lowered ATP levels, and KUS121 and esculetin nearly equally suppressed this reduction in ATP levels (Fig. 3a). Under these conditions, KUS121 and esculetin inhibited AMPK activation (evidenced by the phosphorylation of AMPK Thr172), as well as CHOP induction, an ER stress marker with cell death-inducing activity (Zinszner et al., 1998) (Fig. 3b). Consistent with these results, KUS121 and esculetin were able to suppress cell death by these mitochondrial respiratory chain inhibitors (Fig. 3c, Fig. S2). Interestingly, KUS121 and esculetin appeared to more strongly prevent cell death induced by MPP+, rotenone, and metformin, which are inhibitors of mitochondrial respiratory chain complex I, than antimycin and oligomycin, which are inhibitors of mitochondrial respiratory chain complex III and V, respectively (Fig. 3c, Fig. S2).

In order to examine the potential benefits of KUSs and esculetin for Parkinson's disease in more detail, we analyzed the neuroprotective efficacies of these compounds with MPP+ exposure. Neuronally differentiated PC12 cells were treated with 75 μM MPP+ for 28 h, and then live cell (or dead cell) numbers were estimated (Fig. 4a, b, Fig. S3). With this treatment, approximately 60% of the cells died. This cell death was not inhibited by KUS11 (CAS No. 146293-60-9) (Fig. 1g), which has no

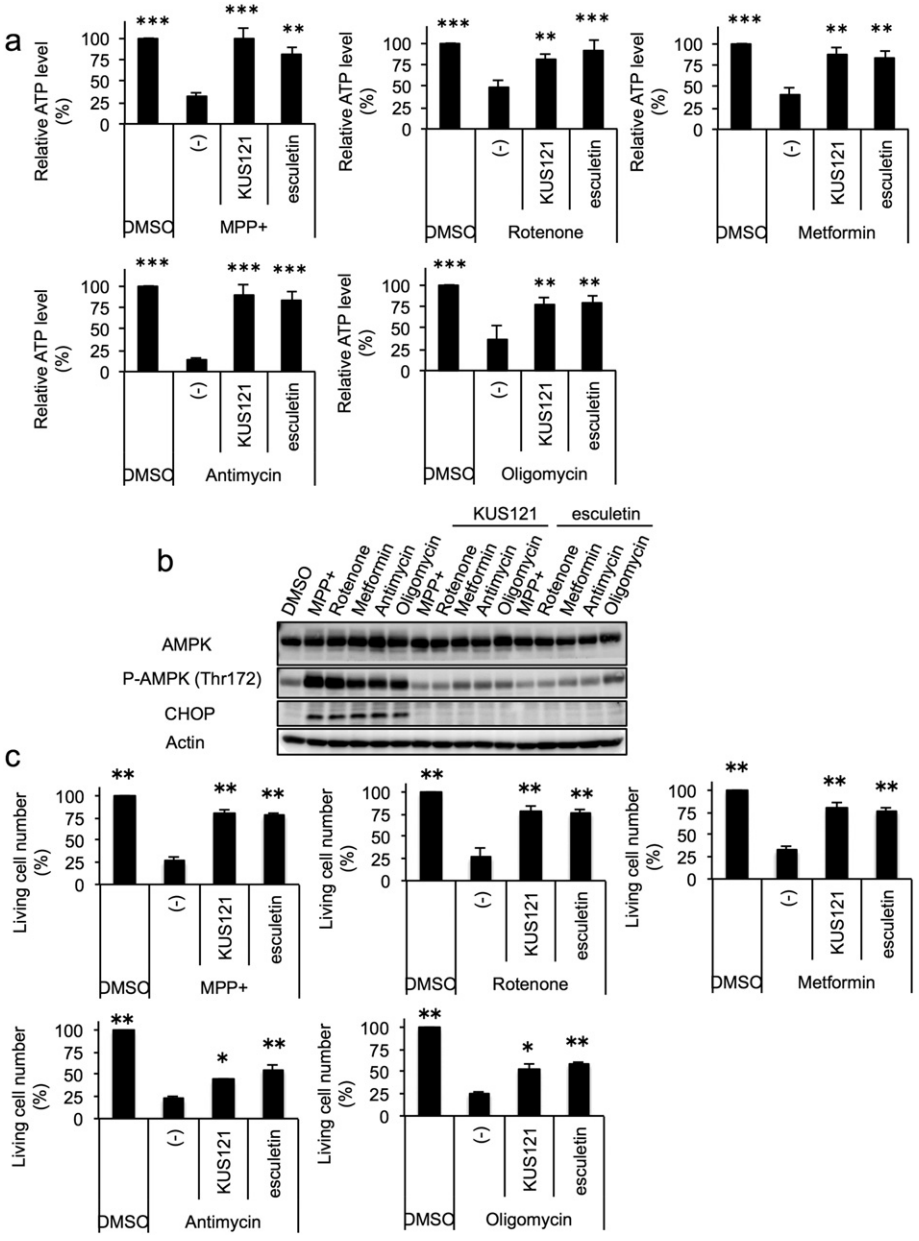

Fig. 3. KUS121 and esculetin prevent mitochondrial dysfunction-induced ATP decrease, ER stress, and cell death in neuronally differentiated PC12 cells. (a) Effects of KUS121 and esculetin on the prevention of ATP decreases induced by mitochondrial respiratory chain complex inhibitors. Neuronally differentiated PC12 cells were incubated for 24 h in the absence (DMSO) or the presence of 50 μM KUS121 or 50 μM esculetin, and then further incubated with each of the mitochondrial respiratory chain complex inhibitors (75 μM MPP+; 10 nM rotenone; 3 mM metformin; 100 nM antimycin; 0.01 μg/ml oligomycin) for 24 h. Then, total ATP amounts and live cell numbers were determined. Mean values of relative ATP levels per cell were calculated and are shown, with values for DMSO alone set at 100%. Error bars indicate standard deviations. $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA with Tukey's post-hoc test ($n = 4$), vs. MPP+, Rotenone, Metformin, Antimycin, or Oligomycin alone (-). (b) Effects of KUS121 and esculetin on the prevention of AMPK phosphorylation and ER stress induced by mitochondrial respiratory chain complex inhibitors. Neuronally differentiated PC12 cells were cultured with the conditions shown in (a), and were subjected to western blot analyses. Actin served as a loading control. (c) Effects of KUS121 and esculetin on the prevention of cell death induced by mitochondrial respiratory chain complex inhibitors. Neuronally differentiated PC12 cells were incubated for 24 h in the absence (DMSO) or the presence of 50 μM KUS121 or 50 μM esculetin, and then further incubated with each of the mitochondrial respiratory chain complex inhibitors shown in (a) for 28 h. Then, live cell numbers were counted by staining with trypan blue. Mean values of relative live cell numbers are shown, with values for DMSO alone set at 100%. Error bars indicate standard deviations. $^{*}P < 0.05$, $^{**}P < 0.01$, ANOVA with Games-Howell post-hoc test ($n = 3$), vs. MPP+, Rotenone, Metformin, Antimycin, or Oligomycin alone (-).