Abschlussbericht zum Projekt

„Der Warburg-Effekt als möglicher Angriffspunkt mariner Substanzen in Prostatakarzinomzellen“

Gunhild von Amsberg

In Vorbereitung auf die geplante Publikation wurde der Abschlussbericht in englischer Sprache abgefasst.

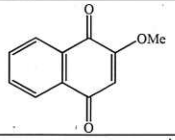
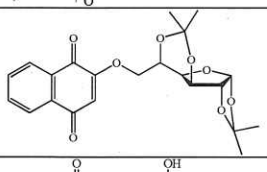
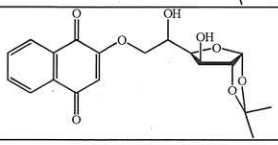
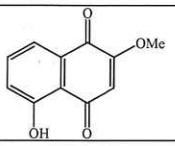
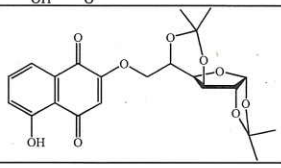
In total we investigated 47 compounds: 23 of 1,4-haphtaquinone-glucose O-conjugates (Table 1A) and 24 of 1,4-haphtaquinone-glucose S-conjugates (Table 1B).

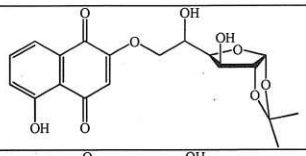
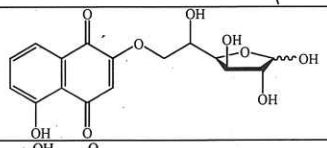
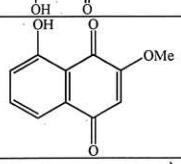
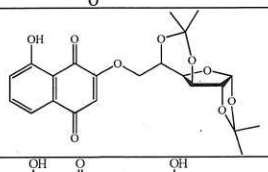
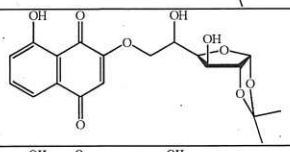
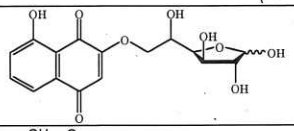
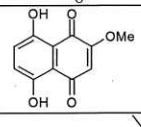
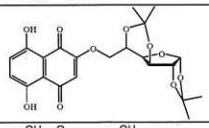
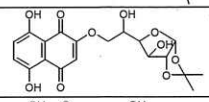
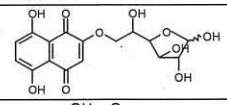
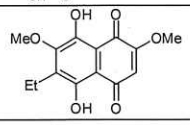
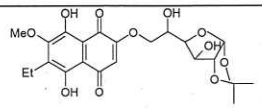
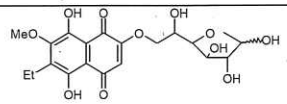
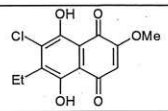
For 1,4-haphtaquinone-glucose O-conjugates and each group consisted of 4 types of derivatives: a) non-conjugated 1,4-haphtaquinone (e.g. Pel-6), b) 1,4-haphtaquinone conjugated with double-protected glucose (e.g. Pel-7), c) 1,4-haphtaquinone conjugated with mono-protected glucose (e.g. Pel-8), 1,4-haphtaquinone conjugated with non-protected glucose (e.g. Pel-9).

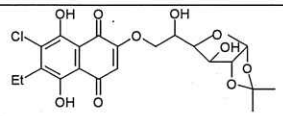
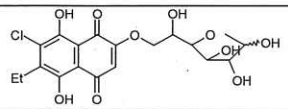
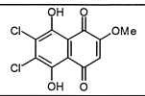
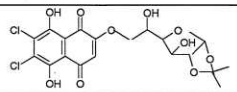
For 1,4-haphtaquinone-glucose S-conjugates and each group consists of 2 types of derivatives: a) 1,4-haphtaquinone conjugated with fully acetylated (protected) glucose (e.g. Sab-1), b) 1,4-haphtaquinone conjugated with non-acetylated (non-protected) glucose (e.g. Sab-2).

Table 1. Structure of the compounds Pel-1 – Pel-28 (**A**), and Sab-1 – Sab-24 (**B**) and its IC_{50} in human cancer PC-3 cells.

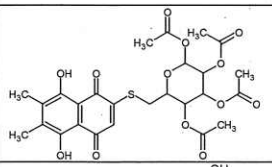
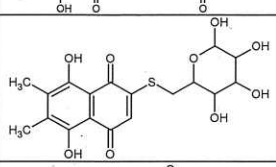
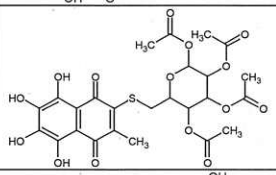
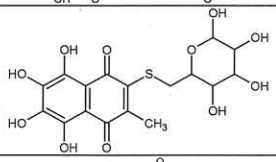
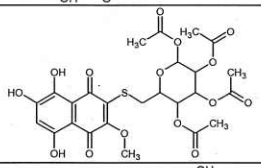
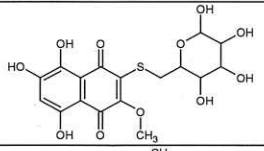
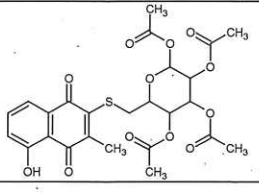
A

