An approach to the rational design of new inhibitors for trypanosoma brucei
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Introduction
**Introduction**

African trypanosomiasis is endemic in an area, known as the “tsetse belt,” extending over one third of the African continent and spanning 36 sub-Saharan countries (figure 1.1). The disease may arise, as a multifocal event throughout equatorial Africa. There are two forms of human African trypanosomiasis or sleeping sickness: gambiense trypanosomiasis, a chronic disease found in Central and West Africa, and rhodesiense trypanosomiasis, a more acute disease found in East and southern Africa.  

Fifty million people, living in or around the 200 disease foci within the tsetse belt, particularly those engaged in hunting, fishing and farming, are at risk of infection. Only five million people are under surveillance. The mean prevalence rates are about 1–2%. Currently, about 20,000 new cases are reported each year.  

African trypanosomiasis is caused by subspecies of the genus, *trypanosomatidae*, consisting of uniflagellated parasitic protozoa. *Trypanosoma brucei brucei* was implicated as the cause of “Nagana” (animal trypanosomiasis) in 1894, and trypanosomes were first demonstrated in human “sleeping sickness” in 1902, although the disease was first described in 1803. The subspecies *Trypanosoma brucei rhodesiense* causes the acute form of African sleeping sickness in East and southern Africa.

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**Figure 1.1**  
Human African trypanosomiasis is transmitted by tsetse flies. The darker colored area is the so-called tsetse belt.
Africa, whereas the less virulent form of trypanosomiasis in Central and West Africa, is caused by *Trypanosoma brucei gambiense*. The parasites are transmitted to humans by the bite of the tsetse fly or *Glossina*. Part of the life cycle of the parasites takes place in the tsetse flies. The existence of a developmental cycle for *T. brucei* in *Glossina* was recognised in 1909. In the mammalian bloodstream, the *T. brucei* population is pleomorphic, consisting of long, slender, dividing forms, intermediate forms, and short stumpy forms (figure 1.2). The slender forms rely on aerobic glycolysis, excreting pyruvate as the sole end-product, for energy generation. Metabolic changes in the stumpy forms probably facilitate their developmental adaptation after ingestion by the fly, whereupon they differentiate and multiply in the tsetse midgut as non-infective coatless “procyclic” trypanomastigote forms. Procyclic forms migrate to the salivary glands where, attached to the epithelial cells, they multiply as a distinct morphological stage, the epimastigote. After cessation of division, the epimastigote starts to express a dense variable surface glycoprotein coat (VSG coat) and this expression leads to detachment and maturation as non-dividing, infective metacyclic trypanomastigotes. The VSG coat accounts for about 10% of the total protein of the bloodstream forms of *T. brucei*. The variability lies in the fact that the parasite can shed its coat and build a new one by expressing another surface glycoprotein. The sequential expression of VSGs results in an ever changing antigenic pattern. Antigenic variation, in the manner and to the extent that it occurs in the African trypanosomes, is unique to the parasites and appears to be the primary mechanism for evasion of the hosts’ immune responses. These conditions arouse deep pessimism about prospects for a trypanosomiasis vaccine.

**Vector Control**

Apart from drug treatment of infected patients the disease is generally fought via its vector, the tsetse fly. The flies are controlled with traps and with aerial insecticide spraying. This last measure is quite costly, of course, and therefore a renewed interest in trapping to control tsetse fly populations is emerging. In Congo, a conical insecticide-imregnated trap has been developed (figure 1.3). Tsetse flies are attracted by the shape, color and movements of the trap hanging from a tree. No tsetse flies were observed over a six month period following its use in some villages in Couloir and Niari disease foci. In Ivory Coast, over 15,000 screens impregnated with the insecticide deltamethrin were placed by farmers in strategic sites in an 83 km² area. This reduced the tsetse population by 98% within one month at a cost of US$ 5.50 per hectare. The possibility to use odours to attract tsetse flies to a trap and thereby increasing its efficiency has been explored in several studies. Octenol, ace-
tone and buffalo urine have been shown to be effective in attracting some tsetse species. Pyramidal traps (Lancien-Gouteux traps), which attract tsetse flies with blue-coloured cloth, do not require the use of chemical attractants or insecticides.\(^6\)

