Vorinostat enhances chemosensitivity to arsenic trioxide in K562 cell line

Nainong Li, Xiaoyan Guan, Fang Li, xiaofan li, Yuanzhong Chen

Objective **:** This study aimed to investigate the chemosensitive augmentation effect and mechanism of HDAC inhibitor Vorinostat (SAHA) in combination with arsenic trioxide (ATO) on proliferation and apoptosis of K562 cells. Methods **:** The CCK-8 assay was used to compare proliferation of the cells. Annexin-V and PI staining by flow cytometry and acridine orange/ethidium bromide stains were used to detect and quantify apoptosis. Western blot was used to detect expression of p21, Akt, pAkt, p210, Acetyl-Histone H3, and Acetyl-Histone H4 proteins. Results **:** SAHA and ATO inhibited proliferation of K562 cells in an additive and time- and dose-dependent manner. SAHA in combination with ATO showed significant apoptosis of K562 cells in comparison to the single drugs alone (p <0.01). Both SAHA and ATO alone and in combination showed lower levels of p210 expression. SAHA and SAHA and ATO combined treatment showed increased levels of Acetyl-Histone H3 and Acetyl-Histone H4 protein expression. SAHA alone showed increased expression of p21, while ATO alone and in combination with SAHA showed no significant change. SAHA and ATO combined therapy showed lower levels of Akt and pAkt protein expression than SAHA or ATO alone. Conclusion **:** SAHA and ATO combined treatment inhibited proliferation, induced apoptosis, and showed a chemosensitive augmentation effect on K562 cells. The mechanism might be associated with increasing histone acetylation levels as well as regulating the Akt signaling pathway.

2 **Vorinostat enhances chemosensitivity to arsenic trioxide in K562 cell line**

4 Running title: combination treatment of SAHA and ATO on K562 cell line

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Abstract:

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32 Objective:This study was aimed to investigate the chemosensitive augmentation effect and mechanism of HDAC

33 inhibitor Vorinostat (SAHA) in combination with arsenic trioxide(ATO)on proliferation and apoptosis of K562

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35 Methods:The CCK-8 assay was used to compare proliferation of the cells. Annexin-V and PI staining by flow

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37 used to detect expression of p21, Akt, pAkt, p210, Acetyl-Histone H3, and Acetyl-Histone H4 proteins.

38 Results: SAHA and ATO inhibited proliferation of K562 cells in an additive and time- and dose-dependent manner.

39 SAHA in combination with ATO showed significant apoptosis of K562 cells in comparison to the single drugs alone

40 (p <0.01). Both SAHA and ATO alone and in combination showed lower levels of p210 expression. SAHA and

41 SAHA and ATO combined treatment showed increased levels of Acetyl-Histone H3 and Acetyl-Histone H4 protein

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45 Conclusion: SAHA and ATO combined treatment inhibited proliferation, induced apoptosis, and showed a

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47 acetylation levels as well as regulating the Akt signaling pathway.

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50 **Keywords:**

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58 **Introduction:**

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60 Histone acetylation and deacetylation comprise one of the common modifications found in epigenetics.

61 Histone acetylation helps transcription factors bind to DNA templates and activates transcription;

62 however, histone deacetylation inhibits transcription. [1-4] In 2006, the second generation of hydroxamic

63 acids histone deacetylase inhibitor (HDACi) SAHA (suberoylanilide hydroxamic acid, Vorinostat) was

64 approved by the FDA for clinical treatment of relapsed or refractory cutaneous T-cell lymphoma. [5]

65 Chronic myeloid leukemia (CML) is a malignant clonal disease derived from hematopoietic stem cells,

66 and it manifests itself through characteristic molecular markers: the bcr-abl fusion gene and the

67 expression of p210 bcr-abl fusion protein. Tyrosine kinase inhibitors (TKI) are the first line of treatment

68 for CML. Although TKIs target the bcr-abl gene, they are unable to eradicate the malignant clone

69 completely. Moreover, drug resistance has been observed in clinical applications. To better eradicate

