The anti-psoriatic drug anthralin accumulates in keratinocyte mitochondria, dissipates mitochondrial membrane potential, and induces apoptosis through a pathway dependent on respiratory competent mitochondria

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SPECIFIC AIMS

The goals of this work were to investigate 1) the effects of the anti-psoriatic drug anthralin on mitochondrial function and the activity of individual respiratory chain complexes in living human keratinocytes; and 2) whether anthralin might be acting on mitochondria to induce keratinocyte apoptosis, thus contributing to its therapeutic efficacy in clearing psoriatic plaques.

PRINCIPAL FINDINGS

1. Anthralin induces disruption of ΔΨm in human keratinocytes

Anthralin induced a dose and time-dependent reduction of ΔΨm (measured as the fluorescence intensity ratio of JC-1 red J-aggregates/green monomers) in human keratinocytes (Fig. 1A). Mitochondria, highlighted by fluorescent JC-1 J-aggregates, appeared swollen, rounded with loss of their filamentous shape, and aggregated around the nucleus after 1 h treatment with anthralin (Fig. 1A).

Flow cytometric analysis of human keratinocytes showed reduction of median TMRM fluorescence in response to anthralin, confirming anthralin-induced dissipation of ΔΨm (Fig. 1B).

2. Anthralin induces cytochrome c release and apoptosis in human keratinocytes

In response to 1–10 μM anthralin for 2–4 h, the normal filamentous and reticulate mitochondrial staining pattern of cytochrome c within keratinocytes was lost (Fig. 1C), there was partial release of cytochrome c into the cytoplasm, and swollen, rounded mitochondria collected in a perinuclear location (Fig. 1C). Complete release of cytochrome c from mitochondria was observed 24–48 h after anthralin treatment (Fig. 1C) and was associated with morphological changes in apoptosis (Fig. 1C).

3. Anthralin induces caspase activation and DNA fragmentation in human keratinocytes

Anthralin induced anti-active caspase 3 positive keratinocytes at 24–48 h, determined by immunostaining and confocal microscopy (Fig. 1D, E). Activation of caspase 3 after anthralin treatment of HaCaT keratinocytes was confirmed using a fluoroogenic caspase assay. Modest activation of caspase 9 by 1 μM anthralin was observed at 12 h in HaCaT keratinocytes, consistent with activation of the intrinsic apoptotic pathway; this was accompanied by activation of caspase 2. Analysis of keratinocyte and HaCaT cell lysates using a cell death ELISA assay showed enrichment of mono- and oligonucleosomes 24–96 h after anthralin treatment, indicating DNA fragmentation.

4. Anthralin localizes to mitochondria

Lambda scanning of keratinocytes by confocal microscopy before and after anthralin treatment revealed a distinct fluorescence spectrum for anthralin; increased fluorescence was detected through a 520–570 nm window within 5 min of anthralin addition. Anthralin rapidly localized to punctuate structures in the cytoplasm of keratinocytes and colocalized with MitoFluor Far Red, indicating that anthralin distributes to mitochondria in living keratinocytes.

5. The effect of anthralin on electron flow through individual complexes of the respiratory chain

Anthralin (5 μM) had no effect on the activities of individual complexes I or IV but induced a clear increase in complex II activity (measured as succinate-ubiquinone reduction).
oxidoreductase, vehicle 149 ±10, anthralin 272 ±23 nmol DCPiP reduced min–1 mg and mitochondrial protein–1, respectively). Malonate decreased complex II activity in the presence of anthralin or vehicle by the same magnitude, suggesting that anthralin does not interfere at the substrate binding site. Anthralin had no effect on succinate dehydrogenase (SDH) activity compared with vehicle, suggesting direct interaction with the ubiquinone pool. A modest increase of a linked assay of succinate cytochrome c oxidoreductase was observed after addition of anthralin. Anthralin could overcome the effect of the specific complex III inhibitor antimycin, indicating that anthralin interacts at the level of the ubiquinone/ubiquinol pool.

6. Role of oxidative phosphorylation in anthralin-induced apoptosis

Anthralin induced dramatic dose-dependent cytotoxicity in p0 osteosarcoma cells as assessed by a sulforhodamine B (SRB) dye assay (Fig. 2A, B). p0 143B cells were significantly more resistant to the cell-killing effects of anthralin after 72 h treatment compared with the parental cell line under identical culture conditions (median EC50 87 and 0.087 μM, respectively, P<0.05, n=5) (Fig. 2B), indicating that a functional respiratory chain and active oxidative respiration are required for cell-killing effects of anthralin in mammalian cells.