**Drug Treatment**

Today, chemotherapy and chemoprophylaxis of African trypanosomiasis rely essentially on four drugs: *pentamidine* (figure 1.4), *suramin* (figure 1.5), *melarsoprol* (figure 1.6A) and *DL-α-difluoromethylornithine* (figure 1.7). *Pentamidine* is used mainly for prophylaxis. The amidino groups in the drugs are the reason for their high polarity and hence their incapability to pass the blood brain barrier. Therefore, it is impossible to use these drugs against sleeping sickness in a late stage of the disease when the trypanosomes have entered the brain. *Suramin* is used for early disease and *melarsoprol* is used for late stage disease when the central nervous system is infected. *DL-α-difluoromethylornithine* is a new drug aimed at the inhibition of the polyamine synthesis.

A great number of diamidines are now known to be trypanocidal.\(^{11}\) All the diamidines are effective as prophylactic agents, however, only diminazene (berenil\(^®\)) is able to effect a cure. *Pentamidine* (see figure 1.4) was discovered as trypanocidal drug in the late 1940’s.\(^1\) The drug is rapidly absorbed by trypanosomes and at once exerts a trypanostatic effect on them, both *in vitro* and *in vivo*. The selective attenuation of the carbohydrate metabolism by inhibiting α-glycero-phosphate dehydrogenase, apparently by opposing the coenzymic effect of spermidine, plays an important role in its mode of action.\(^{16}\) The parasites are finally killed by the host’s defensive forces within some days. One indirect factor that helps the cure is the following: those parasites that survive the direct effect of the drug remain infective, but they have a large number of drug-containing granules. When some parasites die, they liberate the drug, and also an antigen which causes the host to make ade-

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**Figure 1.4**

Structural formulas of some diamidines currently under investigation as drug for the treatment of human trypanosomiasis.

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quate amounts of antibodies against the remaining trypanosomes. This indirect process is believed to explain the slow onset of the action of the diamidine drugs, and their prolonged prophylactic effect.\textsuperscript{15}

The diamidines have also severe side effects like hepatoxicity\textsuperscript{11} and induction of hypotension.\textsuperscript{12} Vasodilatation mediated by histamine release seems the most probable cause, since some diamidines, like e.g. \textit{pentamidine}, are known to be potent histamine releasers.\textsuperscript{12,14} Several other diamidino compounds have antagonistic activity at $\alpha_1$- and $\alpha_2$-adrenoceptors \textit{in vitro}. The concentrations at which these compounds antagonise these receptors is comparable with the concentrations found in plasma of \textit{pentamidine} in clinical studies. Therefore, blockade of postsynaptic $\alpha$-adrenoceptors of vascular smooth muscle might be involved in their hypotensive effects.

Apart from severe side effects, resistance to most of these drugs has been described. Multiple drug resistance of \textit{T.vivax} is prevalent along the coast in Kenya, and exists in Somalia.\textsuperscript{13} 1,3,5-Triacetylbenzene tris(guanylhydrazone) (figure 1.4) is a new experimental drug based on the structure of diminazene. Kaminsky and Zweygarth\textsuperscript{27} studied this drug in a new \textit{in vitro} assay and found an EC$_{50}$ value for \textit{T.b. brucei} between 0.3 to 3.5 $\mu$g/ml, whereas diminazene had an EC$_{50}$ value between 0.046 and 5.263 $\mu$g/ml. The variation is caused by different isolates of trypanosomes.

**Suramin**

Paul Ehrlich (1854-1915) was the first to look for trypanocidal drugs. In 1904 he cured trypanosome-infected mice with trypan red (figure 1.5), which thereby became the first man-made chemotherapeutic agent. \textit{Suramin} (figure 1.5) was discovered in 1920 as drug against trypanosomiasis.\textsuperscript{15} It is a highly effective analogue of trypan red. The drug was later used for prophylaxis after it was found that a single dose gave immunity for three months.

The structure of the drug is remarkably specific in that the removal of the two methyl groups abolishes activity completely. Nevertheless, quite un-
related polyanions, such as dextran sulphate, have also strong trypanocidal activity.