70 the CML clone and overcome the drug resistance, we hypothesized that SAHA augments

71 chemosensitivity when combined with Arsenic trioxide (ATO), another widely used chemoreagent for

72 clinical treatment of hematologic malignancies. Thereby, we investigated the anti-leukemia effect of

73 SAHA combined with ATO on the K562 cell line.

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82 **Material and Methods**

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84   1. Reagent and cell lines

85   SAHA was kindly provided by Dr. Defu Zeng (City of Hope National Medical Center, US, 91010).

86   DMSO was diluted to a concentration of 10 mmol / L. ATO was purchased from Heilongjiang Harbin

87   Medical University Pharmaceutical Co., Ltd., and diluted to a concentration of 2 mmol / L. Both SAHA

88   and ATO were stored at -20 °C. RPMI 1640 was purchased from Gibco; fetal bovine serum was

89   purchased from TBD; penicillin / streptomycin was purchased from Hyclone; an apoptosis detection kit

90   (Annexin-V-FITC, PI double staining) was purchased from Roche; Protein Extraction reagents were

91   purchased from Wuhan Boster Biological Co., Ltd.; antibodies against c-abl, p21, Acetyl-Histone H3

92   and Acetyl-Histone H4, and other proteins were purchased from Cell Signaling Technology. The K562

93   cell line was preserved and cultured in Fujian Institute of Hematology with a standard protocol as

94   previously described. [6,7]

95

96   2. Proliferation assay

97   A CCK-8 assay was used to detect cell line proliferation. A positive control, negative control and the

98   drug treated group (SAHA: 0.25,0.5,1,2 μmol / L; ATO: 1,2,4,8 μmol / L) were detected. The assay

99   was completed in triplicate for each group. Next, 5×10^4 cells / ml were seeded into a 96-well culture

100   plate to a final volume of 100 μl. After 24, 48, and 72 h of treatment, 10 μl of CCK-8 was added into

101   each well and incubated for 1-4 h at 37 ° C in a 5% CO_2 incubator. The absorbance at 450 nm was

102   measured using a microplate reader. The inhibition rate was calculated using the following equation:

103   Inhibition rate = [(control group - experimental group) / blank group] × 100%. A proliferation curve was

104   plotted based on the drug concentration and the proliferation inhibition rate. The Q value was

105   calculated to measure the combined effect of SAHA and ATO. Q was calculated using the following

106   equation: Q = E (ab) / (Ea + Eb-Ea × Eb) as previously described. [6,7] Generally, Ea and Eb are the

107   single drug inhibition rates; E (ab) is associated with the inhibition rate of SAHA and ATO. Q > 1.15

108   was considered to be a synergistic effect, 0.85 > Q > 1.15 an additive effect, and Q < 0.85 an

109   antagonistic effect.

110

111 3. Annexin-V and PI staining

112 Apoptosis was detected by Annexin-V and PI staining. Generally, K562 cells(1×10^6) and the

113 appropriate drugs were co-incubated for 48 h. Cells were harvested after a single wash with PBS.

114 Next, binding buffer (100 μl) was added to resuspending cells.   Annexin-V (2 μl) and PI (2 μl) were

115 added, and the cells were incubated at room temperature for 10-15 min in the dark. K562 cell were

116 detected by flow cytometry. Annexin-V$^-$/ PI$^-$ cells were living cells, and Annexin-V $^+$ / PI$^-$ cells were early

117 apoptotic cells. Annexin-V $^+$ / PI $^+$ were late apoptotic cells. This experiment was repeated three times.

118

119 4. AO / EB fluorescence staining

120 K562 cells and the drugs were co-incubated as described above. Suspended cells (95 μl, 5×10^6/ml)

121 were mixed with AO / EB (5 μl). Immediately after mixing, one drop of suspended cells was placed on

122 a clean glass slide and observed by fluorescence microscopy at an excitation wavelength of 490 nm.

