented the concentration-dependent doxorubicin-induced overexpression of the adhesion molecule (Abou El Hassan et al., 2003b)

In addition, *in vivo*, it was demonstrated by Bruynzeel *et al*. that treatment with doxorubicin induces an increase of $N^{\varepsilon}$-(carboxymethyl) lysine (CML) in intramyocardial arteries in mice (Bruynzeel et al., 2007a). The induced increase in CML, which is regarded as a biomarker for local endogenous stress, was found to be reduced by the anti-inflammatory agents, ketoprofen and dexamethasone, and by monoHER. These findings confirm the role of inflammation in doxorubicin-induced

cardiotoxicity and indicate that monoHER has - besides its antioxidant activity, radical scavenging and iron chelating properties - anti-inflammatory properties which could also be involved in the protection against cardiac damage.

The cardioprotective effect of monoHER could also be explained by the inhibition of the enzyme carbonyl reductase 1 (CBR1). The enzyme reduces doxorubicin into the metabolite doxorubicinol which is more cardiotoxic than the parent compound.

By promoting the antioxidant defense, the heart is able to adapt against oxidative stress caused by doxorubicin. The involvement of an adaptive response in the protection against doxorubicin-induced cardiotoxicity by monoHER should further be investigated.

The possible involvement of the major metabolite 4'-O methylmonoHER was studied (Lemmens et al., 2015). It was concluded that the antioxidant activity of monoHER drastically decreased after methylation at 4'-O position which indicates that the contribution to the protective effect by the metabolite is relatively low.

Based on the promising results with monoHER observed in preclinical experiments, a phase II study was performed to investigate the cardioprotective effect of monoHER on doxorubicin-induced cardiotoxicity in cancer patients (Bruynzeel et al., 2007c). MonoHER did not significantly protect against doxorubicin- induced cardiotoxicity in these cancer patients. The dose should be further investigated.

***Ischemia***

The anti-inflammatory effect of monoHER was also observed in a study on ischemia-reperfusion injury in mice. Inflammation and oxidative stress play a crucial role in the ischemia-reperfusion injury. The monoHER treatment significantly attenuated myocardial neutrophil influx and significantly reduced infarct size after reperfusion (De Celle et al., 2004).

***Cancer***

The effect of monoHER on the antitumor activity of doxorubicin was observed in the clinical phase II study (Bruynzeel et al., 2007c). An intriguing observation was that three of the four patients diagnosed with soft tissue sarcomas experienced objective remissions, while the fourth had stable disease. This 75% response rate is much higher than the expected remission of approximately 25% (Santoro et al., 1995). It is therefore suggestive that monoHER enhances the antitumor activity of doxorubicin in soft tissue sarcomas.

The effect of monoHER on the enhancement of the antitumor activity of doxorubicin was investigated *in vitro,* in a soft tissue sarcoma cell line. Chemotherapeutic agents are known to induce the transcription factor nuclear factor-κB (NF-κB), which causes drug resistance in cancer cells. MonoHER prevented the induction of NF-κB by doxorubicin in soft tissue sarcoma cell line, WLS-160 (Jacobs et al., 2011a). In this way cancer cells are sensitized to doxorubicin via the down-regulation of the NF-κB activation by monoHER. MonoHER may also be valuable in the treatment of other tumors that are resistant via NF-κB activation.

***Other pharmacological properties of monoHER***

Several other pharmacological properties of monoHER have been described. It has also been shown that monoHER has an effect on enzymes that intervene in the metabolism of mucopolysaccharides in human and bovine veins. The presence of monoHER (1 mg/ml) inhibited the enzyme β-glucuronidase extracted from human veins for 12.5% and for 70.3% when extracted from bovine veins, while monoHER did not significantly influence the activity of the enzyme N-acetylglucosaminidase obtained from both veins (Niebes and Laszt, 1971). MonoHER also affected prostaglandin synthesis in guinea pig lung tissue and human skin (Gosta, 1973).

**Future research**

The protective effect of monoHER against the cardiotoxicity induced by doxorubicin is still enigmatic. In the future the involvement of metabolites in the protective effect should further be investigated as well as the adaptive response.

**Conclusion**

MonoHER a derivative of rutin, a natural flavonoid present in food, has versatile beneficial health effects. Besides his antioxidant activity, monoHER also has anti-inflammatory and anti-tumor properties. It was shown that monoHER can support the body to adapt to endogenous or exogenous threats. Because of these beneficial effects, monoHER can be used in the treatment of several diseases. We can conclude that monoHER is a nutraceutical that forms a bridge between nutrition and drugs.

## References

Abou El Hassan, M.A., Kedde, M.A., Zwiers, U.T., Tourn, E., Haenen, G.R., Bast, A., van der Vijgh, W.J., 2003a. Bioavailability and pharmacokinetics of the cardioprotecting flavonoid 7-monohydroxyethylrutoside in mice. Cancer Chemotherapy and Pharmacology 52, 371-376.

Abou El Hassan, M.A., Verheul, H.M., Jorna, A.S., Schalkwijk, C., van Bezu, J., van der Vijgh, W.J., Bast, A., 2003b. The new cardioprotector Monohydroxyethylrutoside protects against doxorubicin-induced inflammatory effects in vitro. British Journal of Cancer 89, 357-362.

Barrow, A., Griffiths, L.A., 1971. The biliary excretion of hydroxyethylrutosides and other flavonoids in the rat. The Biochemical journal 125, 24P-25P.

Barrow, A., Griffiths, L.A., 1974. Metabolism of the hydroxyethylrutosides. II. Excretion and metabolism of 3',4',7-tri-O-(beta-hydroxyethyl) rutoside and related compounds in laboratory animals after parenteral administration. Xenobiotica 4, 1-16.

Bast, A., Haenen, G.R., Bruynzeel, A.M., Van der Vijgh, W.J., 2007. Protection by flavonoids against anthracycline cardiotoxicity: from chemistry to clinical trials. Cardiovascular Toxicology 7, 154-159.

Berte, F., 1974. Toxicite chronique chiens. Internal Report Zyma SA.

Bruynzeel, A.M., Abou El Hassan, M.A., Schalkwijk, C., Berkhof, J., Bast, A., Niessen, H.W., van der Vijgh, W.J., 2007a. Anti-inflammatory agents and monoHER protect against DOX-induced cardiotoxicity and accumulation of CML in mice. British Journal of Cancer 96, 937-943.