It has long been known that suramin abolishes the infectivity of trypanosomes without affecting their mobility. The electron microscope shows that the drug strikes hard and specifically at the parasite’s ribosomes, which lose their polysomal character and form the so-called “cytoplasmic granules”. Fundamentally the attack seems to be on RNA-polymerase; the nucleus, nucleolus and the kinetoplast remain unaffected and the synthesis of DNA continues.\textsuperscript{16} Suramin is not suitable for large-scale field use because it has to be administered intravenously and it is sometimes associated with severe side effects. Like pentamidine it does not pass the blood brain barrier due to its polar character caused by the sulphonic acid groups that are ionised under physiological conditions. Suramin is therefore not suitable for treatment of late stage trypanosomiasis.

The first arsenical drug used against human trypanosomiasis was atoxyl (figure 1.6A). This discovery influenced Ehrlich to commence prolonged experimentation with aromatic arsenicals.\textsuperscript{15} In 1909 he discovered that trivalent arsenicals were trypanocidal in the test-tube and Ehrlich suggested that pentavalent arsenicals (like atoxyl) would also be found active if they were reduced to the trivalent drug first. He showed that pentavalent arsenicals were active \textit{in vitro} when they were first incubated with reducing tissue. This result explains their activity \textit{in vivo}. In 1920 a new arsenical drug was discovered: tryparsamide (figure 1.6A), a pentavalent arsenical drug derived from atoxyl but less toxic. Tryparsamide was effective against the \textit{T.b. gambiense} as it cured the cerebral form of the disease. Tryparsamide, however, is not effective against \textit{T.b. rhodesiense}.\textsuperscript{16} Many related compounds were subsequently synthesised, but only a few have found their way into therapeutic use.

\textbf{Melarsoprol}
A new class of arsenicals, melaminyl-substituted phenylarsonates, of which melarsen oxide was the first, was introduced by Friedheim.\textsuperscript{17,18} Melarsoprol (figure 1.6A) was introduced in the 1940s.\textsuperscript{20} It is used against the effectors of human sleeping sickness and the animal trypanosomiasis; \textit{T.b. brucei}, \textit{T.b evansi} and \textit{T.b. equiperidum}. Melarsoprol is still the drug of choice in late stage trypanosomiasis. It is, however, associated with severe side effects, which are occasionally fatal. Melarsoprol-resistant trypanosomes have also been reported.

The arsenicals were the first drugs to be recognized as acting through the formation of covalent bonds. Ehrlich suggested that some arsenicals combine with essential mercapto-groups in the parasite. Arsenicals can bind to the cysteine thiol groups in proteins. Melarsenoxide was demonstrated to form a stable adduct with the trypanosomatid metabolite trypanothione; abbreviated as Mel T (figure 1.6B).\textsuperscript{20} When bloodstream \textit{T.brucei} is incubated with either melarsenoxide or melarsoprol, Mel T is the only arsenical derivative detectable in acid soluble extracts of cells. Trypanothione in this therefore regarded as a primary target for aromatic arsenical derivatives in African trypanosomes. Having formed within the cell, the Mel T complex might act to guide the arsenical drug toward its ultimate cellular target. Alternatively, the complex might itself be directly toxic. The latter effect is illustrated by the ability of Mel T to inhibit Try(S)\textsubscript{2} reductase activity.\textsuperscript{20} A mean intracellular concentration of 17 µM Mel T could be achieved before cell lysis was observed. This is twice the \textit{K_i} for Mel T for \textit{T. brucei} Try(S)\textsubscript{2} reductase and this concentration would have caused considerable inhibition.

Zweygarth and Kaminsky\textsuperscript{19} reported a new arsenical drug, Mel Cy (Cymelarsan) (figure 1.6C). Like melarsoprol, the drug is in fact a pro-drug because the two cysteamine molecules have to be released from Mel Cy before the compound is active. In chemotherapeutic studies it showed varying degrees of susceptibility in six different \textit{T.b.brucei} and \textit{T.b.evansi} stocks. The susceptibility varied from 4-27 nM.

\textbf{DL-\textalpha-Difluoromethylornithine} In all mammalian cells and in many other eucaryotic cells, decarboxylation of ornithine by ornithine decarboxylase (ODC) is an obligatory step in the biosynthesis of polyamines such as putrescine, spermidine, and spermine, which are ubiquitous in living cells and are thought to play important roles in cell division and differentiation.\textsuperscript{21,22} Hence, inhibition of this enzyme with specific enzyme-activated irreversible inhibitors such as \textit{DL-\textalpha-Difluoromethylornithine} (DFMO) appeared to be an approach to control abnormalities of these cellular activities (figure 1.7).