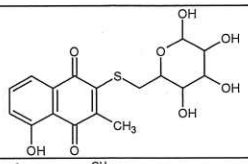
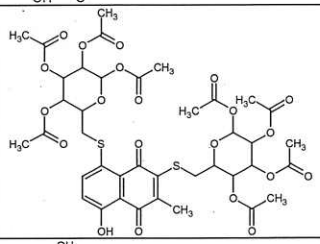
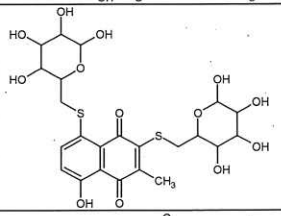
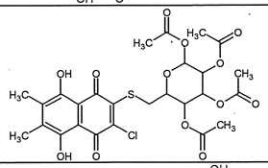
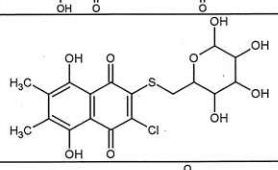
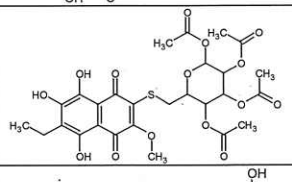
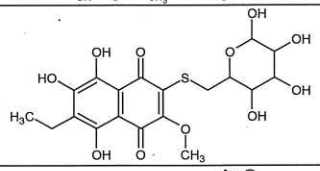
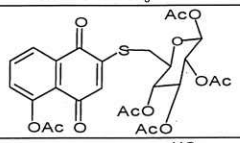
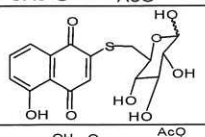
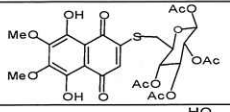
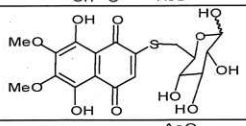
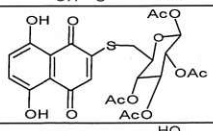
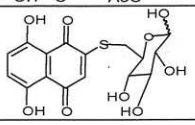
Code	formula	IC_{50} [µM] (PC-3 cells)
Pel-1		9.3
Pel-2		13.1
Pel-3		24.25
Pel-4	Not synthesized	-
Pel-5		4.46
Pel-6		4.51

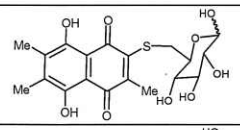
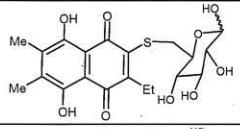
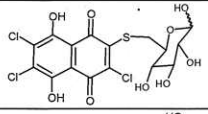
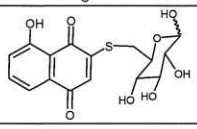
Pel-7		7.82
Pel-8		36.9
Pel-9		2.85
Pel-10		4.17
Pel-11		2.84
Pel-12		27.3
Pel-13		5.38
Pel-14		17.7
Pel-15		6.17
Pel-16		>100
Pel-17		21.8
Pel-18	Not synthesized	-
Pel-19		27.9
Pel-20		>100
Pel-21		31.2
Pel-22	Not synthesized	-

Pel-23		7.78
Pel-24		>100
Pel-25		0.91
Pel-26	Not synthesized	-
Pel-27		3.04
Pel-28	Not synthesized	-

B

Code	Formula	IC_{50} [μM] (PC-3 cells)
Sab-1		3.09
Sab-2		2.53
Sab-3		>100
Sab-4		>100
Sab-5		81.6
Sab-6		37.5
Sab-7		1.01

Sab-8		23.8
Sab-9		0.81
Sab-10		>100
Sab-11		0.94
Sab-12		37.6
Sab-13		64.3
Sab-14		57.0
Sab-15		4.14
Sab-16		50
Sab-17		1.60
Sab-18		8.08
Sab-19		0.70
Sab-20		21.6

Sab-21		3.22
Sab-22		10.9
Sab-23		41.5
Sab-24		39.8

First, we performed screening examinations of the compounds in order to investigate their cytotoxicity in and selectively for prostate cancer cells. Thus, we examined the cytotoxicity of the synthesized compounds in human prostate cancer PC-3 cells and human non-cancer PNT-2 cells, and compared the respective IC_{50} values. Results are presented in Figure 1.

For the further investigations compounds were selected due to the following criteria:

a) selectivity index IC_{50}(PNT-2) / IC_{50}(PC-3) ≥ 2

b) cytotoxic effect in human cancer PC-3 cells with IC_{50}(PC-3) ≤ 50 μM.

Consequently, compounds Pel-8, Pel-12, and Sab-18 were chosen. Along with these substances for the future investigations we also selected their mono-protected derivatives Pel-7 and Pel-11 (for Pel-8 and Pel-11, correspondently) and Sab-17 (for Sab-18). These compounds (Pel-7, Pel-11, and Sab-17) possess strong cytotoxic activity in cancer cells, even though its selectivity towards cancer cells in primary screening (see Figure 1) was lower in comparison with the selectivity of the correspondent unprotected derivatives (Pel-8, Pel-12, and Sab-18)