Bruynzeel, A.M., Abou El Hassan, M.A., Torun, E., Bast, A., van der Vijgh, W.J., Kruyt, F.A., 2007b. Caspase-dependent and -independent suppression of apoptosis by monoHER in Doxorubicin treated cells. British Journal of Cancer 96, 450-456.

Bruynzeel, A.M., Mul, P.P., Berkhof, J., Bast, A., Niessen, H.W., van der Vijgh, W.J., 2006. The influence of the time interval between monoHER and doxorubicin administration on the protection against doxorubicin-induced cardiotoxicity in mice. Cancer Chemotherapy and Pharmacology 58, 699-702.

Bruynzeel, A.M., Niessen, H.W., Bronzwaer, J.G., van der Hoeven, J.J., Berkhof, J., Bast, A., van der Vijgh, W.J., van Groeningen, C.J., 2007c. The effect of monohydroxyethylrutoside on doxorubicin-induced cardiotoxicity in patients treated for metastatic cancer in a phase II study. British Journal of Cancer 97, 1084-1089.

Chesterman, H., Heywood, R., Fuller, R., 1973. Z12007: preliminary toxicity study in the Beagle dog. Internal Report Zyma SA.

Davies, R., Collins, C., 1973a. Acute oral toxicity to beagle dogs of Z12007. Internal Report Zyma SA.

Davies, R., Collins, C., 1973b. Acute oral toxicity to mice of Z12007. Internal Report Zyma SA.

De Celle, T., Heeringa, P., Strzelecka, A.E., Bast, A., Smits, J.F., Janssen, B.J., 2004. Sustained protective effects of 7-monohydroxyethylrutoside in an in vivo model of cardiac ischemia-reperfusion. European Journal of Pharmacology 494, 205-212.

de Franceschi, L., Turrini, F., Honczarenko, M., Ayi, K., Rivera, A., Fleming, M.D., Law, T., Mannu, F., Kuypers, F.A., Bast, A., van der Vijgh, W.J., Brugnara, C., 2004. In vivo reduction of erythrocyte oxidant stress in a murine model of beta-thalassemia. Haematologica 89, 1287-1298.

Deepa, P.R., Varalakshmi, P., 2006. Influence of a low-molecular-weight heparin derivative on the nitric oxide levels and apoptotic DNA damage in adriamycin-induced cardiac and renal toxicity. Toxicology 217, 176-183.

Gabor, M., 1981. The effect of O-(beta-hydroxyethyl)-rutosides (HR) on the skin capillary resistance of rats. Arzneimittel-Forschung 31, 442-445.

Gosta, A., 1973. Effects of HR and its derivates on protaglandin biosynthesis in guinea pig lung tissue and human skin. Internal report Zyma SA.

Hackett, A.M., Griffiths, L.A., 1977. The disposition and metabolism of 3',4',7-tri-O-(beta-hydroxyethyl)rutoside and 7-mono-O-(beta-hydroxyethyl)rutoside in the mouse. Xenobiotica 7, 641-651.

Haenen, G.R., Jansen, F.P., Bast, A., 1993. The antioxidant properties of five O-(-β-Hydroxyethyl)-Rutosides of the flavonoid mixture Venoruton. Phlebology Supplements 1, 10-17.

Hecker, J.F., 1990. Survival of intravenous chemotherapy infusion sites. British Journal of Cancer 62, 660-662.

Hladovec, J., 1977a. Antithrombotic effects of some flavonoids alone and combined with acetylsalicylic acid. Arzneimittel-Forschung 27, 1989-1992.

Hladovec, J., 1977b. Differentiation of in vitro and in vivo effects of a flavonoid on endothelial cell counts. Arzneimittel-Forschung 27, 1142-1144.

Hladovec, J., 1978. Endothelial injury by nicotine and its prevention. Experientia 34, 1585-1586.

Horenstein, M.S., Vander Heide, R.S., L'Ecuyer, T.J., 2000. Molecular basis of anthracycline-induced cardiotoxicity and its prevention. Molecular Genetics and Metabolism 71, 436-444.

Hou, M., Chrysis, D., Nurmio, M., Parvinen, M., Eksborg, S., Soder, O., Jahnukainen, K., 2005. Doxorubicin induces apoptosis in germ line stem cells in the immature rat testis and amifostine cannot protect against this cytotoxicity. Cancer Research 65, 9999-10005.

Hunter, B., Bridges, J., Cherry, C., 1973. Preliminary assessment of Z12007 toxicity to rats in oral administration for four weeks. Internal Report Zyma SA.

Jacobs, H., Bast, A., Peters, G.J., van der Vijgh, W.J., Haenen, G.R., 2011a. The semisynthetic flavonoid monoHER sensitises human soft tissue sarcoma cells to doxorubicin-induced apoptosis via inhibition of nuclear factor-kappaB. British Journal of Cancer 104, 437-440.

Jacobs, H., Koek, G.H., Peters, R., Moalin, M., Tack, J., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2011b. Differences in pharmacological activities of the antioxidant flavonoid monoHER in humans and mice are caused by variations in its metabolic profile. Clinical Pharmacology and Therapeutics 90, 852-859.

Jacobs, H., Peters, R., den Hartog, G.J., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2011c. Identification of the metabolites of the antioxidant flavonoid 7-mono-O-(beta-hydroxyethyl)-rutoside in mice. Drug Metabolism and Disposition 39, 750-756.

Jacobs, H., van der Vijgh, W.J., Koek, G.H., Draaisma, G.J., Moalin, M., van Strijdonck, G.P., Bast, A., Haenen, G.R., 2009. Characterization of the glutathione conjugate of the semisynthetic flavonoid monoHER. Free Radical Biology and Medicine 46, 1567-1573.

Julicher, R.H., Sterrenberg, L., Haenen, G.R., Bast, A., Noordhoek, J., 1988. The effect of chronic adriamycin treatment on heart kidney and liver tissue of male and female rat. Archives of Toxicology 61, 275-281.

Kendall, S., Towart, R., Michel, C.C., 1993. Effects of hydroxyethylrutosides on the permeability of microvessels in the frog mesentery. British journal of pharmacology 110, 199-206.

Koek, G.H., Liedorp, P.R., Bast, A., 2011. The role of oxidative stress in non-alcoholic steatohepatitis. Clinica Chimica Acta 412, 1297-1305.