123 Cells with green fluorescence in the nucleus and cytoplasm were normal cells; cells with yellow-green

124 fluorescence in the nucleus or cytoplasm were apoptotic cells; cells with red fluorescence in nucleus

125 were necrotic cells.

126

127 5. Western Blot

128 K562 cells were co-cultured as described above. After the cells were lysed, the supernatant was

129 collected and quantified. Total protein (40 μg) was transferred to a PVDF membrane after SDS-PAGE

130 electrophoresis. After blocking at room temperature for 2 h, the primary antibodies (against p210, p21,

131 Acetyl-Histone H3, Acetyl-Histone H4,Akt and pAkt) were added and incubated at 4 °C overnight.

132 After washing, the secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG) was

133 added and incubated for 2 h. The chemiluminescence reaction was performed. β-actin was used as

134 an internal control.

135

136 6. Statistical analysis

137 Statistical analysis was performed with SPSS 11.5. Means were compared by the Dunnett-t test, with

138 P <0.05 considered to be a significant difference.

139

140 **Results**

141

142 **SAHA in combination with ATO showed an additive effect on the inhibition of proliferation**

143

144 Compared with the control group, SAHA and ATO inhibited proliferation of K562 cells in a time- and

145 dose-dependent manner. After the cells were treated with SAHA and ATO, cell proliferation was

146 significantly inhibited (Fig. 1). SAHA in combination with ATO showed an additive effect on the

147 inhibition of proliferation (Table 1).

148

149 **SAHA in combination with ATO showed more apoptosis than the single drugs alone**

150

151 Annexin-V-FITC/PI double staining flow cytometry can distinguish early apoptotic cells, late apoptotic

152 cells and necrotic cells. In the lower left quadrant are normal cells; in the lower right quadrant are early

153 apoptotic cells; in the upper right quadrant are late apoptotic cells; in the upper-left quadrant are

154 necrotic cells. The results showed that 95% of the cells in the control group were living cells; the single

155 drug apoptosis rates increased with increasing drug concentration. The K562 cells apoptosis rates

156 were 10.85 ± 0.65％, 29.65 ± 1.75％, and 84.00 ± 0.80％, 48 h after treatment with SAHA (2 μmol/L),

157 ATO (8 μmol/L) and 8 μmol/L ATO combined with 2 μmol/L SAHA, respectively. SAHA in combination

158 with ATO showed significant apoptosis of K562 cells compared to the single drugs alone (p <0.01, Fig.

159 2A).

160

161 The apoptosis results were confirmed by AO/EB staining. In the control group, K562 cells showed

162 uniform cell size and morphology and homogeneous green fluorescence in the nucleus and cytoplasm.

163 In contrast, 48 h after SAHA and ATO combined treatment, we found differences in cell size and

164 morphology. The nucleus was dense, showing yellow-green fluorescent debris. Additionally, SAHA in

165 combination with ATO showed more apoptosis characteristics than the single drugs alone (Fig. 2B-E).

166

167 **SAHA in combination with ATO showed pronounced changes in protein expression**

168

169 Both SAHA and ATO alone and in the combination group showed lower levels of p210 expression and.

170 The SAHA group and the combination group showed increased levels of Acetyl-Histone H3 and

171 Acetyl-Histone H4 protein expression. SAHA alone showed increased levels of p21 WAF1/CIP1

172 expression, while ATO alone and in the combination group showed no significant changes. The

173 combination group showed lower levels of Akt and pAkt protein expression than SAHA or ATO alone

174 (Fig. 3).