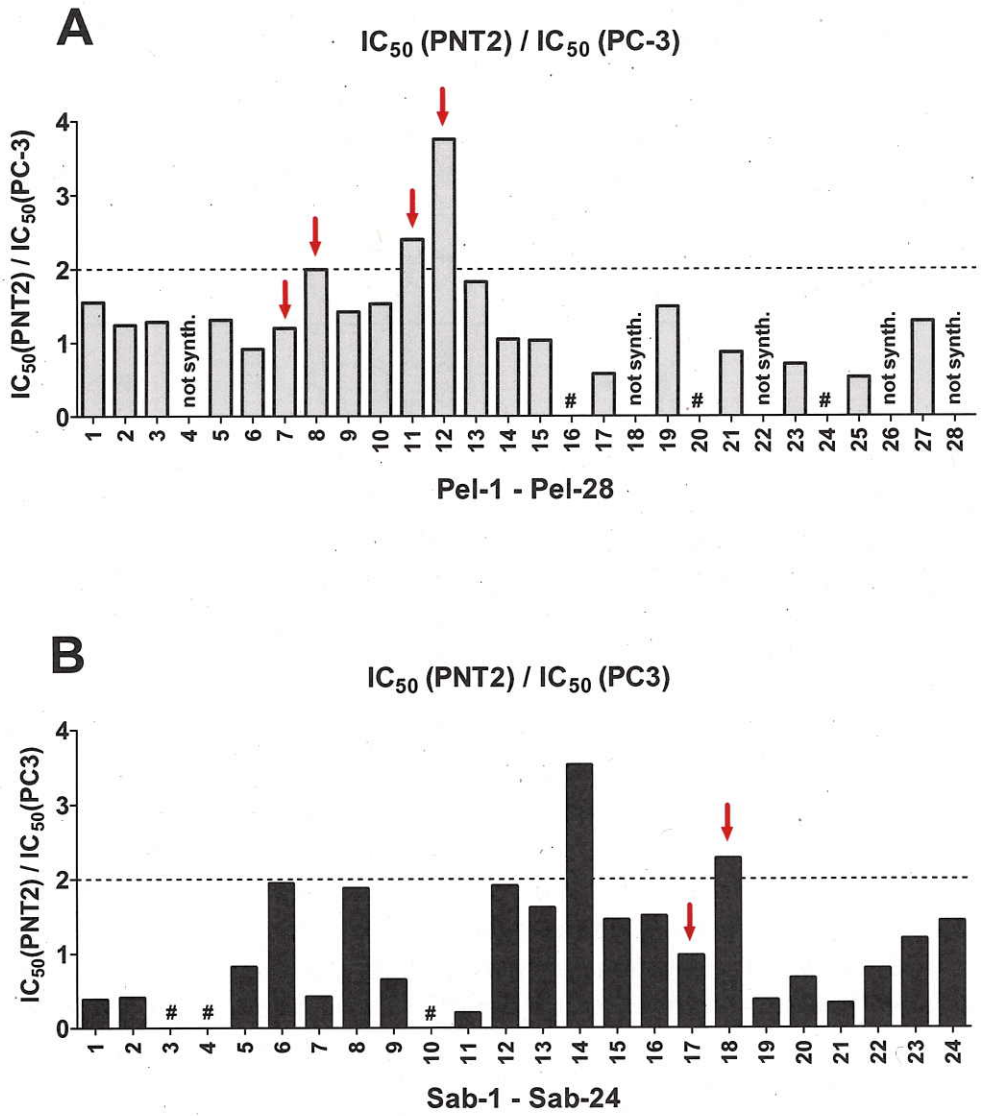


Figure 1. Index of selectivity (IC_{50}(PNT-2) / IC_{50}(PC-3)) of the investigated compounds: 1,4-haphtaquinone-glucose O-conjugates (**A**) and 1,4-haphtaquinone-glucose S-conjugates (**B**). "not synth." – compound was not synthesized. "#" - IC_{50}(PC-3) ≥ 100 μM. Compounds with a selectivity index IC_{50}(PNT-2) / IC_{50}(PC-3) ≥ 2 and IC_{50} ≤ 50 μM (in PC-3 cells), and/or high cytotoxic activity in cancer cells were selected for the further examinations (marked with the red arrows).

In a next step, we examined the activity and selectivity of the chosen compounds in five different prostate cancer cell lines as well as in five non-cancer human cell lines (including normal prostate and non-prostate cell lines). The compounds exhibited a certain degree of selective cytotoxicity in human prostate cancer cells. Interestingly, the elimination of protective groups strongly increased the selectivity of the molecules towards cancer cells while in contrast at the same time the absolute IC_{50} values decreased.

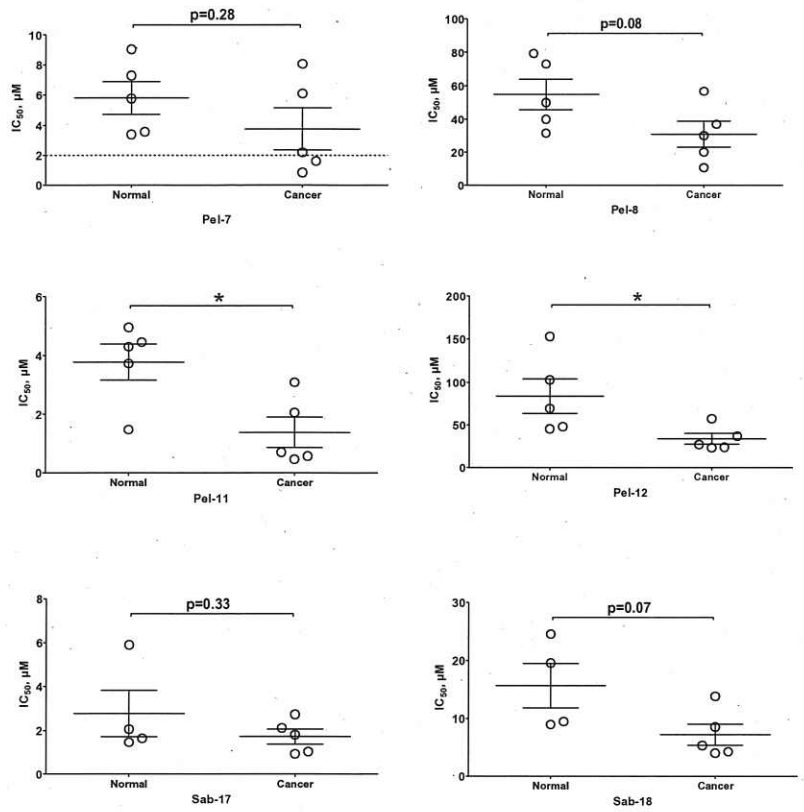


Figure 2. Selectivity of the investigated compounds: IC_{50}s in prostate cancer cells ("Cancer") as well as in non-cancer ("Normal") cells. * - $p \leq 0.05$ (Student's t-test).