Lecomte, J., Van Cauwenberge, H., 1974. Some pharmacologic properties of mono-(O-beta-hydroxyethyl)-7-rutoside in the rat. Archives internationales de pharmacodynamie et de therapie 208, 317-327.

Lemmens, K.J., Sthijns, M.M., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014a. The antioxidant flavonoid monoHER provides efficient protection and induces the innate Nrf2 mediated adaptation in endothelial cells subjected to oxidative stress. PharmaNutrition 2, 69-74.

Lemmens, K.J., van de Wier, B., Koek, G.H., Kohler, E., Drittij, M.J., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014b. The flavonoid monoHER promotes the adaptation to oxidative stress during the onset of NAFLD. Biochemical and Biophysical Research Communications.

Lemmens, K.J., van de Wier, B., Vaes, N., Ghosh, M., van Zandvoort, M.A., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014c. The flavonoid 7-mono-O-(beta-hydroxyethyl)-rutoside is able to protect endothelial cells by a direct antioxidant effect. Toxicology in vitro 28, 538-543.

Lemmens, K.J., Herst, P.M., Housmans, B.A., Moalin, M., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2015. The contribution of the major metabolite 4'-O-methylmonoHER to the antioxidant activity of the flavonoid monoHER. submitted

Lipshultz, S.E., Lipsitz, S.R., Sallan, S.E., Dalton, V.M., Mone, S.M., Gelber, R.D., Colan, S.D., 2005. Chronic progressive cardiac dysfunction years after doxorubicin therapy for childhood acute lymphoblastic leukemia. Journal of Clinical Oncology 23, 2629-2636.

Lueschner, F., 1974. Uber den Einfluss von Z12007, Mono-7-HR, Charge 1020- in diesem Bericht kurz "Mono-7-HR" genannt- auf die trachtige Kaninchen und den Foetus bei Verabreichung per Magensonde. Internal Report Zyma SA.

Mirkovitch, V., 1977. The influence of monoHER on experimental venous thrombosis in dogs. Internal report.

Neumann, H.A., Carlsson, K., Brom, G.H., 1992. Uptake and localisation of O-(beta-hydroxyethyl)-rutosides in the venous wall, measured by laser scanning microscopy. European Journal of Clinical Pharmacology 43, 423-426.

Niebes, P., Laszt, L., 1971. Influence in vitro of a series of flavonoids on certain enzymes in the metabolism of mucopolysaccharides in the human and bovine saphenous vein. Angiologica 8, 297-302.

Origa, R., Galanello, R., 2011. Pathophysiology of beta thalassaemia. Pediatric endocrinology reviews : PER 8 Suppl 2, 263-270.

Rolo, A.P., Teodoro, J.S., Palmeira, C.M., 2012. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. Free Radical Biology & Medicine 52, 59-69.

Santoro, A., Tursz, T., Mouridsen, H., Verweij, J., Steward, W., Somers, R., Buesa, J., Casali, P., Spooner, D., Rankin, E., et al., 1995. Doxorubicin versus CYVADIC versus doxorubicin plus ifosfamide in first-line treatment of advanced soft tissue sarcomas: a randomized study of the European Organization for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group. Journal of Clinical Oncology 13, 1537-1545.

Singal, P.K., Iliskovic, N., 1998. Doxorubicin-induced cardiomyopathy. The New England journal of medicine 339, 900-905.

van Acker, F.A., van Acker, S.A., Kramer, K., Haenen, G.R., Bast, A., van der Vijgh, W.J., 2000. 7-monohydroxyethylrutoside protects against chronic doxorubicin-induced cardiotoxicity when administered only once per week. Clinical Cancer Research 6, 1337-1341.

van Acker, S.A., Boven, E., Kuiper, K., van den Berg, D.J., Grimbergen, J.A., Kramer, K., Bast, A., van der Vijgh, W.J., 1997. Monohydroxyethylrutoside, a dose-dependent cardioprotective agent, does not affect the antitumor activity of doxorubicin. Clinical Cancer Research 3, 1747-1754.

van Acker, S.A., Kramer, K., Grimbergen, J.A., van den Berg, D.J., van der Vijgh, W.J., Bast, A., 1995. Monohydroxyethylrutoside as protector against chronic doxorubicin-induced cardiotoxicity. British Journal of Pharmacology 115, 1260-1264.

van Acker, S.A., Towart, R., Husken, B.C., De Jong, J., van der Vijgh, W.J., Bast, A., 1993. The protective effect of Venoruton and its main constituents on acute doxorubicin-induced cardiotoxicity. Phlebology Supplements 1, 31-32.

van Acker, S.A., van Balen, G.P., van den Berg, D.J., Bast, A., van der Vijgh, W.J., 1998. Influence of iron chelation on the antioxidant activity of flavonoids. Biochemical Pharmacology 56, 935-943.

van Acker, S.A., van den Berg, D.J., Tromp, M.N., Griffioen, D.H., van Bennekom, W.P., van der Vijgh, W.J., Bast, A., 1996. Structural aspects of antioxidant activity of flavonoids. Free Radical Biology & Medicine 20, 331-342.

Willems, A.M., Bruynzeel, A.M., Kedde, M.A., van Groeningen, C.J., Bast, A., van der Vijgh, W.J., 2006. A phase I study of monohydroxyethylrutoside in healthy volunteers. Cancer Chemotherapy and Pharmacology 57, 678-684.

Xu, M.F., Tang, P.L., Qian, Z.M., Ashraf, M., 2001. Effects by doxorubicin on the myocardium are mediated by oxygen free radicals. Life sciences 68, 889-901.

Chapter 8

# Summary and general discussion

Flavonoid intake is associated with beneficial health effects. The antioxidant activity plays a crucial role in the protective action of flavonoids (**chapter 1**). The aim of this thesis was to further elaborate on the molecular mechanism of the antioxidant effect of flavonoids. The focus was put on the antioxidant, flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER), quercetin and their 4'-O-methyl metabolites.

The flavonoid monoHER is a potent protector against cardiotoxicity induced by doxorubicin. The potential relevance of the direct scavenging activity of monoHER was studied in **chapter 2**.
MonoHER protected against intracellular oxidative stress in human umbilical vein endothelial cells ($EC_{50}$ = 60 nM), which confirmed the involvement of a direct scavenging activity by therapeutically achievable concentrations. This direct effect of monoHER was due to its perfect location on three levels. On a molecular level, monoHER is located at the site of radical formation where it site-specifically scavenges the radical involved. On a biochemical level, monoHER is located at a pivotal position in the antioxidant network. On a cellular level, monoHER is located in the endothelial and smooth muscle cells in the vascular wall.
This explains that monoHER displays a physiologically important direct antioxidant effect.

