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Characterization of toxicologically significant efflux transporters and their interactions with drugs

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Abbreviations and symbols

ABC - ATP-binding cassette transporter
ADME - absorption-distribution-metabolism-excretion
ATP - adenosine triphosphate
BBB - blood-brain-barrier
BCRP - breast cancer resistance protein, (MXR, ABCP, ABCG2)
Caco-2 - colorectal carcinoma cell line
HEK293 - human embryonic kidney cells
HL60 - human promyelocytic leukemia cells
HT - high-throughput
K562 - human myelogenous leukemia cells
MCF7 - human breast adenocarcinoma cells
MDCKII - Madin-Darby canine kidney strain II
MDR - multidrug resistance
MDR1 - multidrug resistance protein 1 (P-gp, ABCB1)
MRP1 - multidrug resistance-associated protein 1 (ABCC1)
P_{app} - apparent permeability co-efficient
Sf9 - *Spodoptera frugiperda* ovarian cells
VT - vesicular transport

1. Introduction and aims

Transporters profoundly affect the homeostasis of physiological substrates, endobiotics and also the pharmacokinetics or ADME properties of drugs, xenobiotics. Therefore, transporters have a profound influence on the plasma and tissue levels of various substrates. Drug-mediated inhibition of transporters, therefore, may lead to increases in both plasma as well as tissue levels of endobiotics and xenobiotics. Victim drugs with narrow therapeutic index are of particular concern, as increases in either plasma or tissue concentrations may lead to toxicity. Drugs as perpetrators can also have detrimental effects if the inhibition of transporters leads to increased level of a co-administered drug (drug-drug interaction) or increased levels of endobiotics (drug-endobiotics interaction).

The ATP-binding cassette (ABC) transporter superfamily and its genes represent one of the largest family of known transmembrane proteins, and its members have been found in almost all living organisms, from bacteria to mammals. In humans, they play an important role in several physiological processes, including the modulation of the permeability of endobiotics or xenobiotics across physiological barriers [1, 2].

Numerous physiological interfaces exist in the human body. The intestinal, liver, kidney and blood-brain-barriers are considered crucial in terms of the ADE (Absorption-Distribution-Excretion) of a drug. Hence, they also define the bioavailability and pharmacokinetics of drugs [3]. It is to be noted that unlike toxicity in non-target cells / tissues, target cell toxicity is not always unwanted (e.g. toxicity in cancer cells) [1, 2]. In this thesis only ABCB1 and ABCG2 are discussed in detail, as they have a remarkable role from a toxicological point of view.

This thesis consists of three parts. In each part, the effects of drug molecules on barriers made up of MDR transporters are described.

The first part is about the interaction of the BBB with **seliciclib.** In this part, the aim was i) to identify the transporter responsible for the low brain exposure of seliciclib, and ii) to characterize seliciclib's interaction with ABCB1.

In the second part, we focused on the interactions of **chlorothiazide** and the impact of this diuretic on the kidney barrier. We investigated i) if the three major apically located intestinal efflux transporters, ABCB1, ABCG2 or ABCC2 can modulate the permeability of

chlorothiazide, ii) the impact of interaction(s) with the above-mentioned efflux transporters, iii) the effect of chlorothiazide on ABCG2-mediated urate transport.

Finally, in the third part, **FTC-Ko analogs** were synthesized, in which the ester linkage was replaced by amide or alkyl chains. We studied the specificity and structure-activity relationships of the molecules. This part was carried out in cooperation with Professor Gábor Tóth's group at the Department of Medical Chemistry, University of Szeged.

2. Materials and Methods

Membrane vesicle preparations expressing the human ABCB1, ABCC2, and ABCG2 transporters were from baculovirus-infected Sf9 cells (MDR1-Sf9, MRP2-Sf9, and BCRP-HAM-Sf9, respectively) and from ABCG2-overexpressing mammalian cells (BCRP-M) and its control (M-Ctrl).

Cells used for the experiments were maintained in supplemented media and were grown under standard conditions (5% CO_2 and 37°C).

The interaction of the test drugs with the actually examined transporter was detected in the membrane-based ATPase and vesicular transport assays.

Dye efflux inhibition assays (CalceinAM and Hoechst 33342) helped in the detection of whether the test drug interact with the tested transporter or not.