In addition, the selectivity towards cancer cells correlated with the level of expression of GLUT-1 (Glucose transporter 1) protein in the cells (Figure 3)

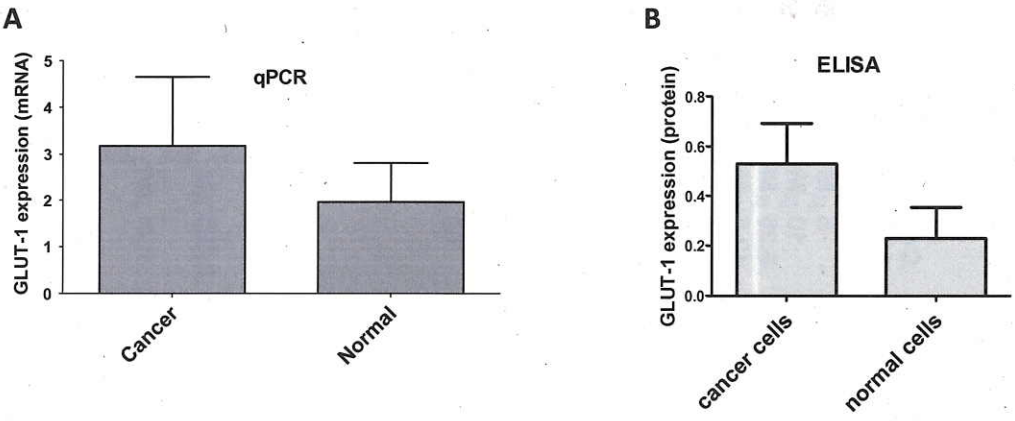


Figure 3. Level of expression of GLUT-1 in human cancer and non-cancer cells, measured by qPCR (GLUT-1 mRNA) (**A**), and ELISA (GLUT-1 protein) (**B**).

Thus, the selectivity of the investigated compounds towards human cancer cells correlated with GLUT-1 expression (Fig. 3). Therefore we have examined the effect of the six selected substances on the glucose uptake in PC-3 cells. A clear inhibition of glucose uptake was detected in cells treated with cytotoxic and sub-cytotoxic concentrations of the compounds, supporting the suggested Warburg effect targeting.

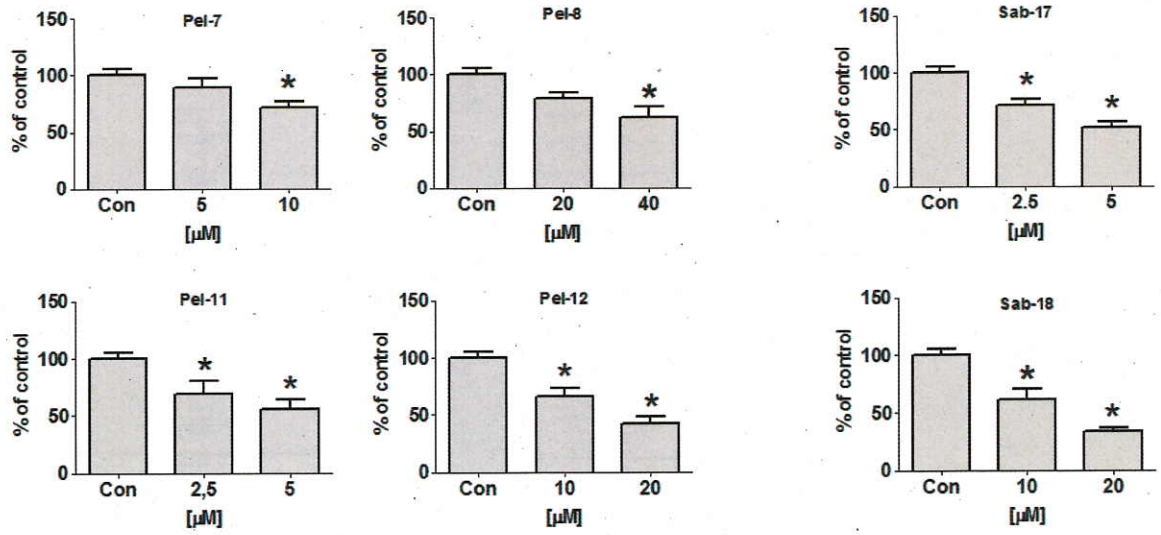


Figure 4. Effect of the selected O- of S-glucoconjugates on the glucose uptake in human cancer PC-3 cells. The experiment was performed using 2-NDBG and fluorescence plate reader. Glucose uptake was normalized to the cell viability measured with MTT assay.

Next, we examined the ability of the selected compounds to inhibit the colony formation of human cancer 22Rv1 cells (*in vivo* prototype of antimetastatic activity). All the substances were able to inhibit the number of colonies at their subcytotoxic or cytotoxic concentrations (Fig. 5)

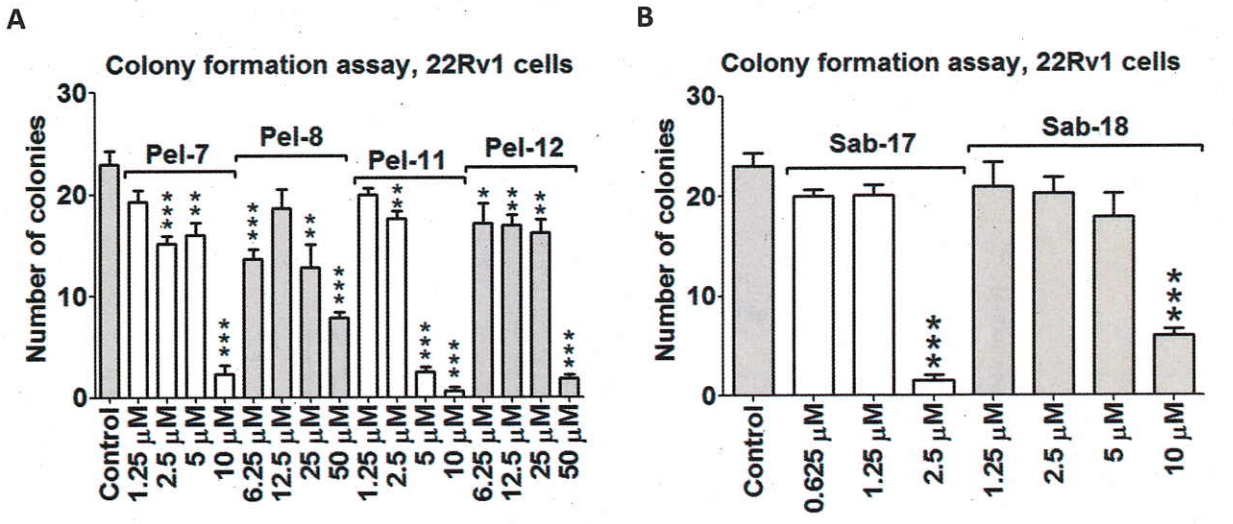


Figure 5. Effect of the O- (**A**) of S-glucoconjugates (**B**) on the colony formation of human cancer 22Rv1 cells after 48 h of treatment.