When ROS are scavenged, they take up an electron or a hydrogen atom from the antioxidant flavonoid. In this way the reactive species are neutralized. In contrast, the flavonoid is oxidized and becomes reactive itself. The reactivity of the oxidized flavonoid is less than that of the radical scavenged which makes it more selective. Flavonoids are reported to selectively channel the reactivity towards thiol groups. Hence, oxidized flavonoids may threat vital cellular compounds containing a critical thiol group.
In **chapter 3** the thiol reactivity of the antioxidant flavonoid quercetin was compared to that of 4'-O-methylquercetin (tamarixetin) towards creatine kinase (CK), a vital protein that contains a critical thiol moiety. Oxidized quercetin and oxidized tamarixetin are reactive towards CK; both oxidized flavonoids adducted the enzyme,

which led to the loss of its function. Oxidized tamarixetin was less thiol toxic than oxidized quercetin, because - rather than adduction to CK - oxidized tamarixetin prefers to pass reactivity to the antioxidant network, *i.e.*, to ascorbate. Apparently, a small structural modification such as the introduction of a methyl group has a great influence on the channeling of the reactivity.

When oxidized flavonoids channel their reactivity toward the thiol containing protein KEAP1, this leads to adaptation (**chapter 4**). In **chapter 4** the effect of antioxidant flavonoid monoHER on the cellular adaptive response in HUVECs exposed to $H_2O_2$ was studied.

It was found that oxidized monoHER adducts KEAP1, which activates the NRF2 antioxidant defense system. NRF2 is released from the KEAP1-NRF2 complex, translocates to the nucleus and upregulates the expression of antioxidant genes, e.g. of *HO-1*. In this way monoHER offers direct protection and induces adaptation against oxidative stress.

Thiol reactivity can lead on the one hand to toxic effects by damaging cellular compounds - as demonstrated in chapter 3 - and on other hand to a beneficial adaptive response. As shown in chapter 4, monoHER added to cells subjected to oxidative stress protects against the damage but does not reduce the adaptive response to oxidative stress while no toxicity is observed. Apparently, the protective and adaptive effect of monoHER prevails over the possible toxicity.

In **chapter 5** the effect of monoHER on oxidative stress as well as the adaptive response in the onset of NAFLD in *Ldlr*$^{-/-}$ mice on a high-fat and high-cholesterol diet was studied. Wild type mice served as a control. The effect of monoHER was compared to that of a placebo.

In the *Ldlr*$^{-/-}$ group, no pronounced effect of monoHER against ROS damage was seen. This could be due to the relatively large variation in the results which is probably because the onset of the disease was studied. In the placebo *Ldlr*$^{-/-}$ group the NRF2 antioxidant defense system was activated, evidenced by a higher *HO-1* and *Gpx3* gene expression, as well as an increased redox status, evidenced by the

higher GSH/GSSG ratio. MonoHER tended to further increase the *HO-1* and *Gpx3* gene expression and the GSH/GSSG ratio in the *Ldlr*$^{-/-}$ group.
Surprisingly, a strong within animal relationship was found that links a relatively low redox status to an improved NRF2 activation in the monoHER *Ldlr*$^{-/-}$ group. This correlation was absent in the placebo and wildtype group. Apparently, monoHER administration results in a relatively potent adaptation. Based on the concept developed in this thesis, this can be explained by the thiol reactivity of oxidized monoHER, formed after scavenging. Oxidized monoHER adducts the thiol-containing protein, KEAP1 and consequently stimulates the NRF2 system. In wildtype mice monoHER did not induce the adaptive response because monoHER itself lacks the thiol reactivity.
From chapter 5 it was concluded that monoHER enforces the endogenous antioxidant shield, to provide protection against NAFLD.

In **chapter 6** the contribution of the major metabolite of monoHER, 4'-O-methylmonoHER (methylmonoHER) to the protective antioxidant effect of monoHER was investigated.
MethylmonoHER was synthesized and the antioxidant activity was evaluated and compared to that of monoHER. In the TEAC, hydroxyl and superoxide radical scavenging activity assays, methylmonoHER was far less potent than monoHER. The thiol reactivity of oxidized methylmonoHER was also less compared to that of monoHER; methylmonoHER was less reactive towards the thiol-containing proteins CK and KEAP1. These findings suggest that methylmonoHER hardly protects against radical damage via scavenging or via activating the NRF2 defense system. This was confirmed in HUVECs; monoHER which was a very potent protector ($EC_{50}$ = 80 nM) whereas methylmonoHER provided minor protection against oxidative stress ($EC_{50}$ > 100 µM).
The results indicate that the involvement of methylmonoHER in the protection against doxorubicin-induced cardiotoxicity by monoHER is relatively low.

The pharmacological effects of monoHER and the potential use as a nutraceutical were reviewed in **chapter 7**.

It was concluded that monoHER is a nutraceutical which has already been applied and of which the use can be extended in the treatment of diseases in which oxidative stress plays a crucial role. MonoHER directly protects against oxidative stress. In the direct protection against oxidative stress, monoHER is converted into a thiol reactive quinone. Interestingly, the thiol reactive quinone can adduct KEAP1 ultimately leading to an adaptive response. In this way the antioxidant monoHER selectively protects cells that are subjected to oxidative stress, which can be seen as an elegant way of targeting the protective effect of this nutraceutical.

**Implications and future perspectives**

The research described in this thesis further unraveled the molecular mechanism of the protective effect of flavonoids i.e. monoHER, quercetin and their 4'-O-methyl metabolites. It was substantiated that flavonoids are able to protect against oxidative stress and also to promote an adaptive response.
Subjects that are still open for research are:

- the *in vivo* adaptive effect of monoHER
- the molecular mechanism behind the anti-inflammatory effect of monoHER
- the protection against doxorubicin-induced cardiotoxicity by metabolites other than 4'-O-methylmonoHER
- the retention of monoHER and its metabolites in the nucleus of the endothelial cells
- the thiol reactivity/toxicity and the channeling of the reactivity of other flavonoids
- the protective and adaptive effects of other flavonoids

# Samenvatting en algemene discussie

De inname van flavonoïden is geassocieerd met gezondheidsbevorderende effecten. De antioxidant activiteit speelt een grote rol in de beschermende werking van flavonoïden (**hoofdstuk 1**). Het doel van dit proefschrift is het verder ontrafelen van het moleculair mechanisme achter het antioxidant effect van flavonoïden. De antioxidant flavonoïden 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER), quercetine en hun 4'-O-methyl metabolieten werden bestudeerd.