175

176

177 **Discussion**

178

179 There are a variety of HDACIs undergoing clinical trials for the treatment of solid tumors and

180 hematological malignancies, especially for the treatment of cutaneous T-cell lymphoma (CTCL),

181 peripheral T-cell lymphoma, and Hodgkin's lymphoma, which have shown promising results. [8] As a

182 novel HDACI, SAHA was approved by the FDA for the treatment of progressive clinical recurrence of

183 CTCL.

184

185 ATO is an important medicine for treating leukemia (particularly for acute promyelocytic leukemia,

186 APL). Additionally, chemotherapy regimens based on ATO are a popular research topic.   In recent

187 years, the mechanism and clinical applications of ATO have been reported, with induction of oxidative

188 stress and DNA damage included.[9,10] Our study mainly focused on the Akt signal pathway. Huang

189 reported that ATRA and arsenic compound-based combination therapy was effective in re-inducing

190 morphological remission in relapsed patients with APL who had previous exposure to ATRA

191 and arsenic compounds, thus producing low molecular remission rates and high risk of secondary

192 relapse. [11] Interestingly, we also found chemosensitive augmentation of SAHA in the NB4 cell line

193 (data not shown), which indicates that combined SAHA and ATO therapy could have clinical

194 applications in the treatment of leukemia.

195

196 The CCK8 results show that individual treatments with SAHA or ATO inhibited K562 cell proliferation

197 in a time- and dose-dependent manner. The combination treatment showed an additive effect in

198 inhibiting proliferation. Additionally, experimental results showed that at certain combined

199 concentrations of SAHA and ATO, there was a greater level of apoptosis than the single drugs alone,

200 which induced apoptosis over 80%. These results indicated that the combined treatment might have

201 beneficial effects including improved chemotherapeutic effects and minimized side effects.

202

203 In this study, both SAHA and the combination group induced increased expression of Acetyl-Histone

204 H3 and Acetyl-Histone H4 when compared to ATO and the control. This outcome indicates that SAHA

205 can significantly increase histone acetylation levels and activate transcription, thereby inducing

206 hematological tumor cell apoptosis. [3,4]

207

208 p21 is an important cell cycle regulatory protein involved in cell growth, differentiation, aging and death.

209 p21 plays a negative role in the regulation of the cell cycle by acting on CDKI. p21 expression was

210 upregulated after treatment with SAHA compared to ATO and the control, thereby blocking the cell

211 cycle and inducing apoptosis. Interestingly, combination therapy did not induce expression of p21.

212 This mechanism needs to be studied further.

213

214 Akt is a serine / threonine protein kinase, which plays a major role in the anti-apoptotic pathway in

215 apoptosis signaling. [12] We found that both the SAHA group and ATO group downregulate Akt and

216 pAkt expression. Compared to the single drug treatment, the combination group downregulated Akt

217 and pAkt expression drastically, indicating that the mechanism may be associated with the regulation

218 of the Akt signaling pathway.

219

220 p210 fusion protein is the primary cause of CML pathogenesis and progression. Furthermore, the

221 p210 fusion protein is the source of the abnormal phenotype of chronic phase CML. We found that

222 both the SAHA group and the ATO group downregulate p210 fusion protein expression. Also the

223 combination group showed downregulation of p210 protein. Given the dosage which we applied by

224 proliferation experiments, both SAHA and ATO in the concentration are very effective in

225 downregulating the p210 fusion protein expression. It could be more effective by the combination.

226 Taken together, these results indicated that both SAHA and ATO can induce cell apoptosis and

227 inhibition of cell proliferation by downregulating the characteristic p210 fusion protein in K562 cells.

228 Combining SAHA with ATO also lowered the expression of p210, which resulted in increased

229 apoptosis.

230

231 In summary, SAHA and ATO can significantly inhibit the proliferation of K562 cells and induce apoptosis.

232 Combined treatment can enhance apoptosis effects within a certain concentration range. The

233 mechanism may be associated with increased histone acetylation and the regulation of the Akt

234 signaling pathway. This study may provide a new combination chemotherapy regimen for the treatment

235 of CML. Further *in vivo* experiments and animal model should be set up to test the enhanced

236 chemosensitivity effect by combining SAHA and ATO. We also need to observe the side effects

237 by combining SAHA and ATO and prepare data for translational medicine work.

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269

270 **Conflict of interest:** The authors declare no conflict of interests.

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Tab.1: SAHA in combination with ATO showed an additive effect on the inhibition of proliferation.