The presence of the appropriate protein was verified by western blotting.

The survival of cells in response to incubation with different concentrations of the test drug was characterized in cytotoxicity assays.

Transport of test drugs from the basolateral to apical compartment and *vica versa* through tight grown cell layer mimicking a barrier was tested in monolayer vectorial transport system.

The solid- or solution-phase synthesis of fumitremorgin analogs are detailed in the article written by Szolomajer-Csikós in 2013 [III].

3. Results and discussion - Applications in drug research and development

3.1. ABCB1 / Seliciclib / BBB

Seliciclib is a selective substrate of ABCB1 but no resistance develops

Seliciclib (R-roscovitine) is a cyclin-dependent kinase inhibitor and a chemotherapeutic drug candidate. It is rapidly cleared from plasma and distributed to organs. The brain level of seliciclib was about 20% of that measured in the plasma, while it was 100% in newborn rats with an immature BBB [4]. This difference may stem from the transporter profile differences of an adult and an immature brain. Efflux transporters expressed in the BBB might be at play.

A part of the work covered in this thesis was to determine whether efflux transporters, thought to limit brain exposure to drugs, would play a role in limiting the brain exposure of seliciclib. We tested seliciclib's interaction with four transporters, ABCB1, ABCG2, ABCC1 and ABCC2.

ATPase assays were carried out to test interactions of seliciclib with efflux transporters present in the BBB. Results indicate that seliciclib interacts with ABCB1, ABCC1 and ABCG2, but not with ABCC2 (Figure 1.). Seliciclib is an activator of ABCB1 but an inhibitor of ABCC1 and ABCG2.

To verify that seliciclib interacts with ABCB1, vesicular transport assay (VT) and calcein assay were carried out (Figure 2.). Seliciclib inhibited the transport of the probe substrate, N-methyl-quinidine (NMQ) in the VT experiment.

After having demonstrated that seliciclib is a transported substrate of ABCB1, we successfully confirmed the transport on MDCKII-MDR1 cells in calcein efflux assay (Figure 2.).

MDCKII wild type and MDCKII-MDR1 cells were applied also in vectorial transport assays. The efflux ratio of approximately 8 decreased to 1.2 when LY335979 was applied.