Anthralin resulted in a uniform reduction in mitochondrial membrane potential in parental 143B p+ cells in a time (30 min–24 h) and concentration (0.1–10 μM)-dependent manner. 143B p0 cells maintain a lower m via glycolytic metabolism but this was unaffected by incubation with anthralin (0.1–10 μM) between 30 min and 24 h.

Anthralin induced cytochrome c release in parental 143B p+ but not 143B p0 cells (Fig. 2C, D). Colocalization of cytochrome c release with late morphological changes of apoptosis was observed in 143B p+ but not p0 cells between 2 and 24 h (Fig. 2C, D). Incubation of 143B p0 cells and 143B p+ cells with anthralin (1–5 μM) for 24 h resulted in caspase 3 activation in 143B p+ but not p0 cells.

Imaging of anthralin within 143B p0 cells and 143B p+ cells showed that anthralin localized to mitochondria in both 143B p0 cells and 143B p+ cells, indicating that the resistance of 143B p0 cells to anthralin-induced apoptosis.
apoptosis could not be accounted for by differences in the intracellular distribution of anthralin.

CONCLUSIONS AND SIGNIFICANCE

Anthralin localizes to mitochondria within living keratinocytes and induces dissipation of $\Delta \Psi_m$, cytochrome $c$ release and caspase 3 activation, resulting in morphological changes of apoptosis. Early reduction of $\Delta \Psi_m$ preceded loss of cytochrome $c$ and morphological changes of apoptosis. Studies of $143B \rho^0$ cells and the isogenic $\rho^+$ cell line indicate cells lacking mitochondrial DNA and a functional respiratory chain are resistant to the apoptotic-inducing effects of anthralin. These data provide strong evidence that a functional respiratory chain is required for anthralin-induced dissipation of $\Delta \Psi_m$, release of cytochrome $c$, and caspase activation.

Early partial release of endogenous cytochrome $c$ was temporally associated with swelling of mitochondria and a decrease in $\Delta \Psi_m$ in keratinocytes in response to anthralin. Retention of a significant pool of cytochrome $c$ in swollen mitochondria in perinuclear location in keratinocytes and $143B \rho^+$ cells was a significant finding of this study. Complete release of cytochrome $c$ observed later was associated with late morphological changes of apoptosis. Apoptosis is an active process requiring high levels of ATP production, at least during the early phases. We hypothesize that early and partial release of cytochrome $c$ may be an important initiating event in anthralin-induced apoptosis, leaving a significant pool of cytochrome $c$ to contribute to oxidative phosphorylation, whereas complete release of cytochrome $c$ is only seen as a late stage event.

Increased complex II activity in response to anthralin in the absence of an effect on SDH activity suggests a direct interaction of anthralin with the ubiquinone pool. This model is strengthened by the reduction potential of the ubiquinone-ubiquinol redox couple ($E^0 = +0.045 \text{ V}$), which favors the transfer of electrons from anthralin ($E^0 = -0.75 \text{ V}$). Our data also suggest that anthralin may be modulating the redox status of the endogenous ubiquinone pool at the level of the ubisemiquinone anion (Fig. 3). In turn, these events lead to increased generation of reactive oxygen species and apoptosis induction, resulting in the preferential death of highly proliferative psoriatic keratinocytes that demand a high energetic requirement from mitochondria. Finally, our data suggest that dithranol-induced apoptosis may contribute to the therapeutic action of dithranol in clearing psoriatic plaques.

Figure 2. $143B \rho^0$ cells lacking functional respiratory chain mitochondria are resistant to anthralin-induced cell death and cytochrome $c$ release. A, B) $143B \rho^+$ or $\rho^0$ cells were treated with anthralin or vehicle for 72 h and cell survival determined using an SRB assay. Data points $\pm$ se from 5 independent experiments. EC$_{50}$ values were calculated for each SRB experiment. Horizontal line shows median value. *P* values were calculated using a 2-tailed Mann Whitney test. C, D) $143B \rho^+$ or $\rho^0$ cells were incubated with 1 mM anthralin or vehicle; Cyt $c$ release and morphological changes of apoptosis were determined by immunostaining with anticytochrome $c$ antibody and labeling with propidium iodide (PI). Cells were visualized by laser scanning confocal microscopy. Representative of at least 3 independent experiments. Scale bar = 20 $\mu$m.

Figure 3. Proposed model for interaction of anthralin with respiratory chain and activation of intrinsic pathway of apoptosis in human keratinocytes.