Agarikon.1 and Agarikon Plus Affect Cell Cycle and Induce Apoptosis in Human Tumor Cell Lines

Boris Jakopovich, Ivan Jakopovich,
Neven Jakopovich

Dr Myko San – Health from Mushrooms
Miramarska c. 109, Zagreb, Croatia

INDEX

- Introduction
- Materials and Methods
- Results
- Conclusions

INTRODUCTION

- Continuation of the study on 6 blended mushroom products and 3 single extracts on 4 human tumor cell lines (Durgo, Jakopovich 2013)

- Neutral Red and MTT proliferation assays demonstrate that blended extracts cause increased tumor cell membrane and mitochondria damage

- This study concentrates on the mechanisms; effects on the cell cycle and inducing apoptosis

Medicinal mushroom metabolites can interfere and modulate all processes related to the 8 steps of carcinogenesis (Petrova 2012):

– inflammation
– cancer cell proliferation
– adhesion
– apoptosis
– angiogenesis
– gene expression
– invasiveness
– metastasis

Apoptosis - programmed cell death - is a crucial tumor suppression mechanism

– eliminates cells with extensive DNA damage (potentially leading to cancer)

- differentiation - cell growth – apoptosis balance

Purpose of the Study

Investigate:

A. proliferation (cell cycle disturbance)

B. induction of apoptosis

– medicinal mushroom extract blends Agarikon Plus and Agarikon.1

– concentration response

– 24 and 48 hour response

– 2 human tumor cell lines: H460 (lung carcinoma) and Caco-2 (colon carcinoma)

– Camptothecin, referent antitumor compound; cytotoxic dose 10 µM used

MATERIALS AND METHODS

Cell lines

- H460 (large cell lung carcinoma)
- Caco-2 (colorectal adenocarcinoma)

Tested extract blends

- Agarikon.1 tablets
- Agarikon Plus

Experimental methods

- Proliferation Assay by MTT
- Cell Cycle Analysis by flow cytometry
- Annexin V Assay for Apoptosis Induction Detection
- Western Blot Analysis

TESTED PRODUCTS

- Agarikon.1 tablets (AG.1)
- proprietary mushroom extract blend from Dr Myko San company
- *Ganoderma lucidum, Lentinus edodes, Grifola frondosa, Pleurotus ostreatus, Agaricus brasiliensis*
- registered med. mushroom supplement
- recommended treatment dose: ~0.1 g/kg bodyweight per day of soluble polysaccharides

- Agarikon Plus extract blend (AG+)
- proprietary mushroom extract blend from DMS
- 10 medicinal mushroom species (inc. *G. lucidum, L. edodes, G. frondosa, P. ostreatus, A. brasiliensis*)
- in liquid form
- Recommended treatment dose: ~0.16g/kg BW per day of soluble polysaccharides

1 Proliferation Assay

- Cells cultured as monolayers, plated in parallel on day 0, at 3 x 10^3 cells/well (H460) and 7 x 10^3 cells/well (Caco-2), depending on doubling times
- AG.1 and AG+ added at 0.001, 0.01, 0.1, 1 and 10 mg/ml concentrations (stock solution for both 40 mg/ml, and 4 x 10^{-3} M/DMSO for camptothecin)
- We used MTT assay to evaluate cell growth rate after 72 hours (absorbance was measured at 570 nm)

2 Cell Cycle Analysis

- seeded at $1x10^5$ cells/well (H460) and $2x10^5$ cells/well (Caco-2), depending on the doubling times
- After 24 hours, AG.1 and AG+ applied at concentrations 0.1 mg/ml and 1 mg/ml; camptothecin (10 μM) for positive control
- After the incubation period, cells were trypsinized, washed with Phosphate Buffer Saline (PBS); stained with propidium iodide (PI) and analyzed on FACScalibur flow cytometer
- Ratio of cells in each cell cycle phase was determined by analyzing the DNA histograms using ModFit LTTM software

3 Annexin V Assay for Apoptosis Induction Detection

- same concentrations used; 0.1 and 10 mg/ml
- cell populations were gated into regions corresponding to live, early apoptotic and late apoptotic/necrotic cells

Annexin V	PI	Cell Region
-	-	Live cells
+	-	Early apoptotic
+	+	Late apoptotic /necrotic

4 Western Blot Analysis

- mushroom extracts (0.1 and 1 mg/ml) were added to well plates after 24 hours
- total proteins were measured using BCA Protein Assay Reagent, separated by SDS-polyacrylamid gel electrophoresis and transferred to PVDF membrane → probing with anticaspase 3, anti-p53, and anti-p21 primary antibodies
- equal loading confirmed using anti-tubulin primary antibody