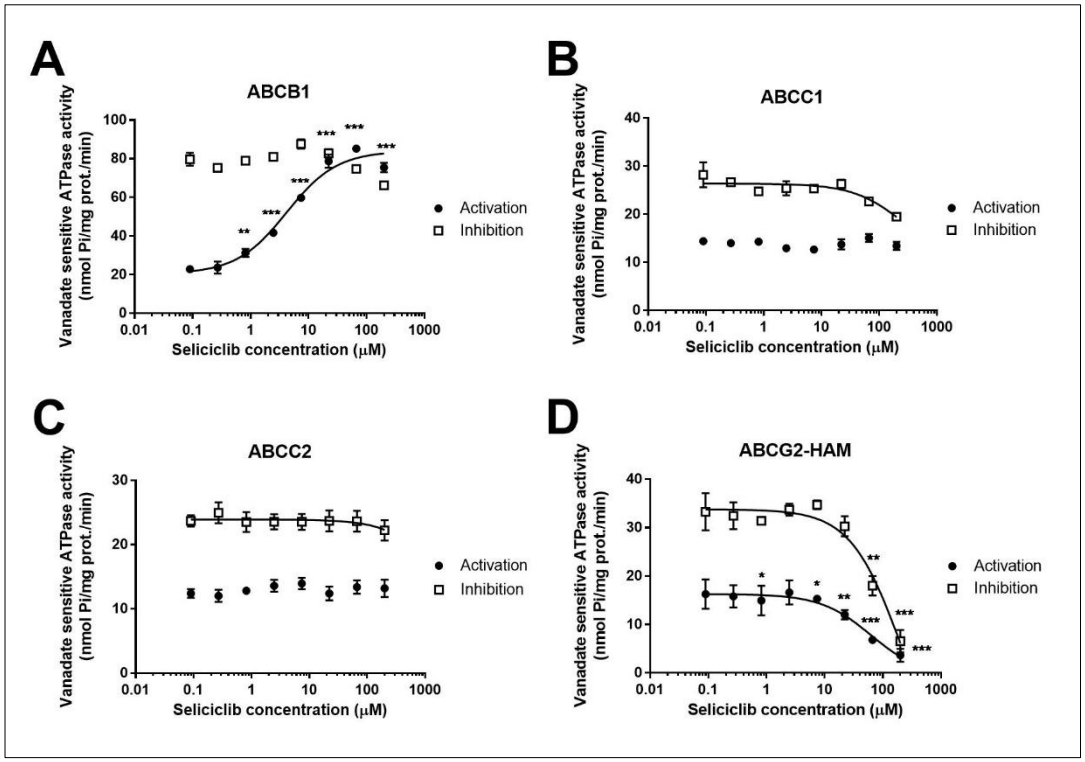

Figure 1. *The vanadate-sensitive ATPase activity of (A) MDR1-Sf9, (B) MRP1-Sf9, (C) MRP2-Sf9 and (D) BCRP-HAM-Sf9 membrane preparations in the presence of seliciclib in activation (solid lines) and inhibition (dotted lines) experiments.*

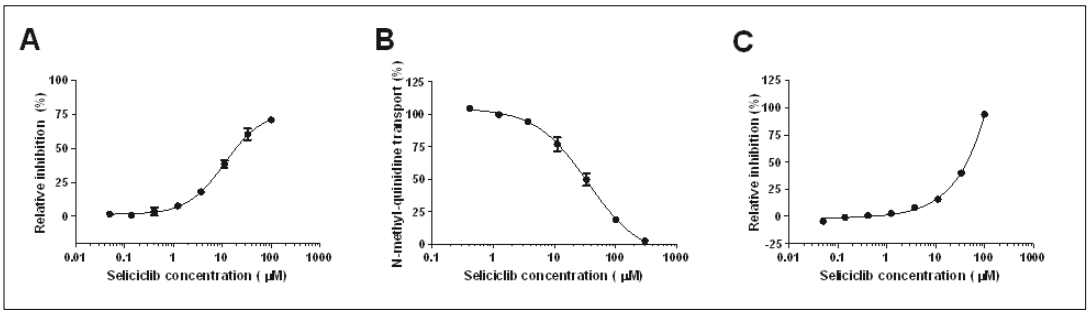

Figure 2. *Inhibition of (A) calcein AM efflux from K562-MDR cells, (B) ATP-dependent NMQ transport into MDR1-K inside-out vesicles and (C) Hoechst 33342 dye efflux from PLB985-BCRP cell by seliciclib.*

Thereafter, we wished to find out whether overexpression of ABCB1 leads to resistance against seliciclib. Our results show that the increased presence of ABCB1 does not imply resistance to seliciclib. Based on these, we can declare that although seliciclib is a transported substrate of ABCB1, this does not provide with protection against the drug even

if the protein is overexpressed. The lack of a protective effect of ABCB1 against cell death may simply be explained by the passive permeability of the drug. There are compounds called ABCB1-inverse because when they are applied, the presence of ABCB1 sensitizes cells to cytotoxicity [5]. So probably ABCB1 plays a dual role in the tested cells. On the one hand, it reduces intracellular drug concentration but on the other hand, sensitizes cells to seliciclib. The two opposing effects might effectively have cancelled each other out in the cell lines used in the present study.

Seliciclib also interacts with ABCG2, most likely in an inhibitory manner, but this interaction is unlikely to be clinically relevant.

In summary, seliciclib is a high-affinity, selective substrate of ABCB1. This interaction is likely to affect disposition of the drug. At the same time, ABCB1 overexpression does not lead to resistance to seliciclib. This means that the drug may be a promising candidate to treat ABCB1 transporter-overexpressing tumors.

3.2. ABCG2 / Chlorothiazide

Chlorothiazide as an approved ABCG2 probe candidate for regulatory studies

Chlorothiazide, a thiazide type diuretic, gets absorbed rapidly following oral administration, yet metabolically stable [6]. Instead of being metabolized, it is eliminated rapidly by the kidneys in the human body [7]. Since this is a BCS/BDDCS Class IV drug, transporter proteins can considerably influence its ADE properties. Chlorothiazide has bioavailabilities around 20-50%. All these indicate the potential role of apically located efflux transporter(s) which may limit the bioavailability of the compound. Based on these, we tested whether chlorothiazide interacted with any of the three most important, apically located efflux ABC transporters found in enterocytes: ABCB1, ABCG2 and ABCC2.