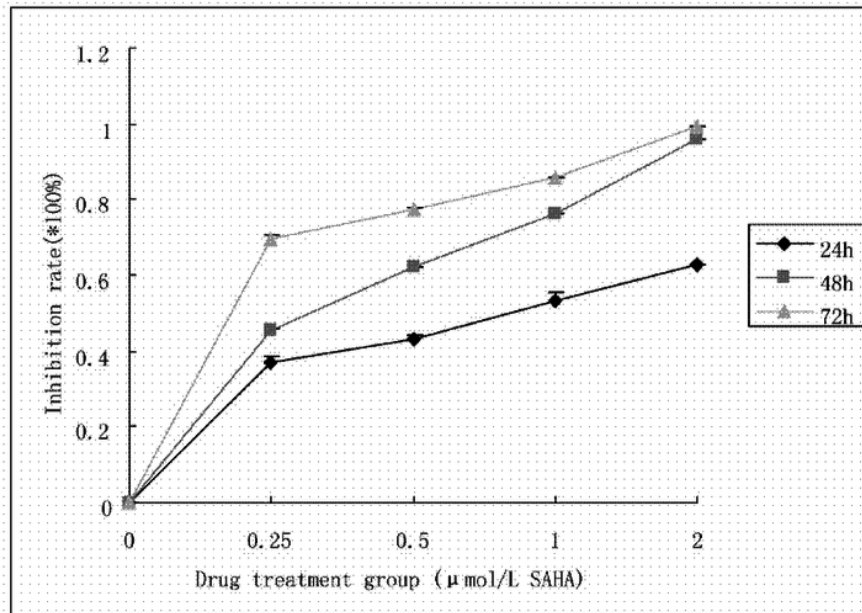
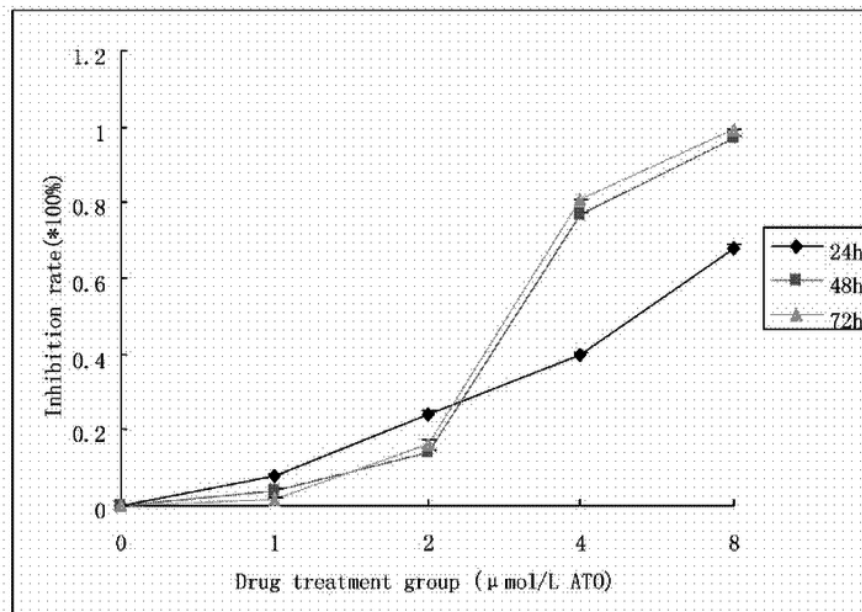
5×10^4 cells / ml were seeded to a 96-well culture plate in a final volume of 100μl. Different concentration of drugs were applied. Inhibition rate was calculated as Fig.1. Q was calculated using the following equation: $Q = E(ab) / (Ea + Eb-Ea \times Eb)$. Ea and Eb are the single drug inhibition rates; E (ab) is associated with the inhibition rate of SAHA and ATO. Q > 1.15 was considered to be a synergistic effect, 0.85 > Q > 1.15 an additive effect, and Q < 0.85 an antagonistic effect.