RESULTS

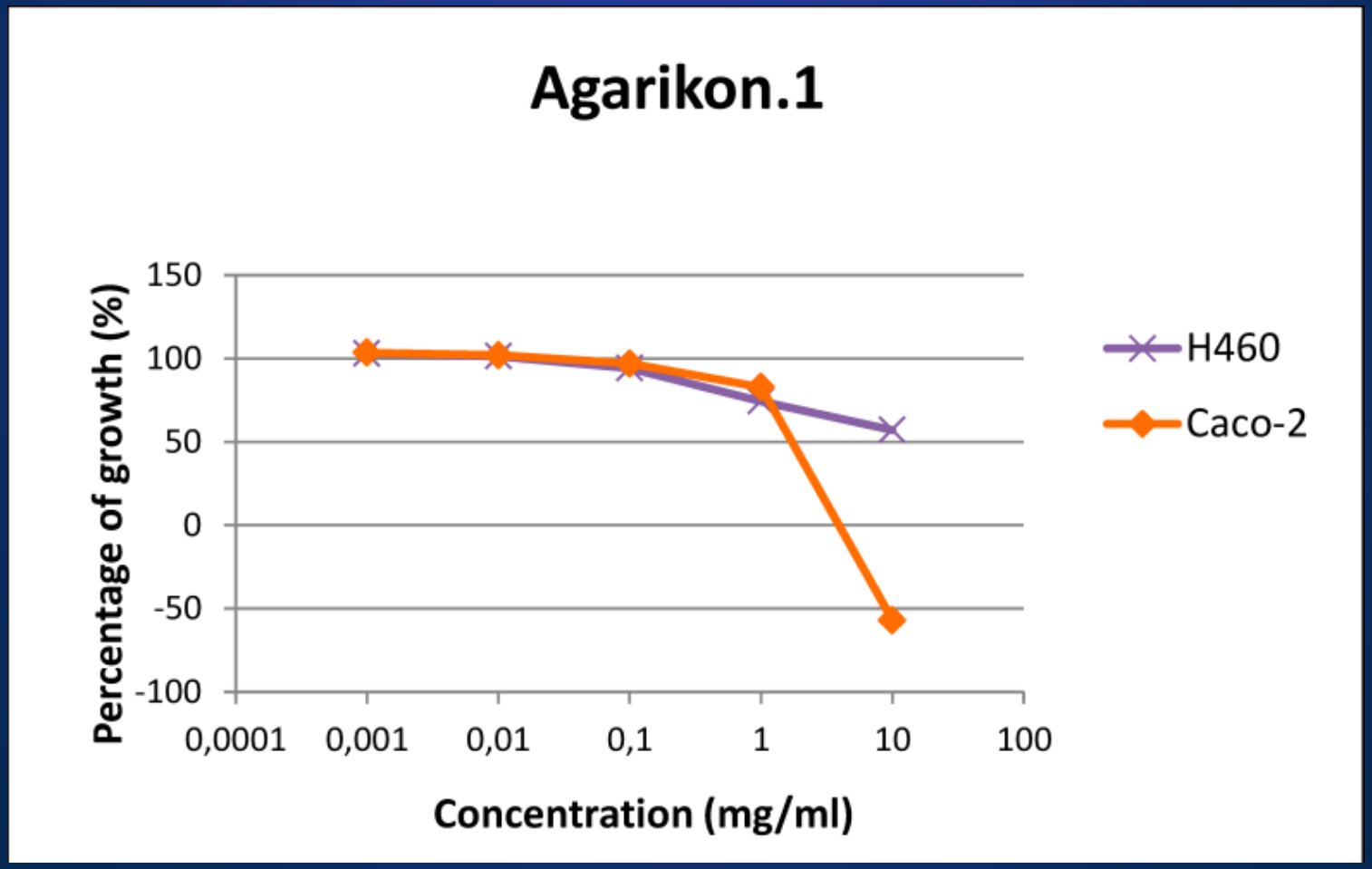
Proliferation Assay

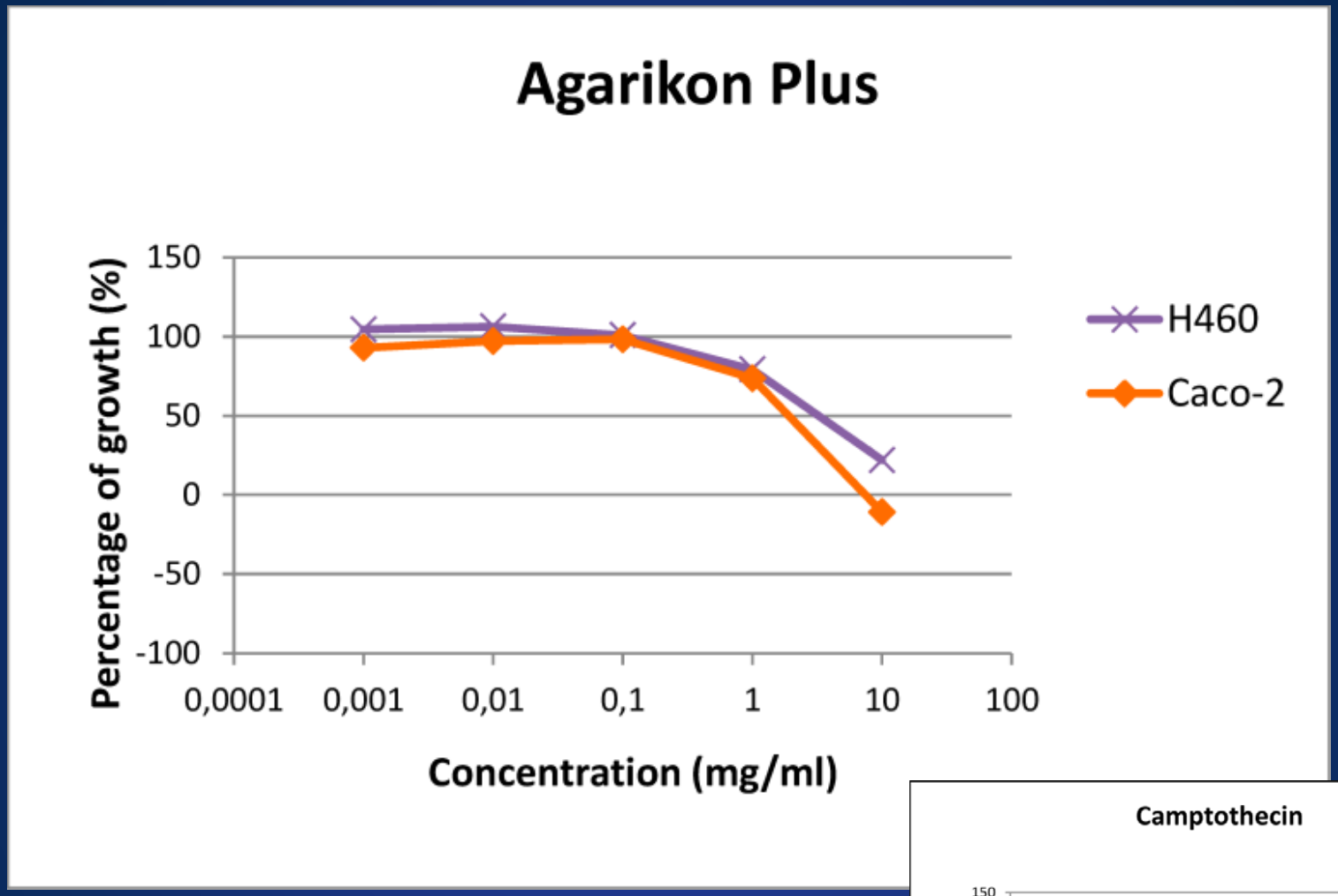
- Agarikon Plus (strong effect at 10 mg/ml, $GI_{50} \approx$ 2-3 mg/ml) and Agarikon.1 inhibit the growth of both tumor cell lines
- H460 cells are more resistant to Agarikon.1 (approaching GI_{50} above 10 mg/ml mass concentration)

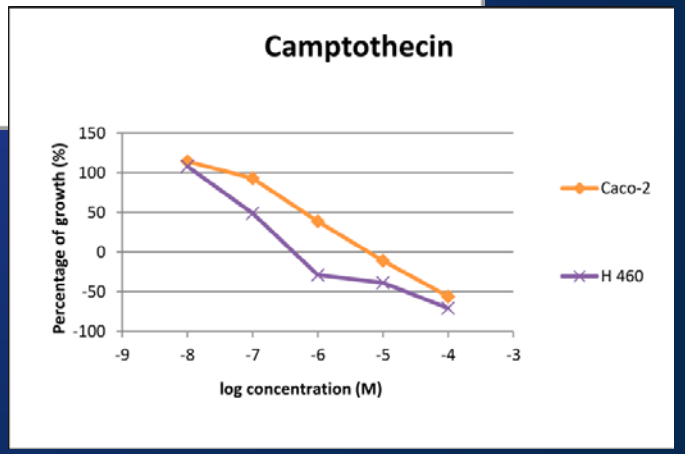
GI_{50}[a] (mg/ml)		
Test agent	Cell lines	
	Caco-2	NCI-H460
Agarikon Plus	1.9 ± 0.1	3.4 ± 1
Agarikon.1	1.6 ± 0.3	≥10

[a] GI_{50}; growth inhibition 50 - the concentration that causes 50% growth inhibition

- Concentration-response curves showing growth inhibition of H460 and Caco-2 cell in vitro after 72 hours after adding Agarikon.1, Agarikon Plus, and camptothecin.