In addition, we investigated the mechanism of cancer cell death induced by the selected compounds. Cell cycle analysis of human cancer 22Rv1 cells treated with the substances for 48 h revealed G2/M phase arrest for all compounds. Strongest effects were observed for cells treated with Sab-17 and Sab-18 (Fig. 6)

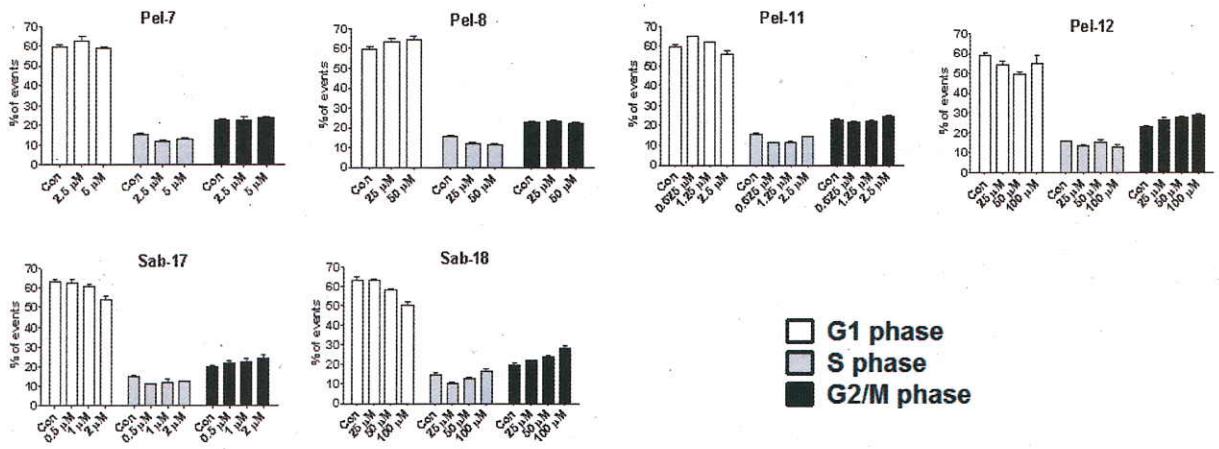


Figure 6. Effect of the O- of S-glucoconjugates on cell cycle progression of human cancer 22Rv1 cells. The analysis was performed using a FACS technique and a PI staining.

Next, we investigated the mechanism of drug-induced cell death. FACS analysis revealed an induction of DNA fragmentation in 22Rv1 cells after a 48 h treatment with the different compounds. Thus, cell death was assumed to be most likely apoptotic (Fig. 7). This effect was most pronounced for S-glucoconjugates.

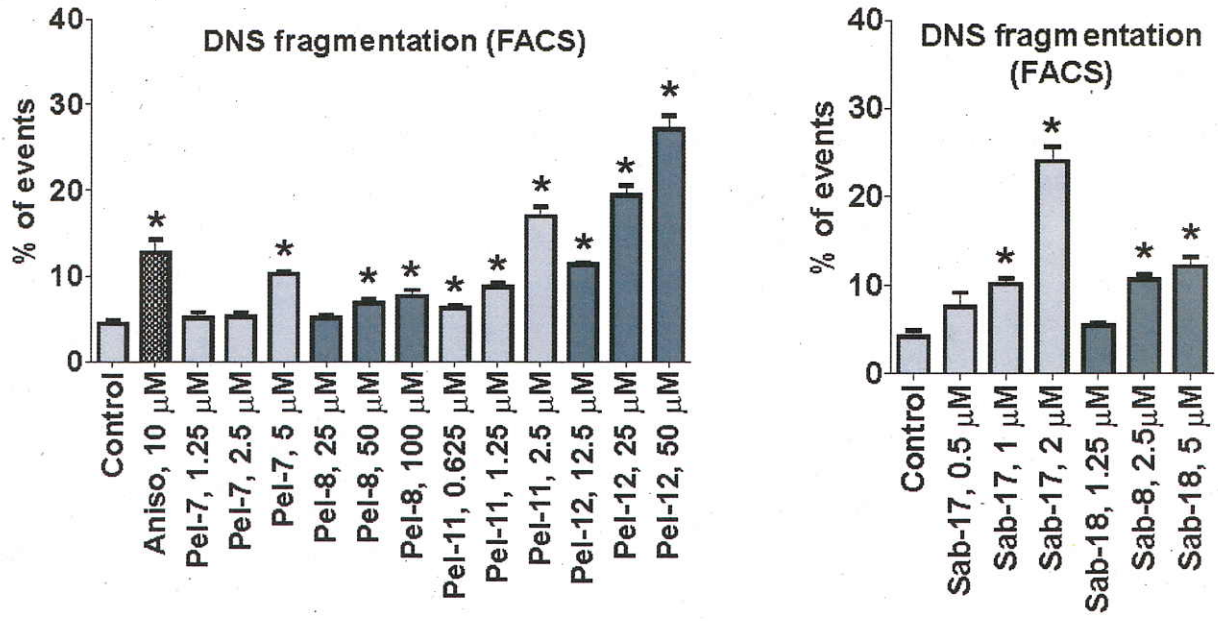


Figure 7. Effect of the O- of S-glucoconjugates on DNA fragmentation of human cancer 22Rv1 cells. The analysis was performed using a FACS technique and a PI staining, cells appeared sub-G0 phase were quantified as apoptotic.