Neither ABCB1 nor ABCC2 showed interaction with chlorothiazide in vesicular transport inhibition assay (Figure 3.). Nevertheless, specific interaction was revealed with ABCG2 (Figure 3.).

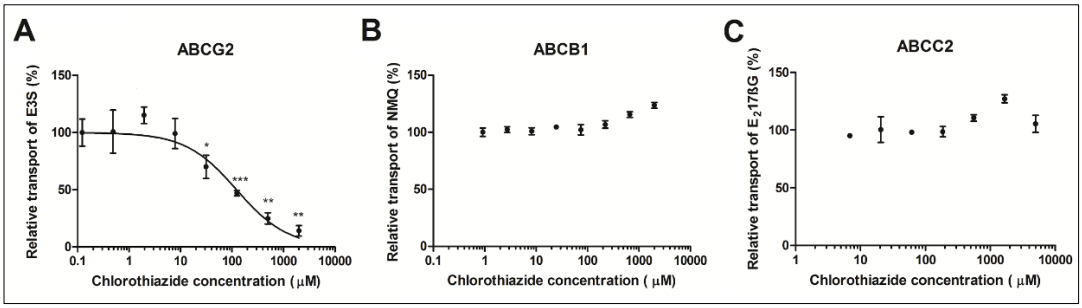

Figure 3. *ATP dependent transport of [3H] Estrone-3-sulfate, [3H] N-methyl-quinidine, and [3H] Estradiol-17-ß-D-glucuronide into (A) BCRP-HAM-Sf9, (B) MDR1-Sf9 and (C) MRP2-Sf9 vesicles, respectively, was measured in the presence of chlorothiazide at concentrations indicated in the figure.*

For further characterization of the interaction of chlorothiazide with ABCG2, we carried out ATPase and VT assays. These experiments confirmed that this diuretic is a transported substrate of ABCG2 (Figure 4.).

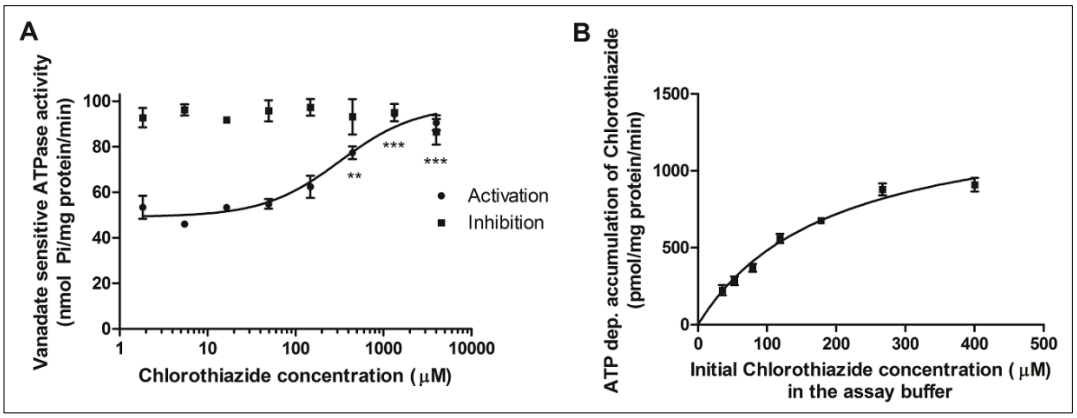

Figure 4. *ABCG2-overexpressing BCRP-M membranes were incubated with increasing concentrations of chlorothiazide and (A) vanadate-sensitive ATPase activity in activation (filled circles) and inhibition mode (filled squares) as well as (B) ATP-dependent chlorothiazide transport into inside-out membrane vesicles was determined.*

Cellular monolayer tests confirmed that the interaction with ABCG2 may modulate the ADME properties of chlorothiazide. Both monolayer test systems applied are extensively characterized. Moreover, they are the most commonly used in vitro tools to predict the absorption of orally administered drugs. These experiments confirmed that ABCG2, an apical efflux transporter in the intestines, is likely to be limiting the absorption of

chlorothiazide, contributing to its low bioavailability. ABCG2 is also expressed in the proximal tubular cells of the kidneys, and may, thus, contribute to renal secretion of the drug.