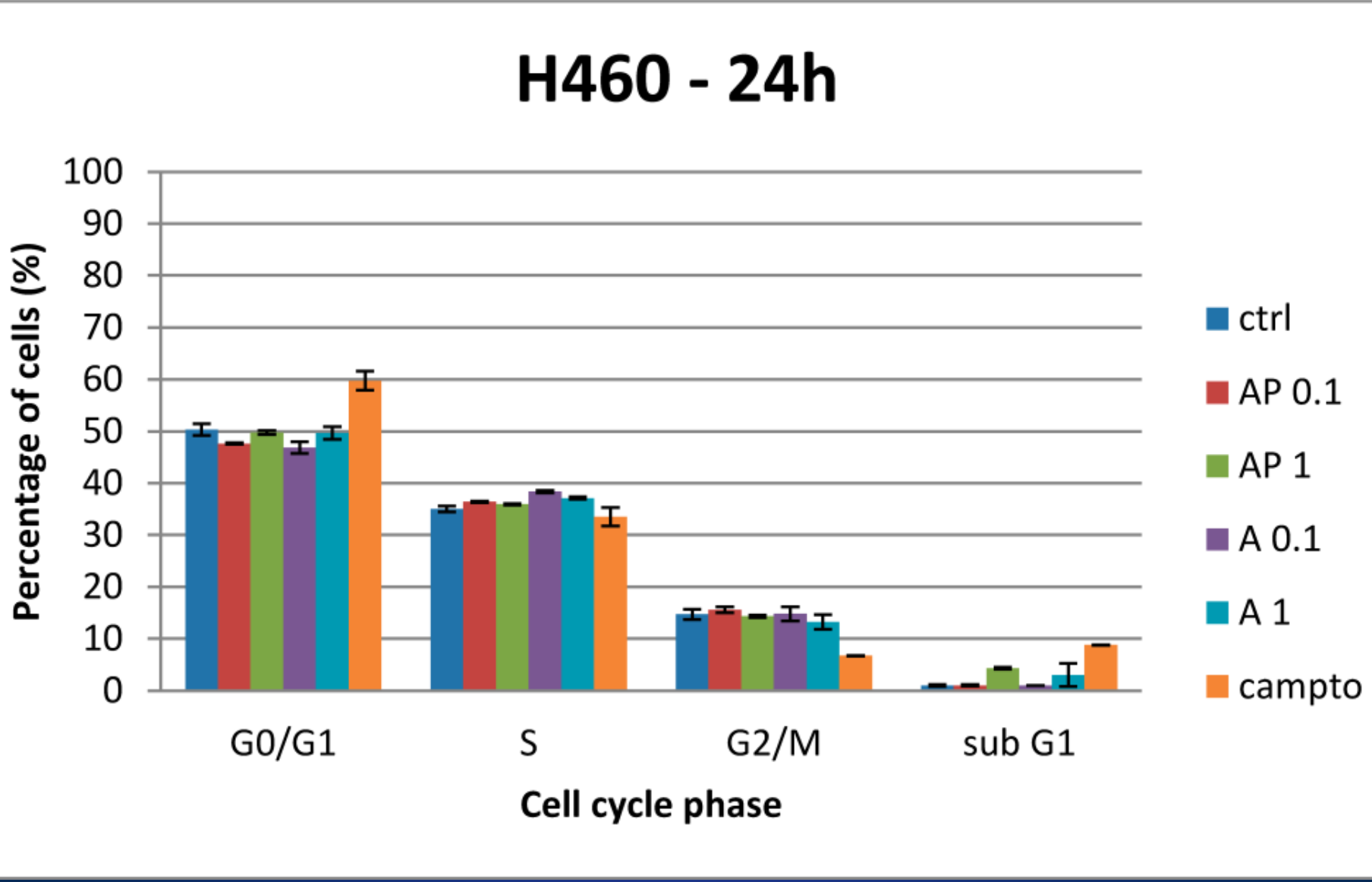




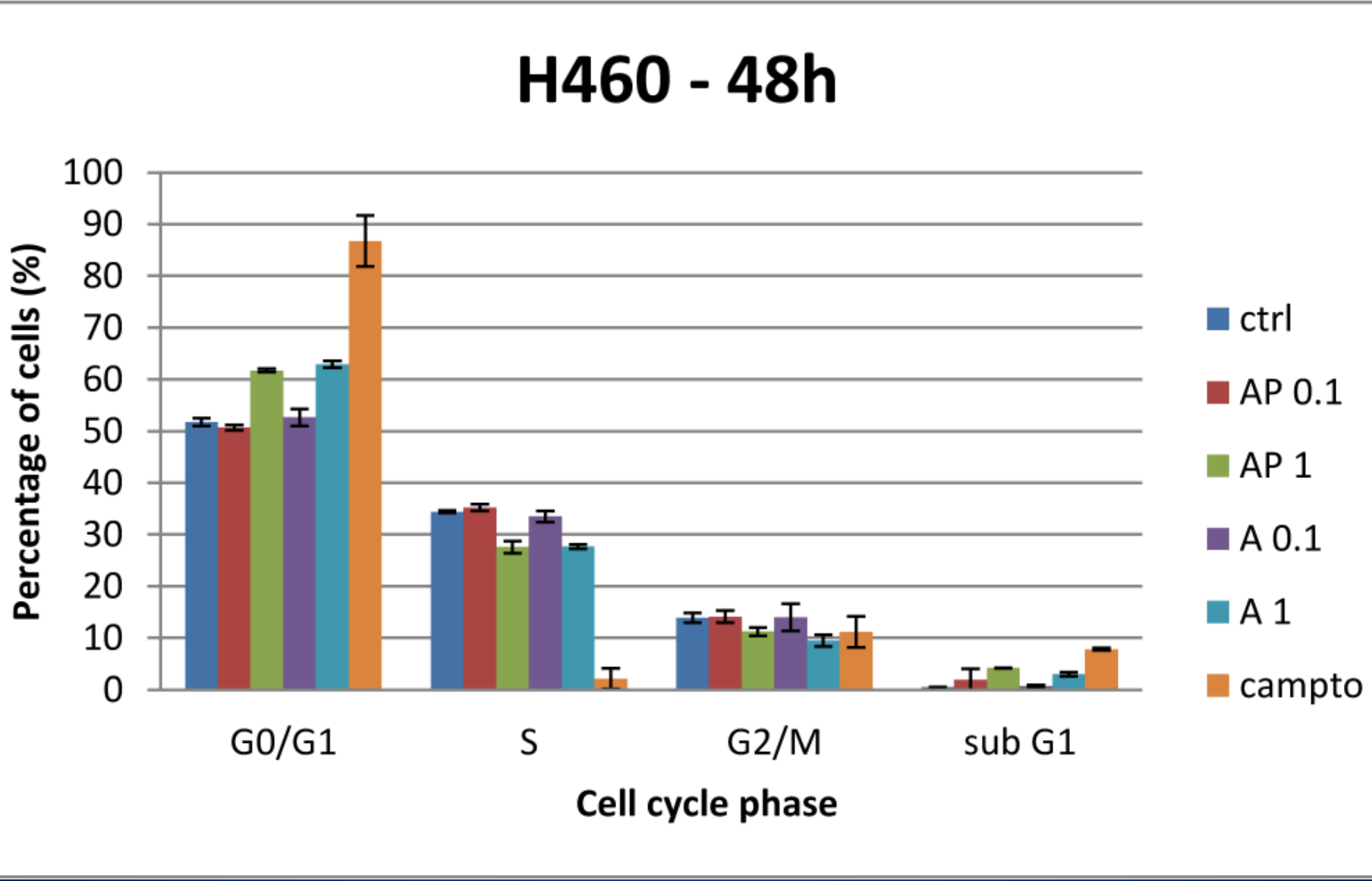
- expected Camptothecin curve confirms valid measurements (positive control)

Cell Cycle Analysis

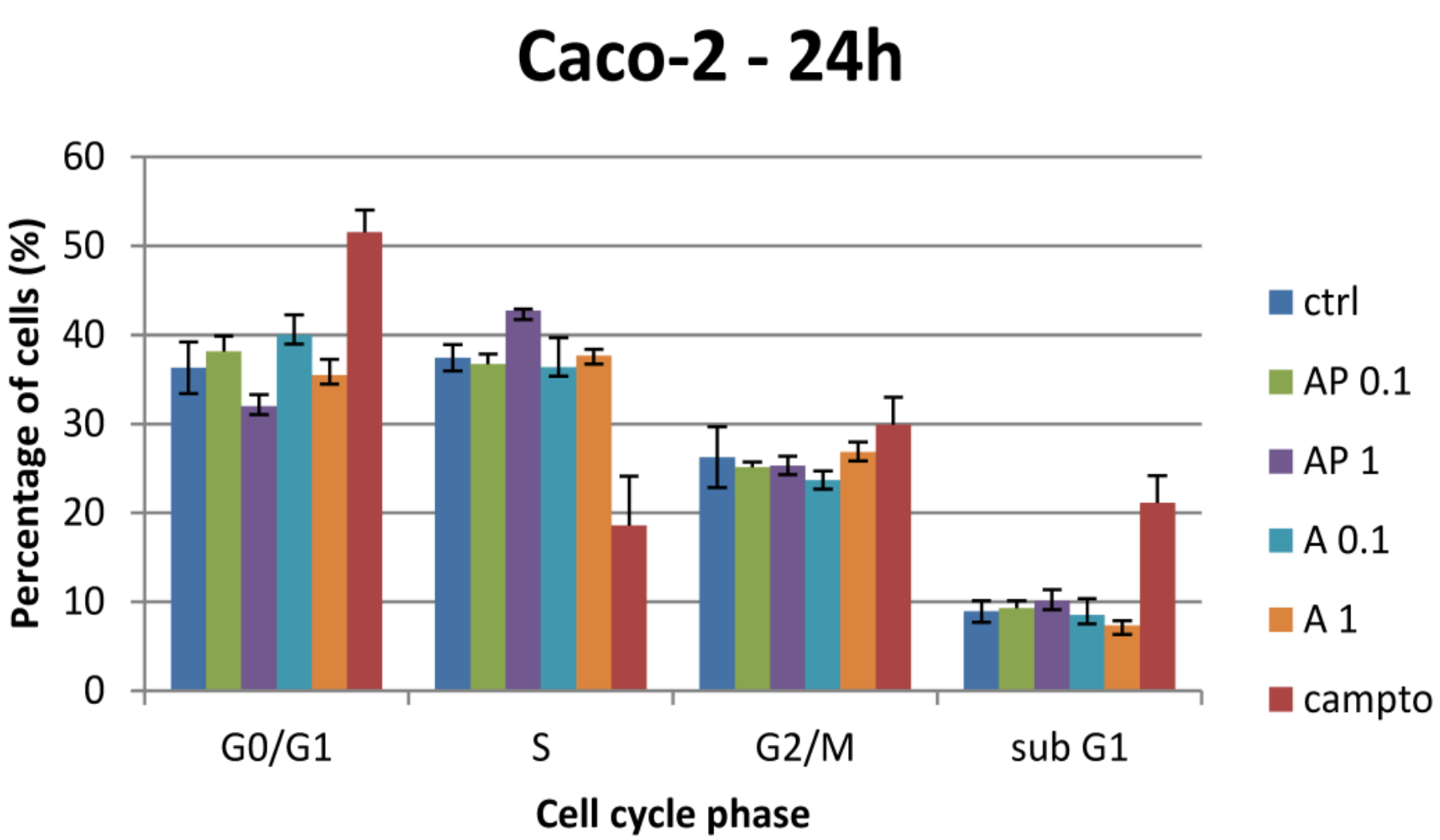
- Distribution of H460 cells by cell cycle phase: G0/G1, S, G2/M; and sub G1 (dead/apoptotic cells)
- treated with Agarikon Plus, Agarikon.1 (at 0.1 and 10 mg/ml), camptothecin at 10 µM.
- measured by flow cytometry at 24 and 48 hours