Then, we evaluated the role of caspases in the drug-induced cancer cell death. Western blotting analysis of the cells treated with the investigated compounds for 48 h revealed typical hallmarks of apoptosis such as caspase-3 and PARP cleavage (Fig. 8). Additionally, the level of pro-apoptotic Bax protein was elevated in cells treated with Pel-7 and Pel-11 (Fig. 8).

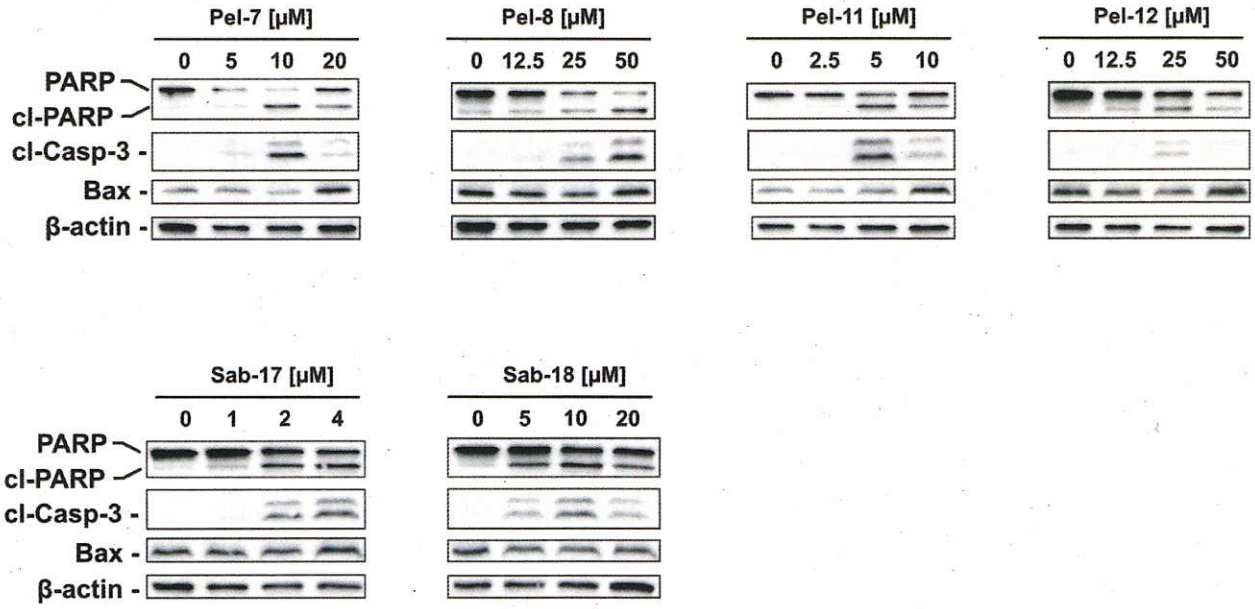


Figure 8. Effect of the O- of S-glucoconjugates on several pro-apoptotic proteins in human cancer 22Rv1 cells after 48 h of treatment in Western Blot Analyses.

Due to the observed activation of caspase-3, we investigated its impact on drug induced apoptosis more closely. We used z-VAD-fmk, a pan-caspase inhibitor, to inhibit caspase activity. In fact, pre-treatment with z-VAD-fmk protected cells from apoptotic cell death, increasing the population of alive cells and decreasing the population of cells undergoing early apoptosis with all six different compounds (Fig. 9, representative pictures for Sab-17 and Sab-18) suggesting a caspase-dependent character of apoptosis.

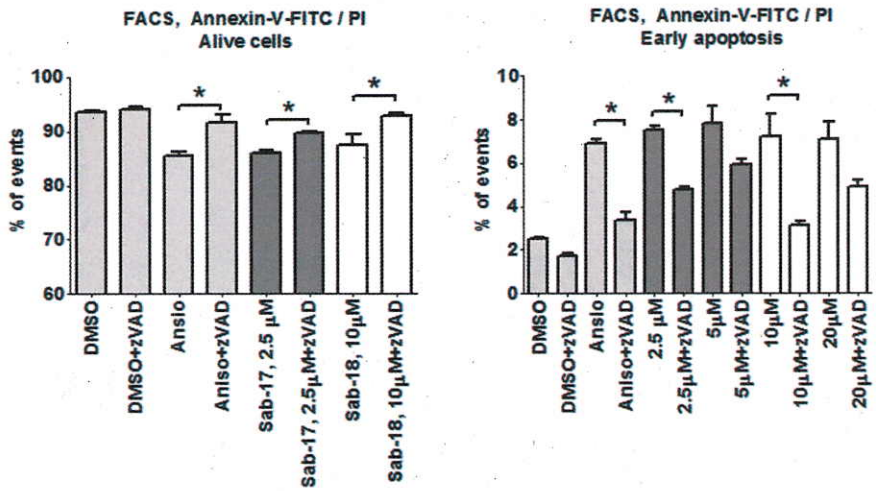


Figure 9. Representative picture – the effect of the S-glucoconjugates on cell viability and phosphatidylserine externalization in human cancer 22Rv1 cells treated with the investigated drugs for 48 h, with or without pre-treatment with z-VAD-fmk for 1 h. The analysis was performed using a FACS technique and annexin-V-FITC / PI double staining. Annexin (+) / PI (–) cells were considered to be apoptotic. Annexin (-) / PI (–) cells were considered to be alive.

Caspase-dependent apoptosis induction in cancer cells can be mediated by mitochondria targeting. This can result in a release of pro-apoptotic mitochondrial proteins like cytochrome C, HtrA2, AIF and others as well as increased ROS (reactive oxygen species) production, which

ultimately leads to caspase-dependent apoptosis. Consequently, we checked the effect of our compounds on ROS levels as one of the hallmarks of mitochondrial-related apoptosis. Indeed, all six compounds were able to induce ROS production in 22Rv1 cells after 2 h of treatment. Thus, mitochondria targeting can be assumed to be a primary molecular event leading to cancer cells death (Fig. 10).