The discovery that polyamines in \textit{T.b. brucei} are also synthesised from ornithine,\textsuperscript{23} suggested testing of DFMO against African trypanosomiasis in rodents infected with \textit{T.b. brucei}, \textit{T.b gambiense}, \textit{T.b. rhodesiense} and
**T.b. congolense.** Apart from a diminished concentration of polyamines also inhibition of protein and nucleic acid synthesis and gross morphological changes associated with cytostasis occurred in trypanosomes.\(^{24}\)

Trypanosomes are more sensitive to DFMO than mammalian cells.\(^{25}\) They may be more sensitive to the effects of polyamine depletion because they contain the unique glutathione spermidine conjugate dihydrotrypanothione. This is the cofactor of trypanothione reductase, that plays an essential role in the detoxification of xenobiotics. Since the arsenical drugs appear to attack this trypanothione metabolism there is a pronounced synergistic effect between Mel B and DFMO.\(^{20}\)

It was found that DFMO notably increased host survival and produced apparent cures. As a result of this, clinical trials were begun in Sudan in 1981 with a dramatically successful outcome. In clinical trials against late stage *T.b.gambiense* infections in the Ivory Coast, Sudan and Zaire, *T.b.gambiense* proved to be susceptible to DFMO. In many instances patients had failed one or more courses of melarsoprol before DFMO treatment.\(^{26}\)

Bacchi et al.\(^{26}\) tested 16 strains of *T.b. rhodesiense* for susceptibility to DFMO and seven were partially or totally refractory to this drug. These results indicate that other novel agents or combinations of agents may be needed for chemotherapy of the East African disease.

The increasing number of pathogenic trypanosomes calls for the development of new drugs. Several biochemical pathways of the parasite’s metabolism are currently under investigation to assess their vulnerability to chemotherapeutic attack. Some lead compounds that have emerged from these investigations are now in the early stages of development.

**Carnithine metabolism**: carnithine, an essential growth factor derived from the metabolism of amino acids methionine and lysine, has been found intracellularly in *T. brucei*. The compound bromoacetylcarnithine (BAC) inhibits parasite carnitine metabolism and prolongs the survival of
trypanosome infected mice.\textsuperscript{1} BACs mode of action is still not understood and further studies will be conducted on this compound.

\textit{Microtubules}: Compounds that disrupt the tubulin structures making up the parasite’s chemical cytoskeleton are under study. A number of phenothiazines are active \textit{in vitro} but have not shown efficacy \textit{in vivo}.

\textit{Glycolytic enzymes}: Trypanosomes are extremely vulnerable to inhibition of their glycolytic metabolism. This was first observed by Clarkson and Brohn\textsuperscript{29} when they treated rats that were infected with trypanosomes, simultaneously with salicylhydroxamic acid (SHAM) and glycerol. They observed that the trypanosomes disappeared within 5 minutes from the bloodstream of the rats. However, they reappeared after several days because all the SHAM and glycerol was removed from the host’s bloodstream before the parasites, hidden in the tissues and organs, were reached (see \textit{Intermezzo I}: The action of SHAM).

Hence the glycolytic metabolism seems to be a useful target metabolism for future trypanocidal drugs. All glycolytic enzymes have been isolated and are being assessed for their potential as target enzyme. One of these enzymes, \textit{i.e.} Triosephosphate Isomerase (abbreviated as TIM), is the target enzyme for the study presented in this thesis.
Trypanosomal Glycolysis

The African trypanosomes belonging to the brucei subgroup are characterised by an unusual flexibility of their single large mitochondrion. In the insect stage, the mitochondrion is fully developed and respiratory chain phosphorylation provides the main source of energy. In the bloodstream and tissue fluids of the vertebrate host, however, mitochondrial biogenesis can be completely repressed. The mitochondrion regresses into a pro-mitochondrion that lacks a functional Kreb’s cycle and the mitochondrial oxidative phosphorylation. The parasite depends entirely on glycolysis for energy production. The dependence on an inefficient energy provision is compensated by an extreme high glucose consumption. In trypanosomes a unique form of compartmentalisation is found: most of the enzymes of the glycolytic pathway are contained within an organelle, the glycosome. These organelles in T. brucei bloodstream trypanomastigotes are extremely homogeneous in size (0.3 µm) and they represent 4% of the total cell volume and 8% of the total protein content. It has been estimated that each trypanosome contains between 200 and 300 glycosomes.