Het flavonoïd monoHER geeft effectieve bescherming tegen doxorubicine geïnduceerde cardiotoxiciteit. De potentiële relevantie van het directe effect van het wegvangen van radicalen door monoHER werd bestudeerd in **hoofdstuk 2**.
MonoHER beschermt tegen intracellulaire oxidatieve stress in humane navelstreng veneuze endotheelcellen (HUVECs) ($EC_{50}$ = 60 nM). Dit bevestigt de betrokkenheid van het directe effect van het wegvangen van radicalen bij therapeutisch haalbare concentraties. Dit directe effect van monoHER komt door de perfecte locatie op 3 vlakken. Op moleculaire vlak is monoHER gelokaliseerd op de plaats waar radicalen gevormd worden. Op deze manier kan monoHER locatie-specifiek de radicalen wegvangen. Op biochemisch vlak bevindt monoHER zich op een gunstige plaats in het antioxidant netwerk. Op cellulair vlak situeert monoHER zich in endotheel- en gladde spiercellen van de veneuze wand.
Dit verklaart waarom monoHER een fysiologisch belangrijk direct antioxidant effect vertoont in de vaten.

Als de reactieve zuurstof deeltjes weggevangen worden, nemen zij een elektron of een waterstof atoom op van het antioxidant flavonoïd. Op deze manier worden de reactieve deeltjes geneutraliseerd. Daarentegen wordt het flavonoïd geoxideerd en zelf reactief. De reactiviteit van het geoxideerde flavonoïd is minder dan die van het weggevangen radicaal waardoor het selectiever wordt. Het is bekend dat flavonoïden selectief hun reactiviteit kanaliseren naar thiolgroepen. Daardoor kunnen geoxideerde flavonoïden een gevaar zijn voor fysiologisch belangrijke cellulaire moleculen die een thiolgroep bevatten.

In **hoofdstuk 3** werd de thiolreactiviteit van het antioxidant flavonoïd quercetine vergeleken met die van 4'-O-methylquercetine (tamarixetine) ten opzichte van creatine kinase (CK), een cruciaal eiwit dat een belangrijke thiolgroep bevat. Geoxideerd quercetine en geoxideerd tamarixetine zijn reactief ten opzichte van CK; beide geoxideerde flavonoïden vormen een adduct met het enzym. Dit leidt tot het verlies in functie van het enzym. Tamarixetine was minder thioltoxisch dan quercetine omdat het geoxideerde tamarixetine liever zijn reactiviteit doorgeeft aan het antioxidant netwerk, i.e. vitamine C, dan een adduct te vormen met CK. Klaarblijkelijk heeft een kleine structurele modificatie, zoals het introduceren van een methylgroep, een groot effect op het kanaliseren van de reactiviteit.

Als geoxideerde flavonoïden hun reactiviteit kanaliseren naar het thiolhoudende eiwit KEAP1, leidt dit tot adaptatie (**hoofdstuk 4**). In **hoofdstuk 4** wordt het effect bestudeerd van de antioxidant monoHER op de cellulaire adaptieve respons in HUVECs blootgesteld aan $H_2O_2$.
Er werd aangetoond dat monoHER een adduct vormt met KEAP1, dat vervolgens het NRF2 antioxidant beschermingssysteem activeert. NRF2 wordt vrijgemaakt uit het KEAP1-NRF2 complex, transloceert naar de kern en induceert de expressie van antioxidant genen, b.v. *HO-1*. Op deze manier biedt monoHER directe bescherming en induceert het adaptatie tegen oxidatieve stress.
Thiolreactiviteit kan aan de ene kant leiden tot toxische effecten door het beschadigen van cellulaire componenten, zoals beschreven in hoofdstuk 3, en aan de andere kant tot een voordelige adaptieve respons. Zoals aangetoond in hoofdstuk 4, beschermt monoHER cellen blootgesteld aan oxidatieve stress tegen schade en reduceert het de adaptieve respons tegen oxidatieve stress niet terwijl er geen toxiciteit veroorzaakt wordt. Het beschermende en adaptieve effect via thiol adductvorming van monoHER treedt blijkbaar eerder op dan de mogelijke thioltoxiciteit.

In **hoofdstuk 5** werd het effect van monoHER bestudeerd op de oxidatieve stress en de adaptieve respons in het beginstadium van NAFLD in *Ldlr*$^{-/-}$ muizen op een hoog vet en hoog cholesterol dieet.
In de *Ldlr*$^{-/-}$ groep was er geen uitgesproken effect van monoHER op de schade door reactieve zuurstof deeltjes. Dit kan verklaard worden door de relatief grote variatie in de resultaten die waarschijnlijk veroorzaakt wordt doordat het beginstadium van de ziekte bestudeerd wordt. In de placebo *Ldlr*$^{-/-}$ groep werd het NRF2 antioxidant beschermingssysteem geactiveerd, aangetoond door een hogere *HO-1* en *Gpx3* genexpressie en een verhoogde redox status, aangetoond door een hogere GSH/GSSG ratio. MonoHER neigde de *HO-1* en *Gpx3* genexpressie en de GSH/GSSG ratio te verhogen in de *Ldlr*$^{-/-}$ groep.
Opmerkelijk was dat er een sterke relatie werd gevonden tussen een lage redox status en een verhoogde NRF2 activatie in de monoHER *Ldlr*$^{-/-}$ groep. Deze correlatie was afwezig in de placebo en wildtype groep. Blijkbaar resulteert de toediening van monoHER tot een relatieve effectieve adaptatie. Gebaseerd op het concept ontwikkeld in dit proefschrift, kan dit verklaard worden door de thiolreactiviteit van het geoxideerd monoHER, gevormd na het wegvangen van radicalen. Geoxideerd monoHER vormt een adduct met het thiolhoudende eiwit, KEAP1 en vervolgens wordt het NRF2 systeem geactiveerd. In de wildtype muis induceerde monoHER de adaptieve respons niet omdat monoHER zelf die thiolreactiviteit niet bezit.
Uit **hoofdstuk 5** kan geconcludeerd worden dat monoHER het endogene antioxidantsysteem versterkt en op deze manier bescherming biedt tegen NAFLD.