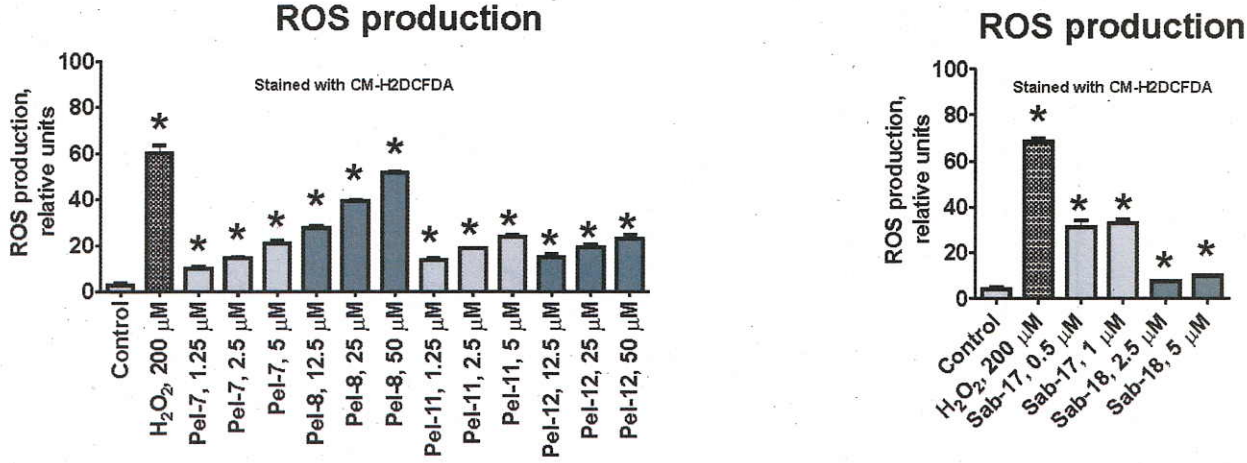


Figure 10. Effect of the O- of S-glucoconjugates on ROS production in human prostate cancer 22Rv1 cells after 2 h of treatment. The analysis was performed using FACS technique and CM-H2DCFDA staining.

Next, we proved the relevance of drug-induced activation of ROS production for the cytotoxic effects of the compounds. Therefore, we co-treated prostate cancer cells with the investigated compounds and a well-established antioxidant, N-acetylcysteine (NAC). The results clearly indicated that the combination with the antioxydant could effectively abolish the cytotoxic effects of the compounds (Fig. 11).

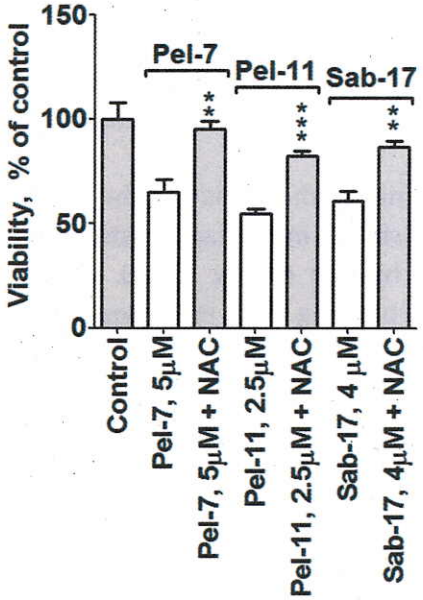


Figure 11. Effect of Pel-7, Pel-11 and Sab-17 on viability of human cancer 22Rv1 cells treated with the investigated drugs for 48 h, with or without NAC. Viability was measured by MTT assay.

Next, we investigated the integrity of mitochondrial membrane via examination of the mitochondria membrane potential (MMP) by specific JC-1 staining in order evaluate the impact of mitochondrial targeting on ROS production. The results clearly show the loss of $\Delta\psi$ in human cancer cells treated with the investigated compounds after 2 h. Thus, mitochondrial targeting is the primary event leading initiating apoptosis (Fig. 12)