The glycolytic enzymes involved in the conversion of glucose and glycerol into phosphoglycerate constitute more than 90% of the organelle’s protein content. The sequestering of the glycolytic enzymes within an organelle may contribute to the high glycolytic flux by locally increasing the substrate concentrations. Indeed, tunnelling of glycolytic metabolites through the glycosomes has been demonstrated in intact T. brucei cells. It has been shown that under conditions of low ionic strength, after disrupting the glycosomal membrane, all glycolytic enzymes, except PGI, stick together and behave as members of a large multienzyme complex.

According to Opperdoes the glycosomal membrane constitutes a barrier to most of the glycolytic intermediates and cofactors. In T. brucei the intracellular concentration of the glycolytic intermediates is not dissimilar to that in cells from other eukaryotes. Since the glycosomes represent only 4% of the cellular volume and contain 20-30% of the glycolytic intermediates, the concentration of the glycolytic intermediates must be at least five times higher than in the cytosol. The concentration of both the active sites and glycolytic metabolites have been estimated to be in the millimolar range in the glycosome. Since each of the enzymes has a $K_m$ for its respective substrate of the same order of magnitude, a high portion of the glycolytic metabolites must be in the protein-bound form. This is a further advantage of compartmentation of the glycolysis. It allows the trypanosome to maintain an extremely high glycolytic flux with a relatively small amount of protein. The glycolytic flux through the T. brucei cell is 0.08 µmol glucose per minute per milligram protein, and for each...
molecule of pyruvate produced one molecule of ATP is synthesized (figure 1.8). 

The glucose metabolism in the bloodstream-form trypanosome differs from glycolysis in eukaryotes in a number of respects: (a) Trypanosomes lack a lactate dehydrogenase and therefore the NADH generated in the glycolysis is reoxidized by molecular oxygen via a dihydroxyacetone phosphate (DHAP): glycerol-3-phosphate (G3P) shuttle combination with a terminal G3P oxidase in the mitochondrion. This oxidase reacts with oxygen without the intervention of pyridine nucleotide coenzymes or cytochromes. Its high activity and specificity for G3P is sufficient to account for the high rate of respiration of bloodstream trypanosomes; (b) pyruvate rather than lactate is the end-product of glycolysis. It is excreted into the host’s bloodstream; (c) under anaerobic conditions, glucose is converted quantitatively into equimolar amounts of pyruvate and glycerol.

Since the production and consumption of ATP and NAD are balanced in the glycosome, this organelle is not involved in the net ATP synthesis. This occurs in the cytosol, where 3-phosphoglyceric acid (3PGA) is converted into pyruvate. Similarly, no net change in NAD takes place in the glycosome during aerobic glycolysis because the NADH produced in the glycosome is reoxidised by glycerol-3-phosphate dehydrogenase which reduces DHAP to G3P which, in its turn, is reoxidised to DHAP in the mitochondrion by the terminal G3P oxidase (figure 1.8). 

Under anaerobic conditions, glucose is metabolised at the same rate as under aerobic conditions, with the formation of equimolar amounts of pyruvate and glycerol. Because of the absence of oxygen, the terminal G3P oxidase in the mitochondrion cannot convert G3P to DHAP. This re-
sults in a drastic increase of the G3P concentration in the glycosome which causes a reversal of the glycerol-kinase reaction. A G3P:ADP transphosphorylase activity of glycerol kinase has indeed been found in *T. brucei* and the relatively high specific activity of glycerol kinase, provides for the synthesis of glycerol and ATP from G3P and ADP. Moreover, glycerol kinase functions indeed under true equilibrium conditions in absence of oxygen, rather than catalyzing the phosphorylation of glycerol to G3P.\(^{33}\) This is facilitated by the ability of the ATP/ADP ratio inside the glycosome to change independently from that in the cytosol. Therefore, under anaerobic conditions as well, no net change in ATP and NAD occurs in the glycosome during glucose metabolism. No ATP is consumed and the dismutation of glucose continues to produce 3-phosphoglycerate (3PGA) which is excreted in the cytosol to be converted to pyruvate with the concomittant production of ATP.