In **hoofdstuk 6** werd de bijdrage van de belangrijkste metaboliet van monoHER, 4'-O-methylmonoHER (methylmonoHER) aan het beschermende antioxidant effect van monoHER bestudeerd.
MethylmonoHER werd gesynthetiseerd en de antioxidant activiteit geëvalueerd en vergeleken met die van monoHER. De TEAC-waarde van methylmonoHER was lager dan die van monoHER en ook in het wegvangen van hydroxyl en superoxide radicalen was methylmonoHER veel minder effectief dan monoHER. De

thiolreactiviteit van geoxideerd methylmonoHER was minder dan die van monoHER; methylmonoHER was minder reactief ten opzichte van de thiolhoudende eiwitten CK en KEAP1. Deze bevindingen suggereren dat methylmonoHER amper bescherming biedt tegen radicaalschade via het wegvangen en het activeren van het NRF2 beschermingssysteem. Dit werd bevestigd in HUVECs; monoHER biedt zeer effectieve bescherming ($EC_{50}$ = 80 nM) tegen oxidatieve stress maar methylmonoHER gaf zeer weinig bescherming ($EC_{50}$ > 100 µM).
De resultaten wijzen erop dat de bijdrage van methylmonoHER in de bescherming tegen de doxorubicine geïnduceerde cardiotoxiciteit door monoHER gering is.

De farmacologische effecten van monoHER en het potentiële gebruik als nutraceutical werd beschreven in **hoofdstuk 7**.
Er werd geconcludeerd dat de nutraceutical monoHER reeds lange tijd wordt toegepast bij een groot scala aan ziektes waarin oxidatieve stress een belangrijke rol speelt. MonoHER biedt directe bescherming tegen oxidatieve stress. In deze directe bescherming ontstaat er een thiolreactief product. Dit thiolreactief product vormt een adduct met KEAP1 dat uiteindelijk leidt tot een adaptieve respons. Op deze manier beschermt de antioxidant monoHER selectief cellen die blootgesteld zijn aan oxidatieve stress. Dit kan gezien worden als een elegante manier van targeting van het beschermende effect van de nutraceutical.

**Implicaties en verder onderzoek**

Het onderzoek beschreven in dit proefschrift ontrafelt het moleculaire mechanisme achter het beschermende effect van flavonoïden i.e. monoHER, quercetin en hun 4'-O-methyl metabolieten. Er werd geconcludeerd dat flavonoïden bescherming kunnen bieden tegen oxidatieve stress en dat ze de adaptieve respons kunnen stimuleren.

Interessante onderwerpen die nog verder onderzocht kunnen worden:

- de adaptieve response van monoHER *in vivo* in de mens
- het moleculaire mechanisme achter het anti-inflammatoire effect van monoHER
- de bescherming tegen de doxorubicine geïnduceerde cardiotoxiciteit door andere metabolieten dan 4'-O-methylmonoHER
- de retentie van monoHER en zijn metabolieten in de kern van endotheelcellen
- de thiolreactiviteit/thioltoxiciteit en het kanaliseren van de reactiviteit van andere flavonoïden
- het beschermende en adaptieve effect van andere flavonoïden

# Valorisatie

Radicalen behoren tot de meest reactieve verbindingen die in de cel worden geproduceerd. Ze kunnen reageren met alle moleculen in het lichaam zoals vetten, eiwitten en het DNA. Om zich te beschermen tegen deze schadelijke stoffen, heeft het lichaam een beschermingssysteem. Dit beschermingssysteem bestaat uit antioxidanten die de radicalen kunnen neutraliseren. Een teveel aan radicalen leidt tot schade die bepaalde ziekten, zoals leverziekten en neurodegeneratieve ziekten kan veroorzaken.

De voeding vormt een rijke bron aan antioxidanten. Een belangrijke groep van antioxidanten in de voeding zijn de flavonoïden. Een flavonoïd rijk dieet beschermt tegen o.a. hart- en vaatziekten, chronische inflammatoire ziekten en kanker. Daarnaast kunnen flavonoïden, vooral in hoge dosering, ook schadelijk zijn.
De precieze werking van deze flavonoïden is niet helemaal duidelijk. In dit proefschrift wordt deze werking verder onderzocht van de flavonoïden monoHER, quercetine en hun gemethyleerde metabolieten. Quercetine is een natuurlijk voorkomend flavonoïd dat wijdverspreid in de voeding voorkomt in o.a. uien en appels. Quercetine kan ook verkregen worden als supplement. MonoHER is een semi-synthetisch flavonoïd afkomstig van het natuurlijke flavonoïd rutine dat chemisch is aangepast om de stabiliteit en bioactiviteit te verhogen.
Flavonoïden staan aan de top van de pikorde van antioxidanten. Zij vangen de radicalen als eerste in. Bij het wegvangen van de radicalen, geeft het flavonoïd een elektron of een waterstof-atoom aan het radicaal waardoor het radicaal geneutraliseerd wordt. Het flavonoïd wordt zelf geoxideerd en daardoor reactief. Deze reactiviteit is minder dan die van het radicaal maar selectief voor thiolgroepen van bv. eiwitten. Deze thiolgroepen zijn essentieel in de werking van eiwitten. Wanneer geoxideerde flavonoïden reageren met de thiolgroepen van eiwitten, zullen de functies van de eiwitten veranderen. Dit kan uiteindelijke leiden tot toxiciteit.
Quercetine laat deze thiolreactiviteit duidelijk zien. Geoxideerd quercetine remt de activiteit van creatine kinase, een enzym belangrijk in de energielevering. Geoxideerd monoHER is ook thiolreactief maar in mindere mate. MonoHER

verkiest de reactiviteit door te geven aan vitamine C dan aan thiolen. Vitamine C is in staat om het monoHER te regenereren.
De thiolreactiviteit van geoxideerd monoHER kan, naast schade, ook leiden tot adaptatie. Het geoxideerde monoHER activeert het antioxidant beschermingssysteem van het lichaam.
MonoHER beschermt dus tegen de schade veroorzaakt door radicalen en zal er voor zorgen dat het lichaam zich aanpast aan de situatie waarin het zich bevindt. Dit kan gezien worden als een elegante manier van gerichte bescherming (‘targeting’).
Het doorgeven van de reactiviteit speelt een belangrijke rol in de werking van flavonoïden. Bij het toedienen van een flavonoïd dient er gekeken te worden in welke richting de reactiviteit wordt doorgegeven. Bij quercetine lijkt dit meer richting toxiciteit te gaan, bij monoHER wordt duidelijk ook adaptatie gezien. Om gezondheidsbevorderende effecten van een flavonoïd te verkrijgen moet er een optimale balans gevonden worden tussen toxiciteit en adaptatie.