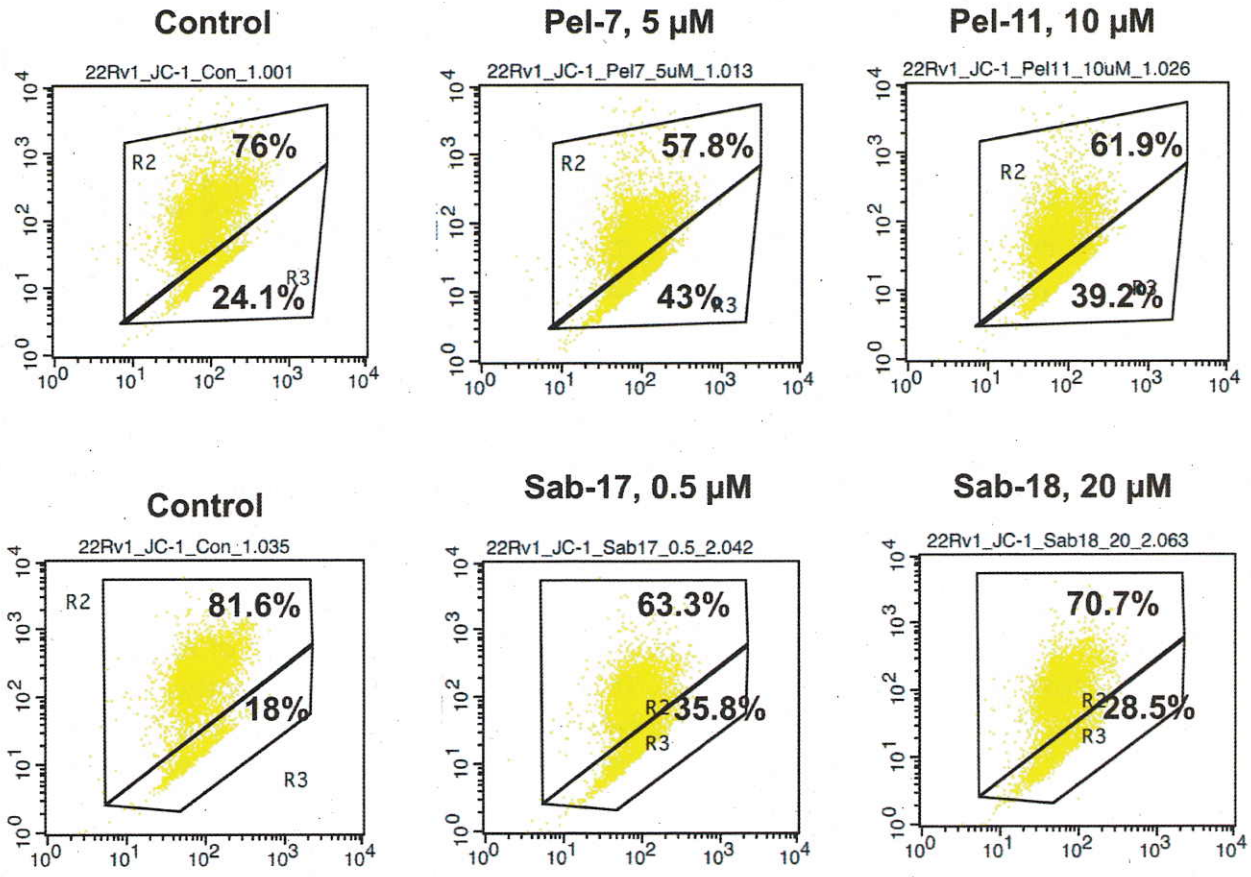


Figure 12. Effect of Pel-7, Pel-11, Sab-17 and Sab-18 on mitochondria membrane integrity in human cancer 22Rv1 cells treated with the substances for 2 h. The analyses were performed using a FACS technique and a JC-1 staining.

Finally, we examined the effect of the compounds on autophagy as one of the main mechanisms of drug resistance in human prostate cancer cells. Indeed, we found up-regulation of LC3B-II levels in the human cancer 22Rv1 cells treated with each of the six investigated compounds by Western blotting. This indicates inhibition of autophagy at the late stages (Fig. 13).

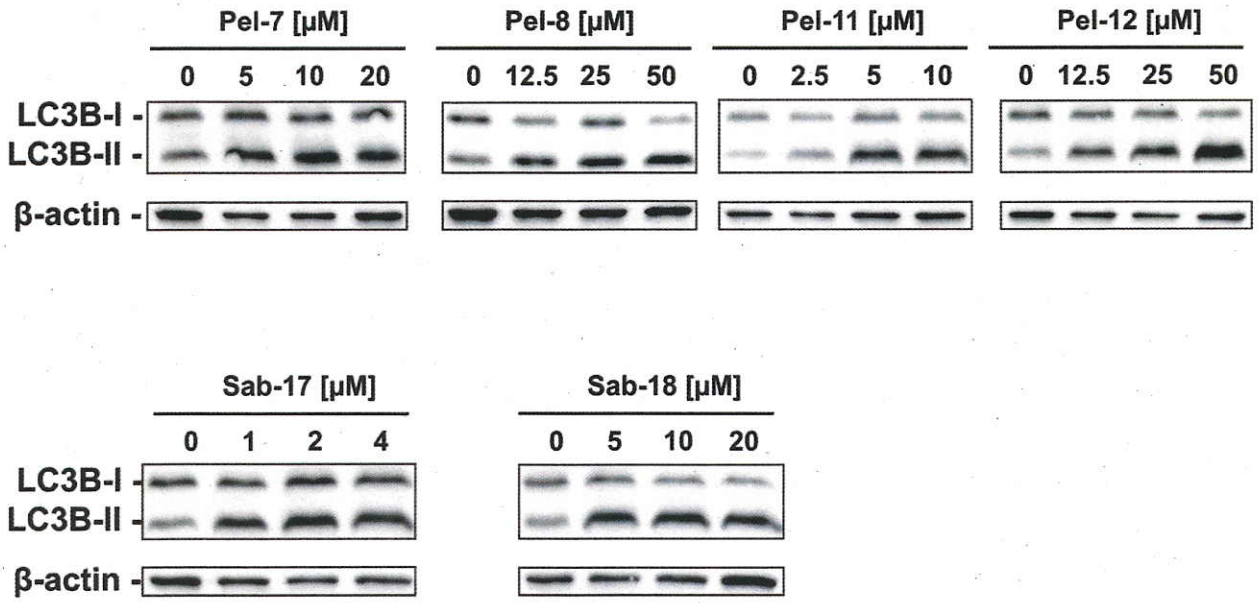


Figure 13. Effect of the O- of S-glucoconjugates on the expressional levels of LC3B-I/II proteins in human cancer 22Rv1 cells after 48 h of treatment. The analyses were performed by Western blotting.

In conclusion, we investigated 47 synthesized compounds including 23 of 1,4-haphtaquinone-glucose O-conjugates and 24 of 1,4-haphtaquinone-glucose S-conjugates. We identified 6 compounds to be selective towards human prostate cancer cells when compared to human prostate non-cancer as well as other normal human cells. The selectivity correlated with the expression of GLUT-1. The compounds were able to inhibit glucose uptake by the cells, indicating Warburg effect targeting. The cyctotoxic effect of the compounds was mainly mediated by mitochondrial targeting. It was associated with a loss of $\Delta\psi$ and caused increased ROS production. This ultimately resulted in caspase-dependent apoptosis of prostate cancer cells. Distinct inhibition of cell cycle progression and late stage autophagy were identified as additional mechanisms of anticancer action of the selected compounds.