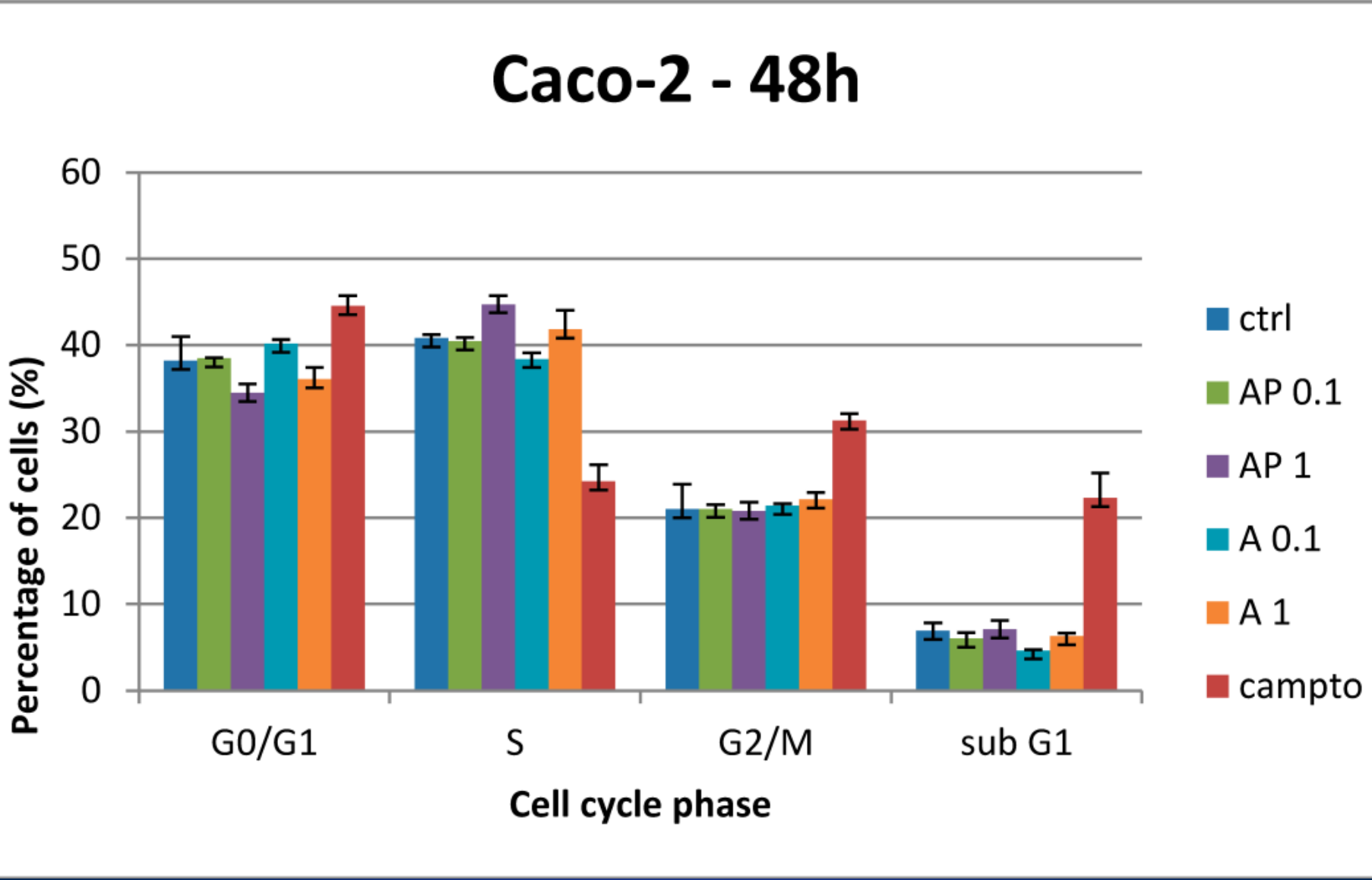
- no significant influence on cell cycle
- moderate increase in apoptotic/dead cells for AG+ at higher concentrations



- AG+ and AG.1 (1mg/ml) induce accumulation of cells in G1, reduction in S, increase in sub G1 (apoptotic/necrotic)



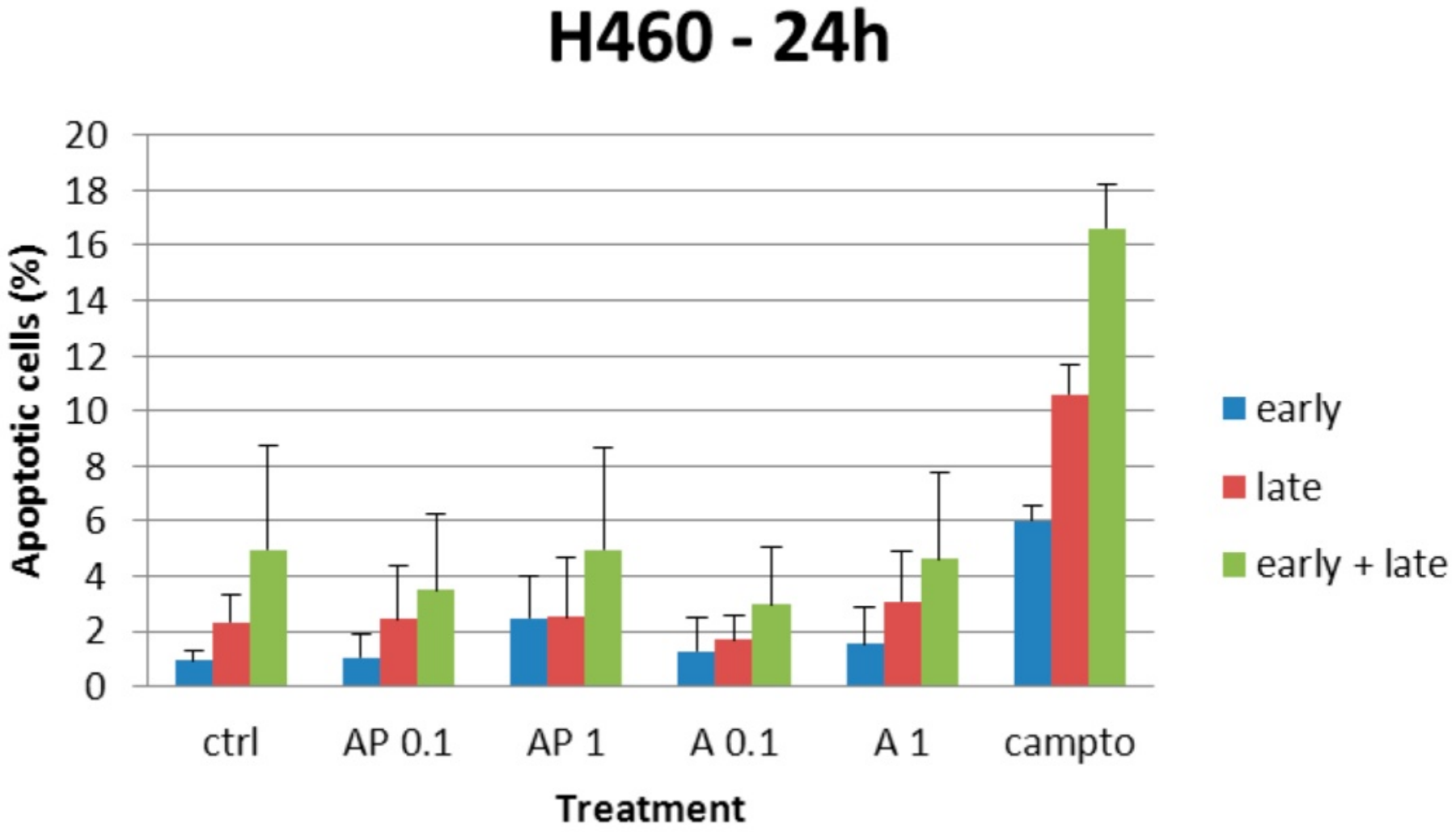
- AG+ (1mg/ml): reduced G1, increased S
- no significant sub G1 influence