**Planning**

In de toekomst moet de antioxidantwerking van andere flavonoïden verder in kaart worden gebracht want elk flavonoïd is anders. Het doorgeven van de reactiviteit en de balans tussen toxiciteit en adaptatie zal hierbij betrokken moeten worden. Als de werking van flavonoïden gekend is, kunnen ze op een optimale manier ingezet worden in behandelingen van ziekten want elke ziekte zal om een ander flavonoïd vragen. Ons onderzoek maakt het mogelijk om het juiste flavonoïd te kiezen voor diverse aandoeningen. Met deze kennis kan de voeding in diverse klinische situaties aangepast worden of kunnen er juist ‘getargete’ geneesmiddelen op basis hiervan ontwikkeld worden.

# Dankwoord

Ik zou iedereen willen bedanken die geholpen heeft om dit boekje tot stand te brengen. Zonder jullie had ik het nooit gekund!

Eerst en vooral een woord van dank aan mijn promotieteam, waaronder mijn promoter Prof. dr. Aalt Bast en copromotores Dr. Guido R.M.M. Haenen en Prof. dr. Wim J.F. van der Vijgh.

**Aalt**, dankjewel voor de goede en enthousiaste begeleiding! De wekelijkse besprekingen waren zeer nuttig en inspirerend. Je stelde me altijd de vraag: 'Wat willen we eigenlijk weten?' waardoor ik door de bomen het bos weer zag. Bedankt dat je nooit je vertrouwen in mij bent verloren!

**Guido**, bij jou is er nooit een drempel. Je staat altijd open voor vragen. Telkens wist je me weer te boeien. Je bent een echte ideeëngenerator. Bij het schrijven van dit boekje heb je me veel geholpen. Ik heb veel van je geleerd. *Je bent een 'reus' en ik heb veel kunnen zien op je schouder.*

Ik ben jullie zeer dankbaar dat ik als postdoc kan beginnen.

**Wim**, ik bewonder je onuitputtelijke interesse voor het onderzoek en de wetenschap. Onze 'meander'-besprekingen samen met Aalt en Guido waren telkens zeer nuttig! Je zorgde ervoor dat we een goede planning opstelden. Mijn manuscripten bekeek je steeds heel gedetailleerd. Je kritische vragen hebben me vaak aan het denken gezet. Ook al zaten we niet vaak samen, ik heb erg veel van je opgestoken. Ik vind het leuk dat we tijdens mijn postdoc-project nog samen kunnen werken.

Hartelijk dank aan de **beoordelingscommissie** voor het beoordelen van dit proefschrift.

Vervolgens wil ik mijn super zus en grote voorbeeld bedanken. **Sofie**, door je eigen ervaring raadde je me aan om een baan te zoeken op de uni. Daardoor ben ik als laborant kunnen beginnen en verder opzoek gegaan naar een interessant promotieonderzoek. Ik heb veel bewondering voor alles wat je doet, en vooral hoe je het doet: precies, nauwkeurig, bedachtzaam,...

Ik heb deze AIO-positie in grote mate te danken aan jou, **Daniëlle**. Je duwde me letterlijk naar binnen bij Aalt. Bedankt voor je uitleg over celkweek, experimenten, het beantwoorden van mijn vragen maar ook de leuke uitstapjes buiten het werk.

My paranymphs **Agnieszka** and **Carmen**! Thanks for the nice time at the department in our room. I could always talk to you about anything! Thanks for the support during my defense. Good luck with your own defenses!

**Bregje**, jou wil ik vooral bedanken voor de leuke samenwerking tijdens de muizenstudie. Er is een interessante publicatie uit ontstaan, hoofdstuk 5. Je was een toffe collega!

**Leonie** en **Marie-Jose**, ook aan jullie bedankt voor de hulp bij de muizenstudie en resultaten in hoofdstuk 6.

Dankjewel **Misha** voor je leuke resultaten waaruit een mooi hoofdstuk (3) is gekomen. Samen met **Roger B.** en **Leonie** hebben jullie gezorgd voor super uitstapjes, fijne babbels en leuke sfeer. Bedankt!

My other roommates **Erik** (dankjewel voor je hulp bij de experimenten, computerproblemen, het beantwoorden van al mijn vragen,…), **Matt** (thanks for the entertainment, especially the singing and gossips ;) ), **Max** (bedankt voor de de hulp bij de synthese van methylmonoHER) and **Nuria** (thanks for the nice chats). Dankjewel **Hilde** om mij het monoHER-project over te dragen.

**Rianne**, **Alie**, **Quan** and **Gesiele** thank you for the nice time in the lab and beyond. Good luck with your defenses!

Bedankt **Roger B.**, **Esther**, **Joy**, **Lou**, **Daniëlle P.** en **Marie-Jose** voor de hulp bij de praktische zaken op het lab en de gezellige sfeer.

Bedankt **Marie-Claire** voor het regelen van afspraken en de administratieve zaken maar ook de leuke praatjes.

In die 4 jaar heb ik de kans gekregen om studenten te begeleiden. **Nathalie**, **Arjan**, **Kelsey**, **Mireille**, **Katerina**, **Frederik**, **Pauline**, **Bas**, bedankt voor jullie hulp. Hopelijk hebben jullie ook iets van mij kunnen leren.

Bedankt **Mitrajit**, **Marc**, **Jeroen** voor de hulp bij de fluorescentiemetingen in de vaten en **Freek** voor de hulp bij de MALDI-TOF metingen.

Ik mocht paranimf zijn bij de promoties van **Sofie**, **Max**, **Matt**, **Danielle**, **Erik** dankjulliewel! Het was een hele eer! Thank you! I was honoured!