2   **Tab.1: SAHA in combination with ATO showed an additive effect on the inhibition of proliferation.**

Concentration (μmol/L)		Inhibition (%)	Q value	additive effect
SAHA	ATO			
0	0	0		
0.25	0	45.33		
0.5	0	62.24		
1	0	76.01		
2	0	95.84		
0	1	3.7		
0	2	14.07		
0	4	76.67		
0	8	96.99		
0.25	1	49.91	0.906	+
0.25	2	55.75	1.051	+
0.25	4	84.08	0.964	+
0.5	1	63.85	1.003	+
0.5	2	70.88	1.049	+
0.5	4	85.41	0.937	+
1	1	84.75	1.102	+
1	2	87.19	1.098	+
1	4	96.01	1.017	+

1

Fig. 1: SAHA and ATO alone showed inhibition of proliferation on K562 cell line.

5×10^4 cells / ml were seeded to a 96-well culture plate in a final volume of 100μl. After 24, 48, and 72 h of treatment, 10 μl of CCK-8 was added into each well and incubated for 1-4 h. The absorbance at 450 nm was measured using a microplate reader. Inhibition rate = [(control group - experimental group) / blank group] × 100%. (A) The proliferation curve of SAHA alone is shown. (B) The proliferation curve of ATO alone is shown.

SAHA group

ATO group

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Apoptosis rate after treatment.