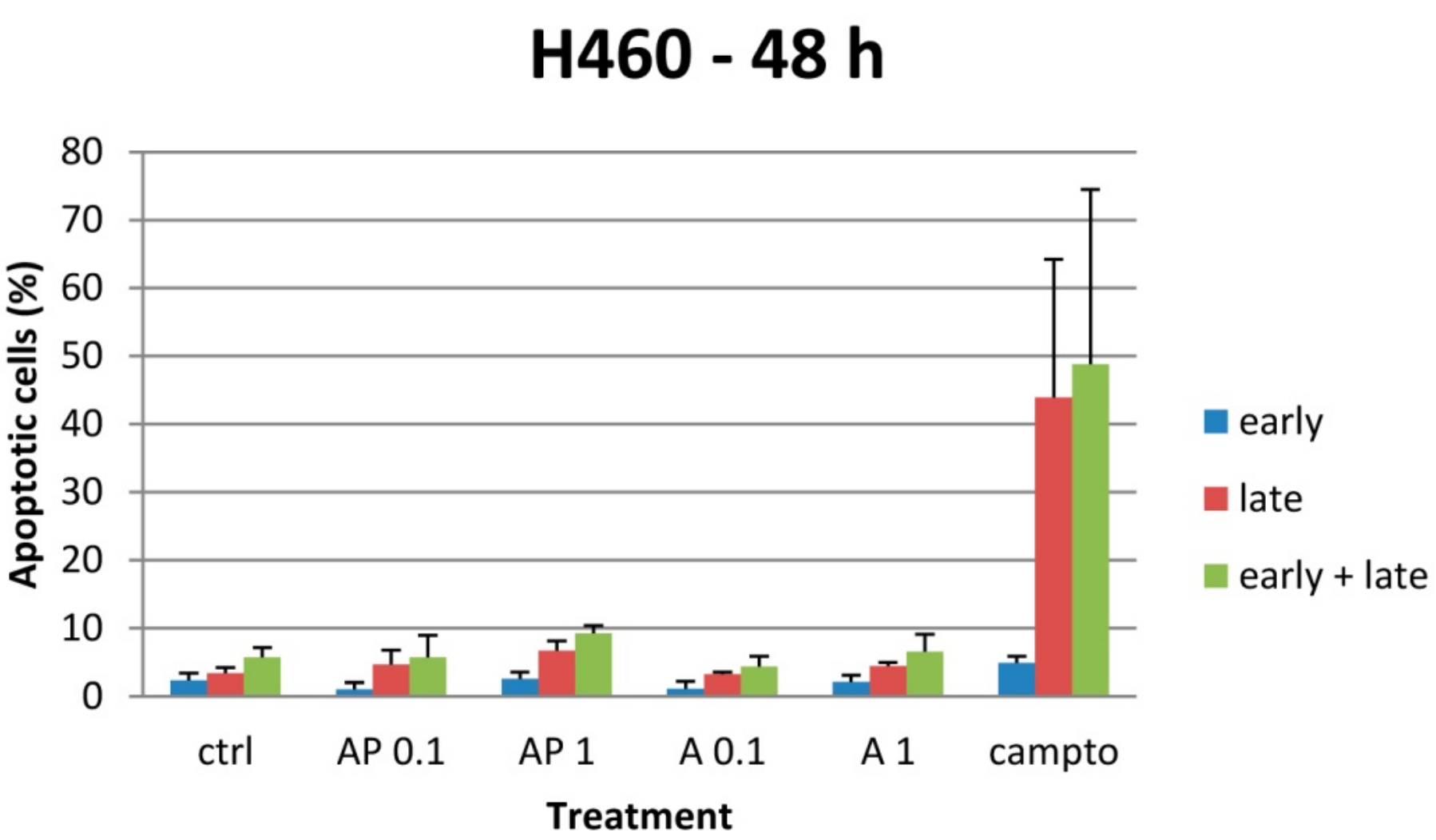
- AG+, AG.1 (1 mg/ml) reduced G1, increased S phase
- no significant sub G1 influence

Apoptosis Induction Detection by Annexin V Assay

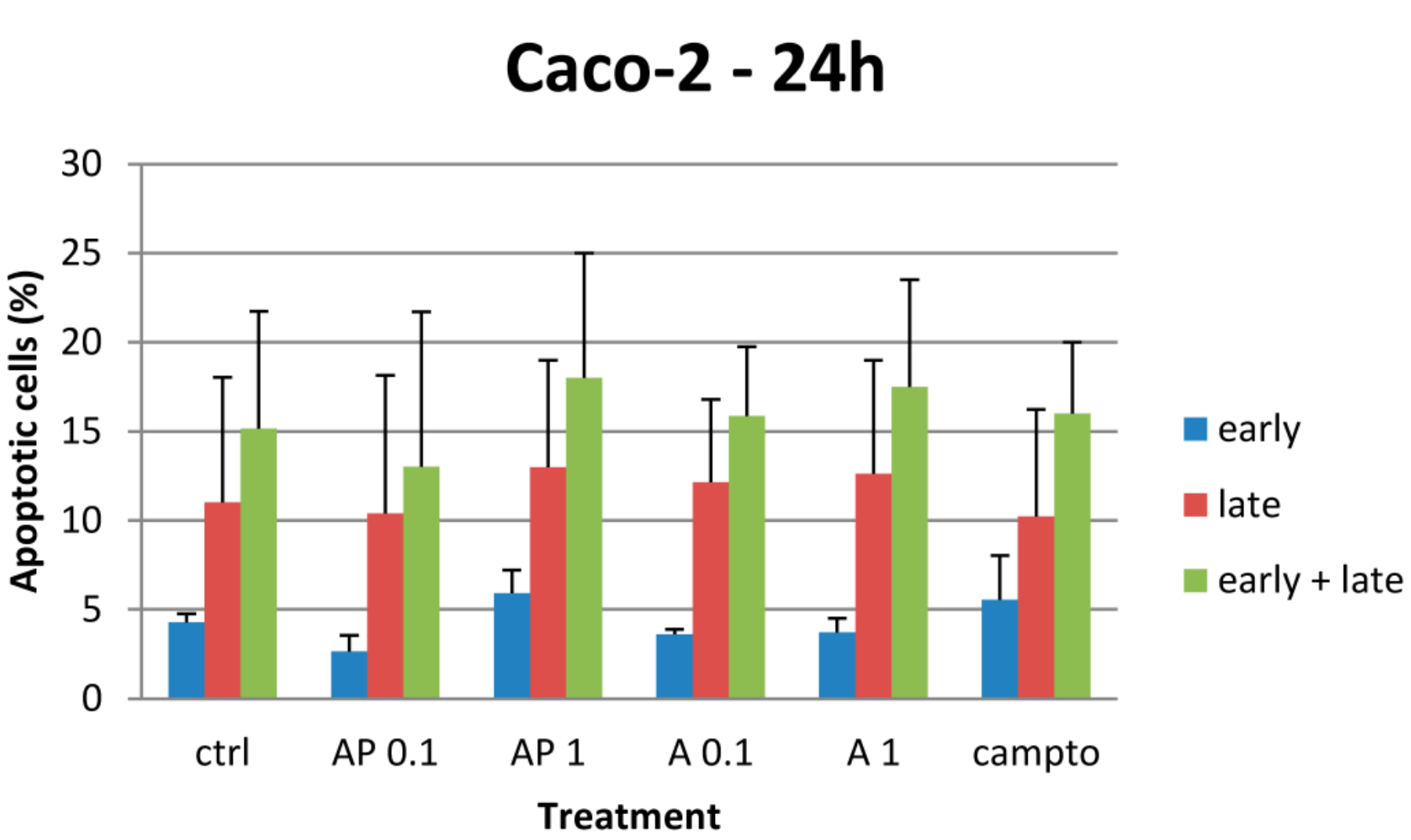
- Ratio of H460 cells in early or late apoptosis, obtained by co-staining with FITC-labeled annexin V and propidium iodide (PI) and analyzed by flow cytometry.