We verified in monolayer efflux experiments that chlorothiazide is a transported substrate of ABCG2. We saw difference in transport values both with MDCKII-BCRP as well as with Caco-2 cells in the presence or absence of Ko143, an ABCG2-specific inhibitor.

To sum this up, we tested if any of the three major intestinal, apically located efflux transporters - ABCB1, ABCG2 or ABCC2 - modulated permeability of the drug in vitro. ABCG2 modulated the permeability of chlorothiazide in all three experimental systems tested. As these experimental systems used in this study comply with the current regulatory guidelines, the results suggest that chlorothiazide is a legitimate ABCG2 probe candidate for regulatory studies. Furthermore, potential inhibition of ABCG2-mediated urate transport may be behind hyperuricaemic activity of chlorothiazide.

3.3. Fumitremorgin C analogs / Inhibition specificity of ABCG2

FTC-Ko analogues: specificity is limited to a single chiral center – the C-6 and a 3S, 6S, 12aS configuration is essential

Molecules that inhibit the function of ABC transporters may improve the efficacy of anticancer agents. Reversal of ABCG2-mediated transport is a promising area of research.

Professor Gábor K Tóth's group at the Department of Medical Chemistry at the University of Szeged synthetized 16 tricyclic and 28 tetracyclic novel analogs devoid of ester linkages and we tested them for ABCG2 inhibition potency and specificity. Hoechst assay was used for the measurement of the ABCG2-inhibitory activity of the synthetized compounds. We used Ko134, a well-known specific ABCG2 inhibitor, as a positive control.

No inhibitory activity was found among the tricyclic analogs, unlikely among tetracyclic analogs. The inhibitory potencies of some tetracyclic derivatives and the diastereoisomeric mixtures were comparable to that of Ko134. All the stereochemically pure compounds in this group had an absolute configuration of 3S, 6S, 12aS. Their diastereoisomeric counterparts (3S, 6R, 12aS) showed no inhibitory activity, with only one exception. One compound of the 3S, 6S, 12aS absolute configuration showed partial activity.

Next, we focused on compounds which showed potent inhibition of ABCG2 and calculated IC50 values (Table 1). Among the compounds 3e2 (R), 3e5 (R), and 3e6 (R), only 3e5 (R) proved to be inhibitory, with a 16-fold difference.

Molecule number	**ABCG2 inhibition**	**ABCG2 inhibition**	**ABCB1 inhibition**	**ABCC1 inhibition**
Compound	10 uM (%)	IC50 (uM)	IC50 (uM)	IC50 (uM)
1c (*3S,6S,12aS*)	118	0.05 ± 0.03	0.75 ± 0.02	0.82 ± 0.04
2c (*3S,6S,12aS*)	113	0.06 ± 0.24	0.84 ± 0.07	0.92 ± 0.08
3e1 (*3S,6R,12aS*)	0	>100	>100	86.0 ± 7.07
3e1 *(3S,6S,12aS*)	113	1.73 ± 0.01	>100	83.5 ± 8.91
3e2 *(3S,6R,12aS)*	7	>100	40.3 ± 5.94	37.6 ± 15.4
3e2 *(3S,6S,12aS)*	131	0.41 ± 0.28	33.4 ± 20.9	4.16 ± 0.82
3e3 *(3S,6R,12aS)*	1	>100	>100	>100
3e3 *(3S,6S,12aS)*	59	24.7 ± 4.4	>100	52.3 ± 10.7
3e4 *(3S,6R,12aS)*	4	>100	28.8 ± 6.21	33.6 ± 0.74
3e4 *(3S,6S,12aS)*	110	0.14 ± 0.03	8.74 ± 2.08	2.66 ± 0.32
3e5 *(3S,6R,12aS)*	101	7.49 ± 1.12	28.6 ± 14.0	42.9 ± 6.27
3e5 *(3S,6S,12aS)*	121	0.45 ± 0.12	13.1 ± 2.40	12.1 ± 2.02
3e6 *(3S,6R,12aS)*	42	16.1 ± 2.25	0.99 ± 0.23	1.34 ± 0.25
3e6 *(3S,6S,12aS)*	117	0.14 ± 0.06	2.58 ± 1.19	0.88 ± 0.09
4d *(3S,6S,12aS)*	110	0.32 ± 0.24	8.38 ± 1.56	6.21 ± 3.81
1c'*(3S,6S,12aS; 3S,6S,12aS)*	88	5.50 ± 4.10	1.79 ± 0.35	2.99 ± 0.25
2c'*(3S,6S,12aS; 3S,6S,12aS)*	96	3.00 ± 2.20	2.75 ± 0.53	3.53 ± 0.44
KO134	111	0.06 ± 0.03	2.04 ± 0.06	5.51 ± 0.88
KO143	118	0.06 ± 0.01	8.74 ± 0.29	9.13 ± 1.07