Dankjewel aan **Antje**, **Gertjan**, **Merel**, **Jiska**, **Roger G**, **Agnes**, **Geja**, **Thalita**, **Kimberly**, **Marieke**, **Frederik-Jan**, **Aggie**, **Edwin**, **Jan**, **Pieter** en de **collega's van farmacologie** voor de tips, fijne samenwerking en leuke sfeer.

De (ex)collega's van toxicogenomics: **Lize**, **Sandra**, **Karen**, **Leen**, **Wim** en **Dennie** bedankt voor de gezelligheid.

Dankjewel **Leo**, **Matty**, **Simone** en **Stijn** dat ik als laborant ben kunnen starten op de uni.

**Chris**, dankjewel voor het ontwerpen van de mooie cover en uitnodiging.

Dank aan **Maria**, **Simon**, **Caroline**, **Pieter**, **Jonathan** en **Robin** voor jullie ondersteuning, goede zorgen en de ontspannende momenten.

Mijn **Mama** en **Papa**, **Sofie** en **Giel**, ik kan de dankbaarheid amper uitdrukken in woorden. Jullie staan altijd klaar voor mij. Jullie toonden steeds veel interesse in mijn werk en hebben me steeds aangemoedigd. Dankjewel voor alle vertrouwen.

Mijn liefje, **Martijn**. Je hebt me door dik en dun gesteund in deze hele periode. Je stelde me altijd gerust: 'Het komt allemaal goed!'. Ik bewonder je geduld en relativeringsvermogen. Ik kijk uit naar meer tijd met jou samen in ons huisje. (Bedankt voor je hulp bij figuur 3 in hoofdstuk 2 ;) )

Bedankt iedereen voor de ondersteuning en hulp maar ook voor de super promotieperiode!

# Curriculum vitae

Kristien Lemmens was born on February the 14th 1986 in Oupeye, Belgium. After finishing secondary school at Provinciale School Voeren, she started in 2004 with her master study; industrial sciences and technology at the Katholieke Hogeschool Limburg, Belgium. She did her internship entitled 'Preparation and optimization of new interfaces for localized surface plasmon resonance (LSPR) and surface-enhanced raman scattering (SERS) studies' at 'Institut d'Electronique de Microélectronique et de Nanotechnologie in Lille, France. She graduated in June 2008 having specialized in biochemistry. After one year of work experience in the industry, she accepted the job of technician at the Department of Epidemiology at Maastricht University. In February 2011 she started as a PhD student at the Department of Toxicology at Maastricht University. The research performed during this project, under supervision of Dr. G. Haenen, Prof. Dr. van der Vijgh and Prof. Dr. A. Bast, is described in this thesis.

# List of publications

**Full papers**

**Lemmens, K.J**., van de Wier, B., Vaes, N., Ghosh, M., van Zandvoort, M.A., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014. The flavonoid 7-mono-O-(beta-hydroxyethyl)-rutoside is able to protect endothelial cells by a direct antioxidant effect. Toxicology in Vitro 28 , 538-543.

**Lemmens, K.J.**, Vrolijk, M.F., Bouwman, F.G., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014. The minor structural difference between the antioxidants quercetin and 4'-O-methylquercetin has a major impact on their selective thiol toxicity. International Journal of Molecular Sciences 15, 7475-7484.

**Lemmens, K.J.**, Sthijns, M.M., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014. The antioxidant flavonoid monoHER provides efficient protection and induces the innate NRF2 mediated adaptation in endothelial cells subjected to oxidative stress. PharmaNutrition 2 , 69-74

**Lemmens, K.J.***, van de Wier, B.*, Koek, G.H., Köhler, E., Drittij, M., van der Vijgh, W.J., Bast, A., Haenen, G.R., 2014. The flavonoid monoHER promotes the adaptation to oxidative stress during the onset of NAFLD. Biochemical and Biophysical Research Communications 456, 179-182

**Lemmens, K.J.**, Herst, P. M., Housmans, B.A., Moalin, M., van der Vijgh, W.J., Bast, A. and Haenen, G.R.. The contribution of the major metabolite 4'-O-methylmonoHER to the antioxidant activity of the flavonoid monoHER. (Submitted)

**Lemmens, K.J.***, Jacobs, H.*, van der Vijgh, W.J., Bast, A. and Haenen, G.R.. The health effects of the nutraceutical 7-mono-O-(β-hydroxyethyl)-rutoside. Bridging the gap between nutrition and medicine. (Submitted)

Sabbe, L.*, Heyninck, K.*, Szarc vel Szic, K., Vander Veken , P., **Lemmens, K.J.**, Lahtela-Kakkonen, M., Naulaerts, S., Op de Beeck, K., Laukens, K., Van Camp, G., Weseler, A.R., Bast, A., Haenen, G.R., Haegeman, G., Vanden Berghe, W.. Withaferin A induces heme oxygenase (HO-1) expression in endothelial cells via activation of the KEAP1/NRF2 pathway. (Submitted)

* equal contribution

**Abstracts**

**Lemmens, K.J.**, Vaes, N., van der Vijgh, W.J., Bast, A., Haenen, G.R.. The antioxidant flavonoid monoHER directly protects against oxidative stress in a cultured endothelial cell line at physiologically achievable concentrations.
Society for Free Radical Research International meeting, September 2012, London, UK
NUTRIM day, December 2012, Maastricht, The Netherlands

**Lemmens, K.J.**, van de Wier, B., Vaes, N., Ghosh, M., van Zandvoort, M.A., van der Vijgh, W.J., Bast, A., Haenen, G.R.. The direct antioxidant effect of the flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside.
Meeting of the Netherlands Society of Toxicology June 2013, Zeist, The Netherlands

**Lemmens, K.J.**, Vrolijk, M.F., Bouwman, F.G., van der Vijgh, W.J., Bast, A., Haenen, G.R.. Difference in thiol reactivity of oxidized quercetin and 4'-O-methylquercetin, tamarixetin towards a thiol containing protein, creatine kinase.
NUTRIM day, December 2013, Maastricht, The Netherlands

**Lemmens, K.J.**, Sthijns, M.M., van der Vijgh, W.J., Bast, A., Haenen, G.R.. The antioxidant flavonoid monoHER provides efficient protection and induces the innate NRF2 mediated adaptation in endothelial cells subjected to oxidative stress.
NUTRIM day, December 2014, Maastricht, The Netherlands