Two phases can be recognised in the glycolytic pathway: an ATP consuming phase and an ATP producing phase. In the ATP consuming phase two molecules of ATP are required for the production of glucose-6-phosphate and of fructose-1,6-bisphosphate.\(^{39,40}\) In figure 1.8 triosephosphate isomerase (TIM) is located roughly in the middle of the glycolytic cascade, at the end of the ATP consuming phase. It lies between the reaction steps catalysed by aldolase and by glyceraldehydephosphate dehydrogenase. Its role in glycolysis is the conversion of dihydroxyacetone phosphate (DHAP) to D-glyceraldehyde-3-phosphate (DGAP).

The conversion of DGAP to pyruvate yields two ATP molecules. Since the production of fructose-1,6-bisphosphate consumes two ATP molecules and because only DGAP is utilised in the glycolytic pathway there would be no net ATP synthesis if not all of the triose units were converted to pyruvate, with concomitant production of ATP. TIM ensures that the glycolysis is an efficient energy producing pathway; it provides for the complete catabolism of the whole hexose unit rather than just half of it.\(^{39}\) Hence, the conversion of DHAP to DGAP by TIM is a key step in glycolysis. TIM seems therefore a suitable target enzyme for future trypanocidal drugs.
Introduction

The study presented in this thesis is part of a larger multidisciplinary project with the common objective of rational development of new drugs against African trypanosomiasis. The glycolytic pathway was chosen as target system.

The strategy was that of a so-called rational drug design cycle (figure 1.9) as outlined by Hol in 1986. After the isolation of the glycolytic enzymes, attempts were made to crystallize them and subsequently the X-ray structures of the enzymes were determined. These three-dimensional structures were the input in the rational drug design cycle. The structures were studied and potential ligands were designed.

Following the design, some potential inhibitors were synthesised and tested for inhibitory activity. If active, the inhibitors were diffused into the crystals of the glycolytic enzymes for which they were designed. In this way a crystal of the complex of the inhibitor with the target enzyme was obtained. Subsequently, the X-ray structure of this complex was solved. The data obtained were evaluated and used in the design of new inhibitors aiming at better inhibitory activity.

Scope of this thesis

The scope of this thesis is the investigation of a rational approach to the design of new inhibitors of TIM. This enzyme had been isolated together with the other glycolytic enzymes present in the glycosome and it was the first enzyme that gave suitable crystals for an X-ray analy-
sis.\textsuperscript{36,37} An X-ray structure of high resolution has been obtained and this structure was studied with computer modelling techniques. The aim of these studies was the rational design of new inhibitors for this enzyme.

A serious disadvantage of TIM as target enzyme is the fact that there is a strong homology in terms of primary and tertiary structure of the active site of TIMs of various species (see chapter 2). This means that an inhibitor designed to bind solely in the active site, is an inhibitor with a comparable affinity for TIMs of all species.

In order to make the inhibitor specific for the trypanosomal enzyme, additional affinity contributions need to be looked for outside the active site in areas specific for the enzyme of this species. The specific amino acid sequence nearest to the active site, the so-called selectivity area, is still 15Å away from the center of the active site. To span this distance is not a trivial task. The objective of this thesis is to design inhibitors that have an anchor in the active site and an extension outside the active site, in order to reach the selectivity area. The general requirements for these new selective inhibitors are being outlined in the next chapter (page 37). The investigation started with a known inhibitor to which some modifications were made. These new, potential inhibitors were synthesized and tested for inhibitory activity. This investigation is described in Chapter 3. In Chapter 4 ensuing studies involving further modifications in order to extend the inhibitors away from the active site of the enzyme are described. The design and synthesis of an entirely new series of potential inhibitors designed \textit{de novo} are described in Chapter 5.

Finally, some variations were made on irreversibly binding inhibitors described in the literature. These inhibitors were subsequently combined with one of the inhibitors described in Chapter 5. This study is described in Chapter 6. In Chapter 7 the conclusions of this study are reported.

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Section I: Introduction

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