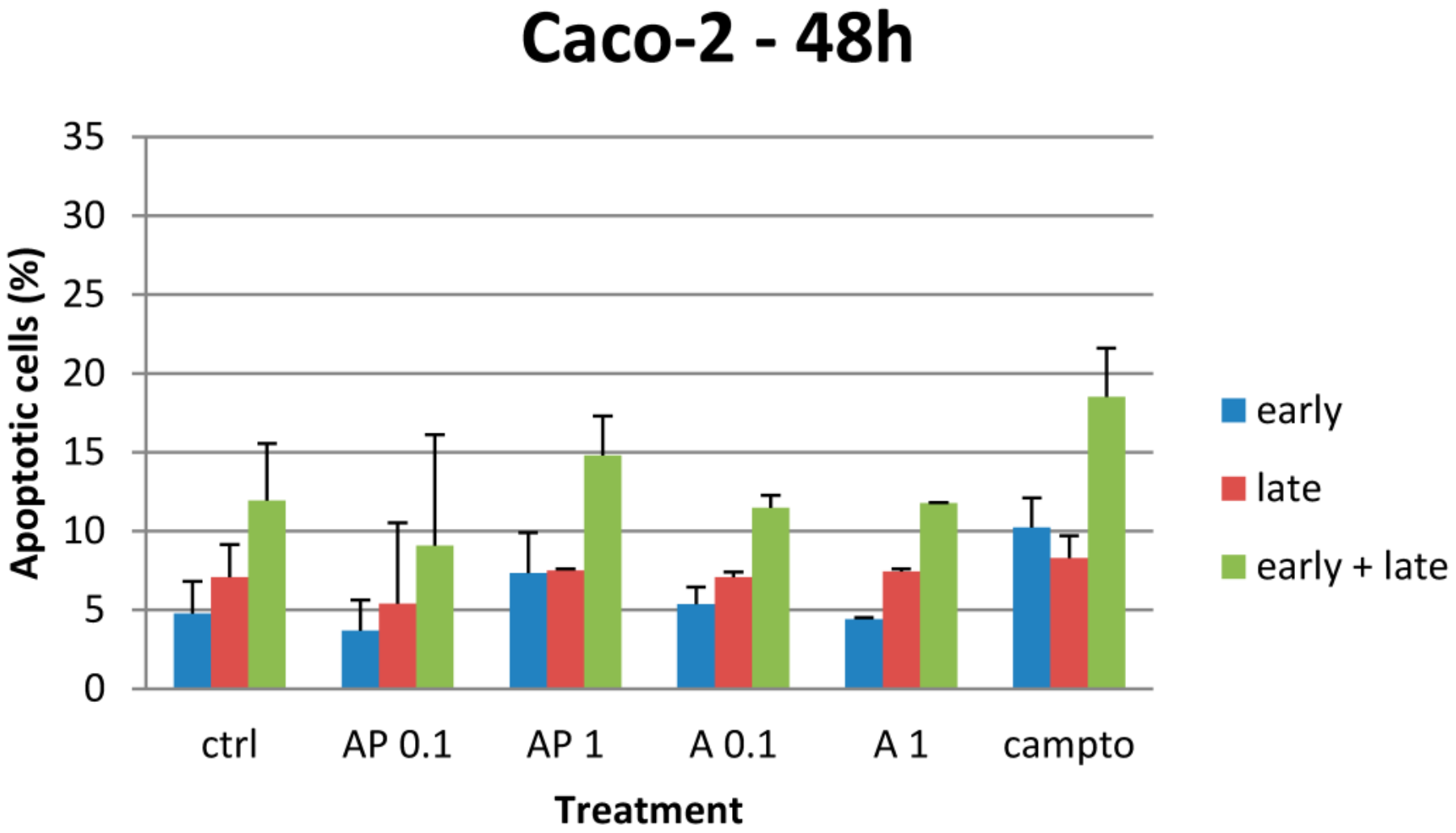
- AG+ (1 mg/ml): moderate increase in early apoptotic cells



- AG+ (1 mg/ml): larger increase in late apoptotic/necrotic cells



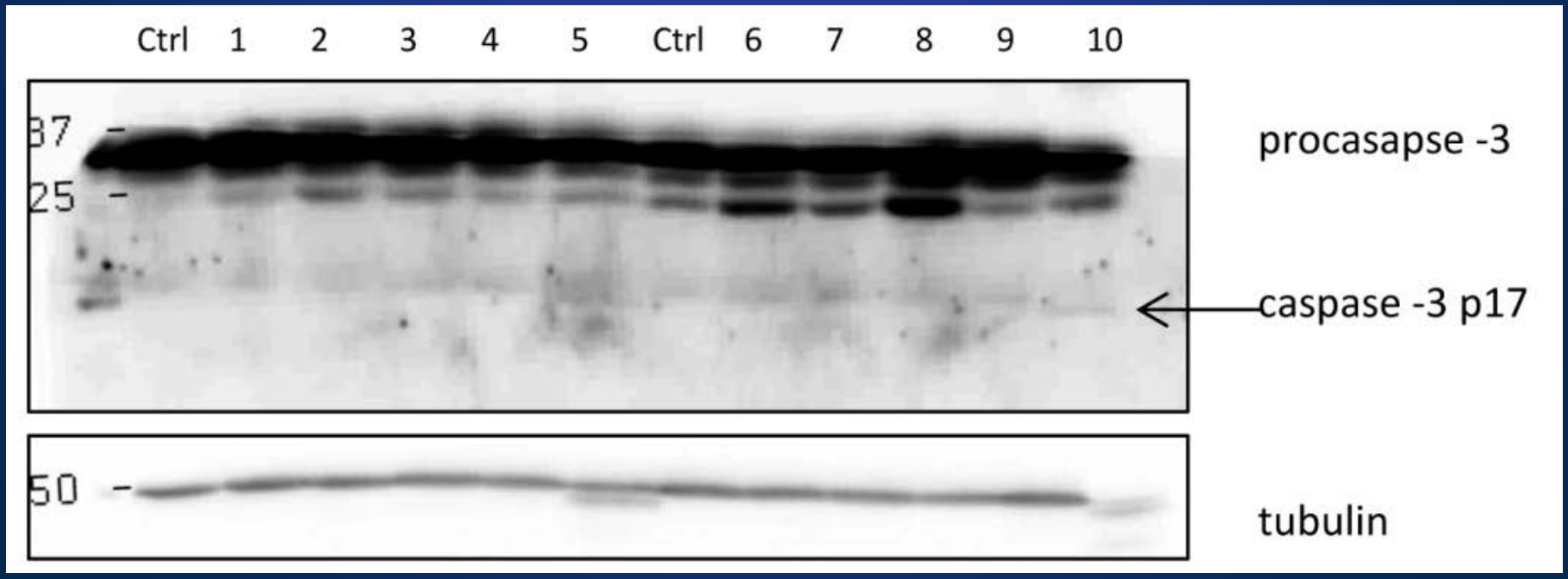
- no significant influence, AG+ (1mg/ml) moderate early apoptotic cell increase



- AG+ (1 mg/ml): moderate increase in induced early apoptosis

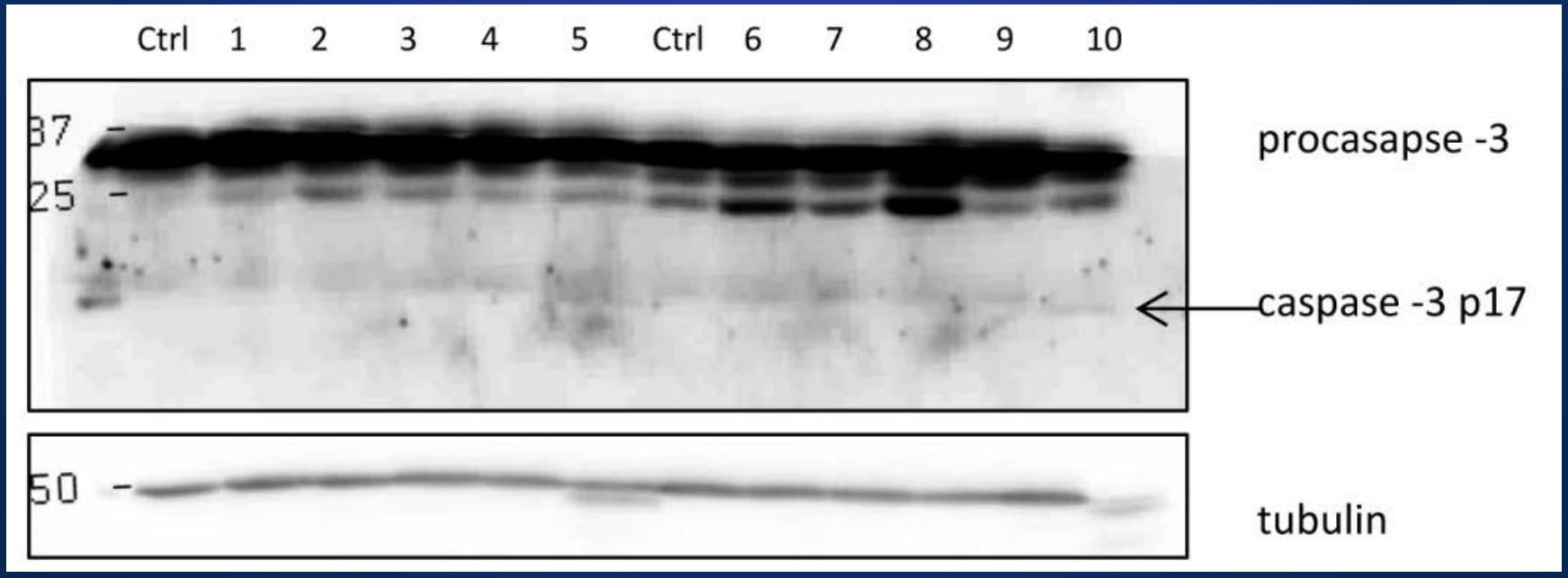
Apoptosis induction detection by caspase-3 cleavage assessment

- Appearance of the 17-kDa subunit (caspase-3 p17) – a major cleaved product of the 32-kDa zymogen procaspase-3 - confirms caspase-3 activation which marks the induction of apoptosis.