Table 1. *Dye efflux inhibition. Data are given as mean±SD.*

The dye efflux calcein assays helped us to determine specificity of ABCG2 inhibition over ABCB1 and ABCC1 inhibitory activity. The noteworthy stereospecificity (3S, 6S, 12aS vs. 3S, 6R, 12aS) observed regarding the inhibition of ABCG2 was completely absent for the inhibition of ABCB1 and ABCC1. In addition, ABCG2 specificity over ABCB1 and ABCC1 was not found for compounds with 3S, 6R, 12aS configuration. As expected [8], the racemate compounds 1c' (S+R) and 2c' (S+R) having phenyl or benzyl instead of isobutyl sidechain at C-6 did not specifically inhibit ABCG2 over ABCB1 and ABCC1 either.

As the tricyclic analogues IIIa-IIId showed no activity in the Hoechst assay, it became clear that the FTC type diketo-piperazine ring structure is fundamental for activity. The C-6 and a 3S, 6S, 12aS configuration is essential. Compounds with 3S, 6R, 12aS configuration were

inactive as expected [8]. The 3e6 (3S,6R,12aS) might be the only exception with its partial activity. But even in that case, the diastereoisomeric pair, 3e6 (3S,6S,12aS), was more than 115-fold more potent. Noticeably, the stereospecific (3S, 6S, 12aS vs 3S, 6R, 12aS) inhibition seen in case of ABCG2 was missing with ABCB1 and ABCC1. In addition, ABCG2 specificity over ABCB1 and ABCC1 was not observed in case of compounds with the 3S, 6R, 12aS configuration. The fact that configuration at position 6 alone ensures specificity for ABCG2 over ABCB1 and ABCC1 has not been described before. Taking into consideration the broad and overlapping substrate specificity of the above-mentioned ABC transporters, it is striking and quite unexpected that this specificity is limited to a single chiral center [9, 10].

The Ko family of fumitremorgin C analogs include potent and selective ABCG2 inhibitors. However, the most potent Ko compounds carry an ester linkage in their side-chain that makes them chemically and metabolically less stable. We have tested novel 16 tricyclic and 28 tetracyclic Fumitremorgin C analogs for ABCG2 inhibition potency and specificity. Among the tetracyclic analogs, we discovered efficacious ABCG2-inhibiting compounds. Compounds with the 3S,6S,12aS configuration showed stereospecificity. Diastereoisomeric pairs with a 3S,6R,12aS configuration showed at least 18-fold less potent inhibition. Such stereospecificity was not observed in ABCB1 and ABCC1 inhibition.

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II. Beéry, E., Rajnai, Z., Abonyi, T., Makai, I., Bánsághi, S., Erdő, F., Sziráki, I., Herédi-Szabó, K., Kis, E., Jani, M., Márki-Zay, J., Tóth, G. K. and Krajcsi, P.: ABCG2 modulates chlorothiazide permeability *in vitro* - characterization of the interaction, *Drug Metab. Pharmacokinet.*, 27:349-353, 2012.

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IX. Rajnai, Z., Méhn, D., Beéry, E., Okyar, A., Jani, M., Fülöp, F., Lévi, F. and Krajcsi, P: The CDK inhibitor roscovitine (Seliciclib) is a selective substrate of human P-glycoprotein (P-gp/ABCB1/MDR1) *Drug Metab. Rev.* 42:299-300, 2010.

[1] In the manuscript these papers will be referred to by the roman numbers