(A) Apoptosis was detected by Annexin-V and PI staining. K562 cells (1×10^6) and the drugs were co-incubated for 48 h. Cells were harvested after a single PBS wash. 100µl of binding buffer was added to resuspend the cells. Annexin-V (2µl) and PI (2µl) was added. After an incubation at room temperature for 10-15 min in the dark, K562 cells were detected using a Flow Machine. The apoptosis rates after different treatments are shown (SA2:2µmol/L SAHA,SA4:4µmol/L SAHA,AS4:4µmol/L ATO,AS8:8µmol/L ATO,SA2+AS8:2µmol/L SAHA in combination with 8µmol/L ATO).**(B-E)** Apoptosis observations using fluorescence microscopy 48 h after treatment, ×400 magnification). K562 cells and the drugs were co-incubated as described in Fig. 2. Suspended cells (95 µl, 5×10^6 / ml) were mixed with AO / EB (5µl). Immediately after mixing, one drop of suspended cells was placed on a clean glass slide and observed by fluorescence microscopy at an excitation wavelength of 490 nm. Cells with green fluorescence in the nucleus and cytoplasm were normal cells; Cells with yellow-green fluorescence in the nucleus or cytoplasm were apoptotic cells; cells with red fluorescence in the nucleus were necrotic cells. (B) control,(C) 2 µmol / L SAHA; (D) 8 µmol / L ATO,(E) 2 µmol / L SAHA in combination with 8 µmol / L ATO.
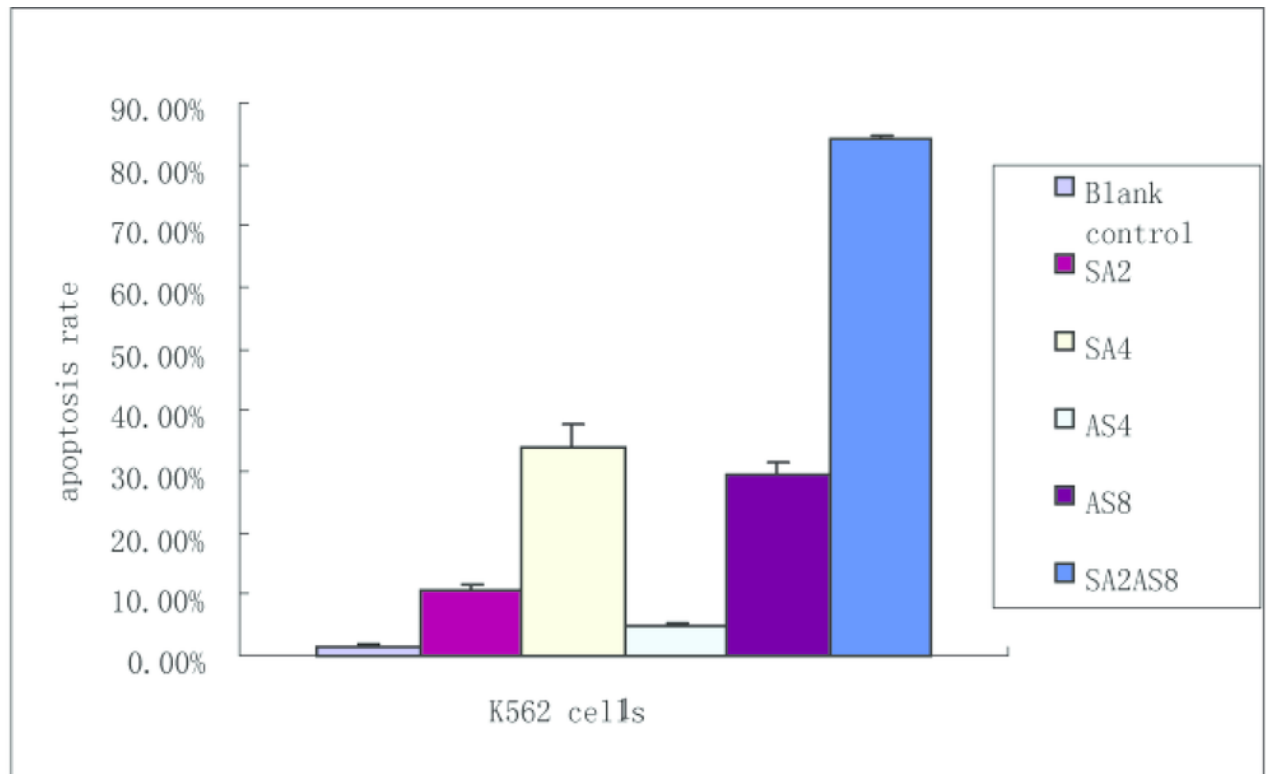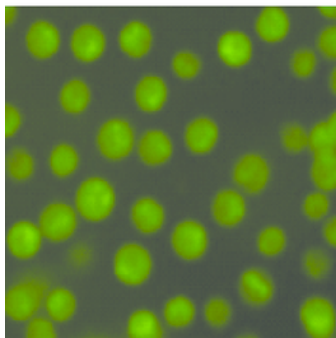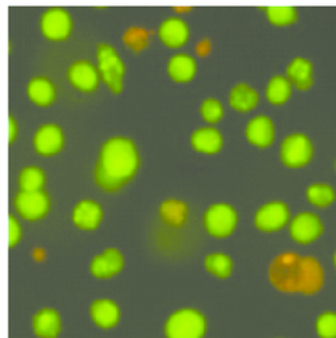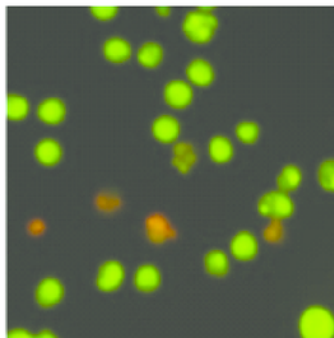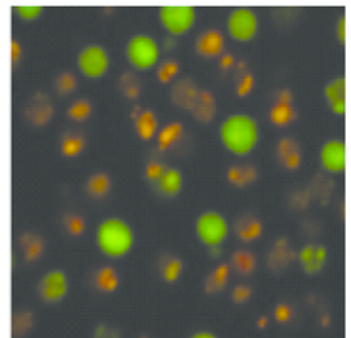
Fig. 3: SAHA in combination with ATO showed pronounced changes in protein expression.

K562 cells were co-cultured as described above. p210, p21, Acetyl-Histone H3, Acetyl-Histone H4, Akt and pAkt were detected by Western blot. β-actin was used as an internal control. (**C**. control,**S**. 2 μmol / L SAHA,**A**. 8μmol / L ATO,**S+A.** 2 μmol / L SAHA in combination with 8 μmol / L ATO)