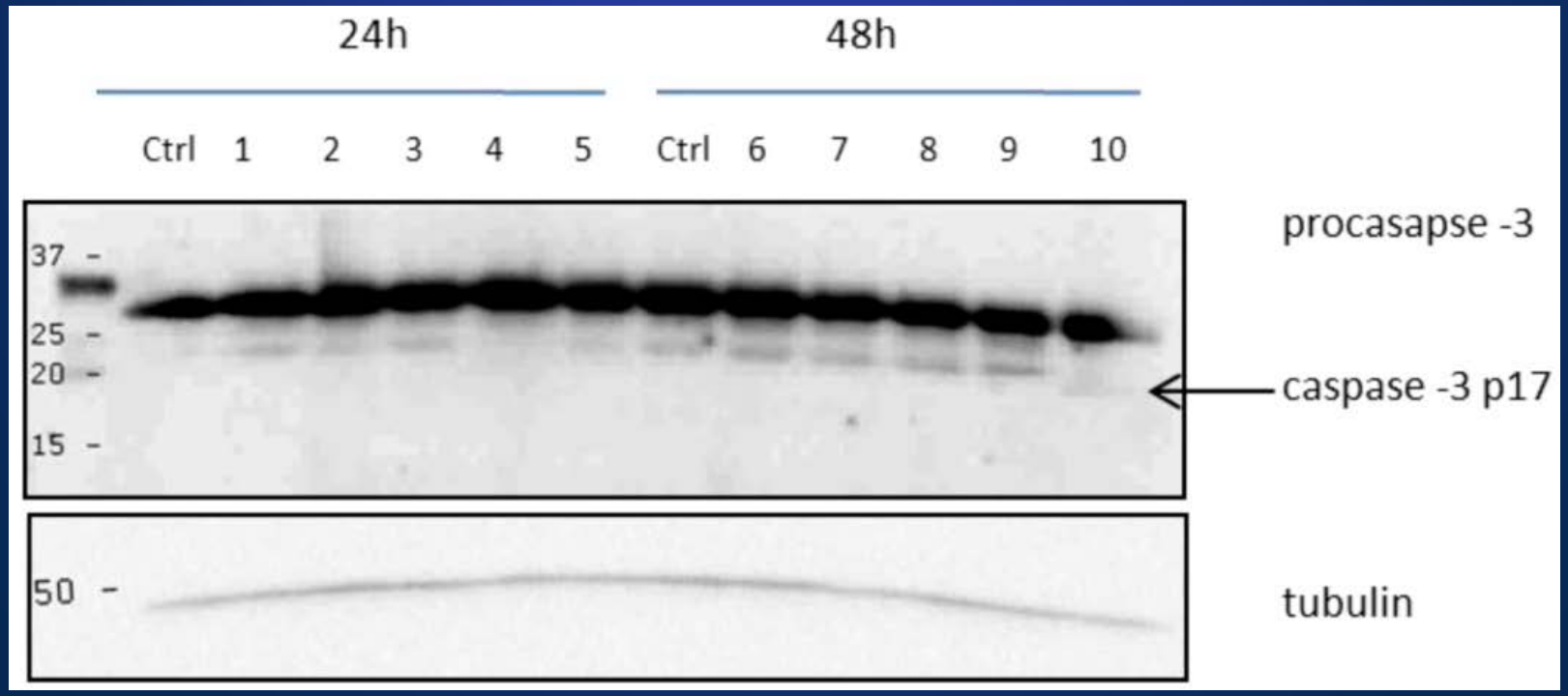
The effect of AG+ and AG.1 on cleavage of procaspase-3 in H460 cells (24; 48 hours)

A. AG+ at 0.1 mg/ml(lanes 1;6), 1 mg/ml (lanes 2;7).
B. AG.1 at 0.1 mg/ml (lanes 3;8), 1 mg/ml (lanes 4;9)
C. Camptothecin at 10μM (lanes 5;10)



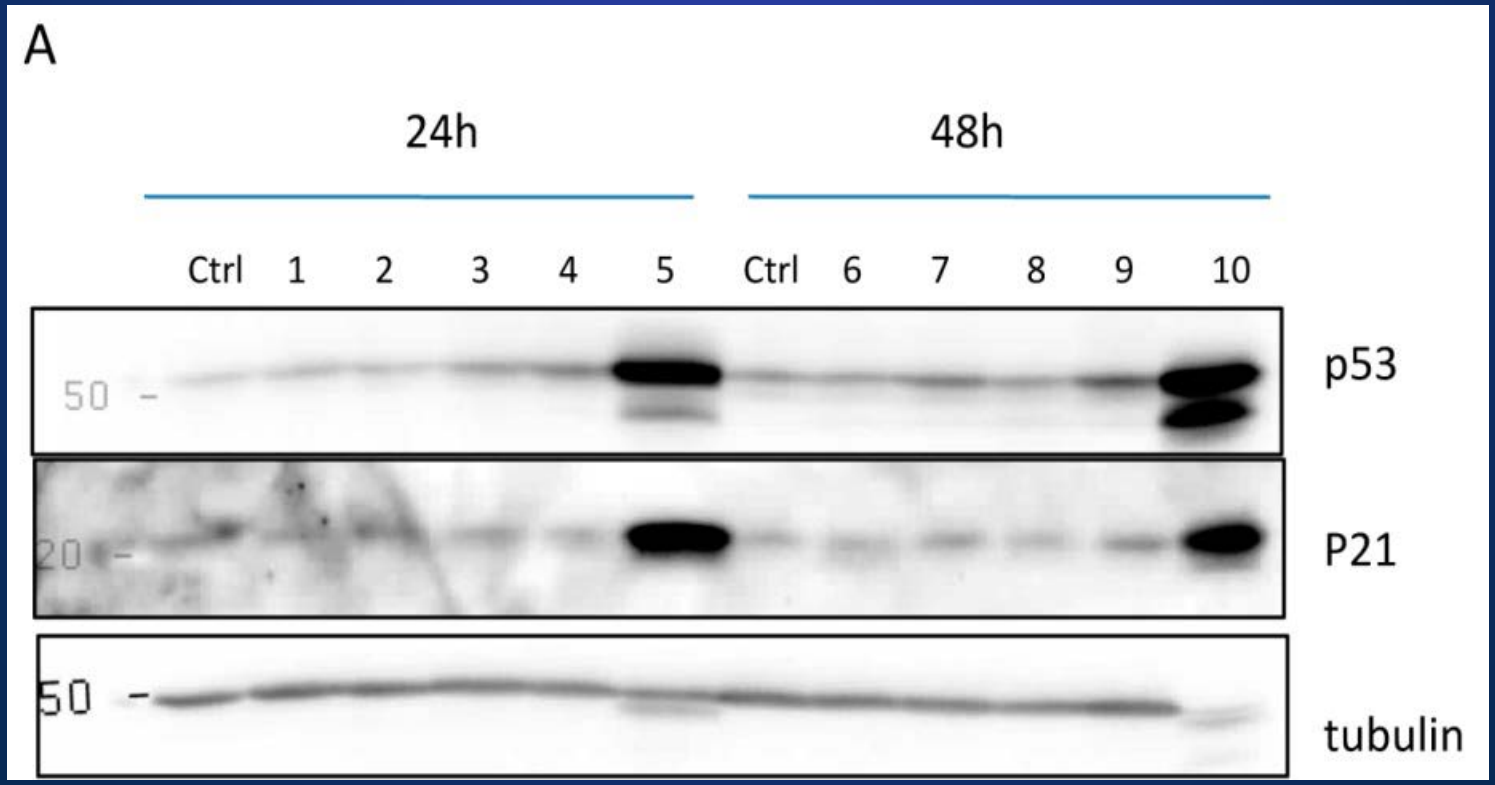
The effect of AG+ and AG.1 on cleavage of procaspase-3 in Caco-2 cells (24; 48 hours)

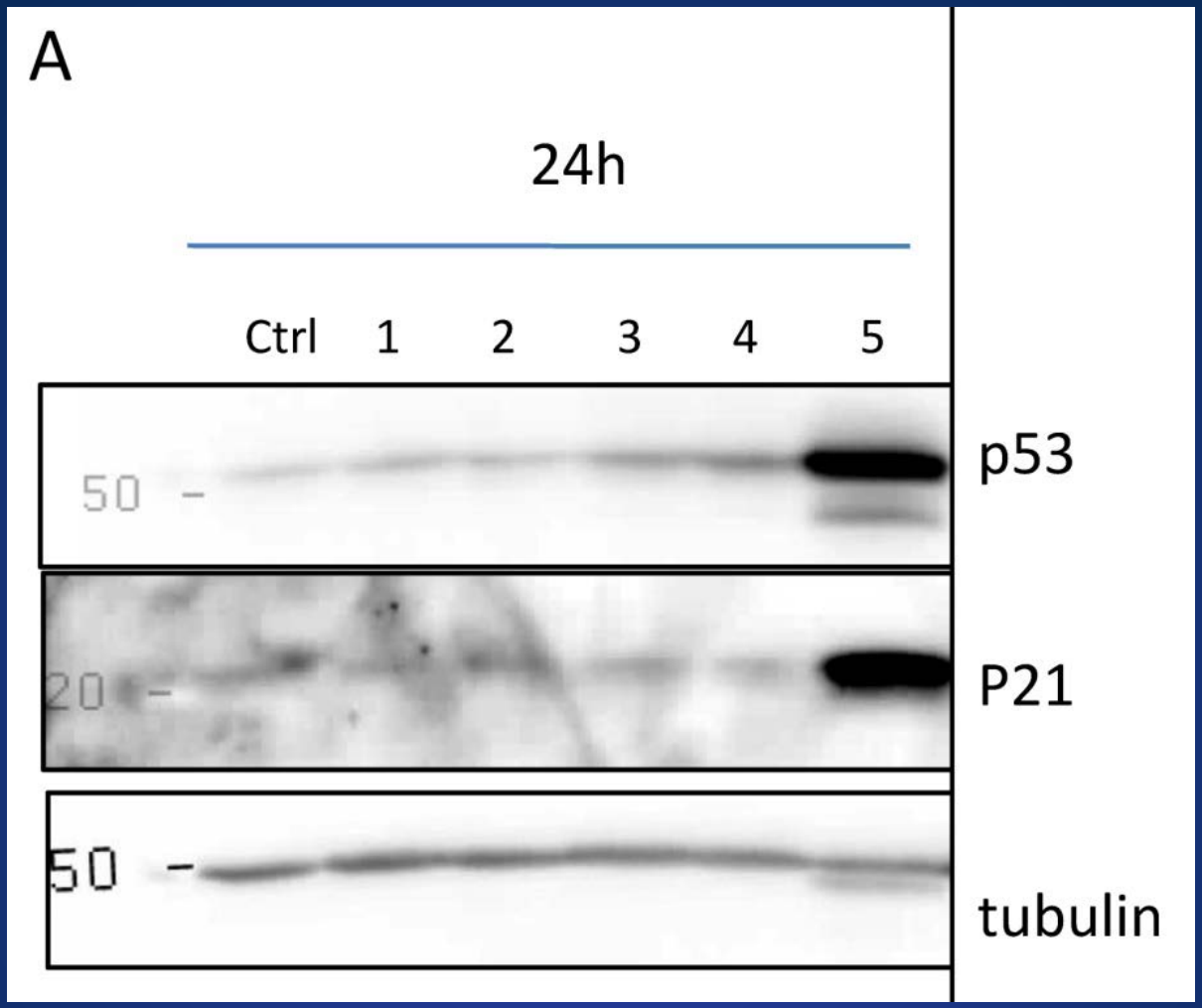
A. AG+ at 0.1 mg/ml (lanes 1;6), 1 mg/ml (lanes 2;7).
B. AG.1 at o.1 mg/ml (lanes 3;8), 1 mg/ml (lanes 4;9)
C. Camptothecin at 10μM (lanes 5;10)



Influence of AG+ and AG.1 on p53 and p21 protein expression

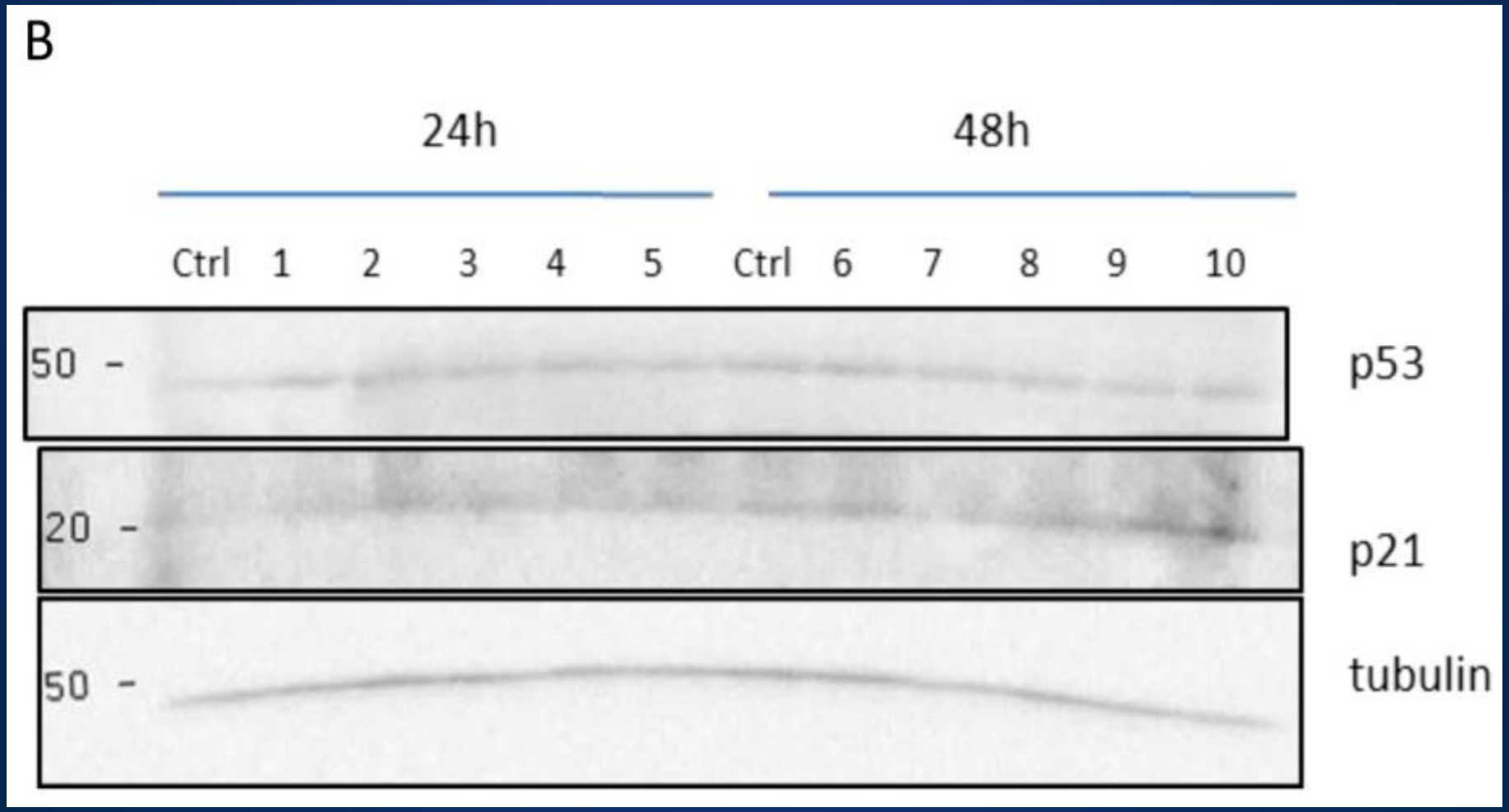
- Agarikon.1, in both concentrations, induced mild p53 protein expression in H460 after 24 hours (A; lanes 3 and 4)





- Both Agarikon Plus (lane 2) and Agarikon.1 (lane 4) at the concentration of 1 mg/ml induced a moderate increase in the expression of both p53 and p21 after 24 hours in H460

- In Caco-2 cells (B), only minor upregulation of p21 protein expression is detected after 48 hour treatment.



CONCLUSIONS

- Agarikon Plus and Agarikon.1 possess antiproliferative, mainly cytostatic activity, on H460 and Caco-2 cells, in the concentration range 1-10 mg/ml
- Both induce cell cycle perturbations, by delaying the progress through the G1 and S phase
- This points to disturbances occurring before or during DNA replication (confirmed by increase in both p53 and p21 protein expression)

CONCLUSIONS (II)

- Although a modest induction of early (after 24 hours) and late (48 hours) apoptosis was noticed by annexin V test, no processing (cleavage) of caspase-3 was detected
- More-pronounced antiproliferative activity (MTT) of tested agents towards Caco-2 line at maximal concentration (10 mg/ml) points to a non-specific cytotoxic effect

Acknowledgements

Dr. Marijeta Kralj

Dr. Ana-Matea Mikecin

Biozyne, Bijenicka c. 54, 10000 Zagreb,
Rudjer Boskovic Institute

Thank you for your attention!

- Send correspondence to: borisjakopovic@yahoo.com

APPENDIX

Percentage of Growth definition

- The percentage of growth (PG) was calculated according to either of the following expressions:
- If $(A_{test} - A_{tzero}) \geq 0$ then:
- $PG = 100 \times (A_{test} - A_{tzero}) / (A_{con}t - A_{tzero})$

- If $(A_{test} - A_{tzero}) < 0$ then:
- $PG = 100 \times (A_{test} - A_{tzero}) / A_{tzero}$
- Where:
- A_{tzero} = the average absorbance before exposure of the cells to the test compound
- A_{test} = the average absorbance after the desired period of time (72 h)
- A_{cont} = the average absorbance after 72 hours with no exposure of cells to the test compound

- The results were presented as concentration response curves and GI50

Camptothecin Proliferation Assay