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# CALORIMETRIC SCREENING TEST FOR DERMATOLOGICALLY ACTIVE DRUGS ON HUMAN SKIN FIBROBLAST-CULTURES

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## Abstract

The influence of anthralin (1,8-dihydroxy-9-anthron) and some of its derivatives on the energy metabolism of human skin fibroblasts in culture has been investigated by means of a batch calorimeter. In the absence of any drug, confluent monolayers produced a fairly constant heat output of 51 $\pm$ 5 pW per cell reflecting metabolism of maintenance. Addition of anthralin or a derivative resulted in different effects on the heat production.

## Introduction

Microcalorimetry is communly used as an analytical tool in microbial cultures (1, 2). Some experiments have been performed with organs and tissues (3), but only a few were reported on in vitro cultures of vertebrate cells (4). Difficulties in manipulating vertebrate cell cultures may be one explanation for this fact. Nevertheless cell cultures seem to be suited for calorimetric investigations of specific drug effects, because no artificial tissue sampling or homogenization is necessary.

This paper describes a calorimetric screening method to investigate the influence of chemical agents used in dermatologic therapy.

## Material and Methods

Cell culture: For all experiments human foreskin fibroblasts of line Flow 7000 from Flow Laboratories (Assnières, France) have been used. The cells were in the twelfth passage and they were adapted to the growth medium over another three passages. The growth medium was prepared from Dulbecco's Modified Eagle's Medium and contained 15% (v/v) fetal calf serum, 25 mM HEPES, 100.000 units penicillin/l, 100 mg streptomycin/l, and 250 $\mu$g amphotericin B/l. The pH was adjusted to 7.4 .

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Cells of precultures were washed in HBSS, trypsinized with HBSS containing 0.3% (w/v) trypsin, suspended in growth medium, centrifuged (5 min at 600g), and resuspended in growth medium (cell density $2 \times 10^5$ cells/ml).

Cell seeding: For the experiments cells were seeded on a plastic foil (Sterilin No 337, Teddington, UK) according to Schaarschmidt & Reichert (5). For this purpose the foil was cut into pieces of 5x7 cm and put into a tenter-frame. About 6 ml of the cell suspension was added. During cell attachment the tenter was kept at 37° C in a steril plastic box and cell growth could be followed microscopically.

For growth experiments the supernatant was removed some hours after seeding and counted for suspended cells in order to estimate the number of cells remaining on the foil. When the influence of drugs was investigated, cells were grown on the foil up to confluency. Then the supernatant was removed.

Calorimetric experiments: The calorimetric measurements were performed in a differential batch calorimeter (type Bioflux, Thermanalyse, Grenoble) at 37° C. One calorimetric vessel (volume 18 ml) was charged with an unseeded foil, the other one with the pretreated foil. The vessels were carefully filled up with 15 ml of growth medium, thermostated at 37° C for 15 min and then inserted into the calorimeter.

In growth experiments the heat production dQ/dt was measured over several days. The power-time curve was recorded with a sensitivity of 62.4 µV/ mW.

Drug treatment: For screening experiments the foil was placed in the calorimetric vessel for at least 5 hours before the drug was added. Then the medium was removed, new growth medium containing different amounts of drugs was added, and the vessels presented to the calorimeter.

Pure preparations of anthralin (dithranol, 1,8-dihydroxy-9-anthron), anthralin dimer, 10-acetyl-anthralin, anthraquinone, and DT 86[1] were dissolved in 17 N acetic acid. The compounds were diluted in the growth medium to give final concentrations of 0.5 to 20 µM compound/l and 1:500 N acetic acid. The pH was

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1) code-name for an anthralin derivative

adjusted to 7.4 using a 10 N NaOH solution. Immediately after preparation the medium was given to the monolayer culture.

Cell count: At the end of the experiment the cells were detached by trypsinization and counted. Cell viability was checked by Trypan Blue exclusion.

## Results

Cultures could be kept alive more than one week. Even occasionally removal and replacement of the foil for microscopic observations did not cause contamination, but allowed further measurements.

Power-time curve of growing fibroblasts: Growing fibroblasts produce a characteristic and reproducible heat pattern (fig. 1). The experiment of fig. 1 was started with an initial cell number of $10^6$. The fibroblasts were aible to divide twice or thrice before reaching confluence. At the end of all experiments cell numbers between $3.5 \times 10^6$ and $4 \times 10^6$ were found, even when the starting cell numbers have been different. The heat output during the constant part of the thermogram (phase III) was 170 to 210 µW. This corresponds to 51±5 pW/cell.

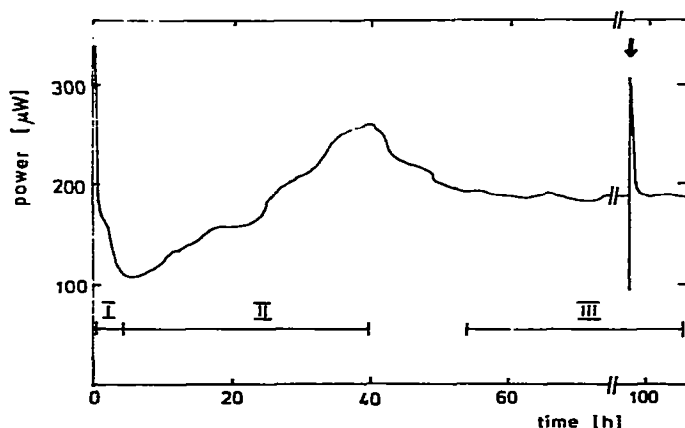


Fig. 1. Power-time curve of growing human skin fibroblasts in culture.
I change from aerobic to anaerobic metabolism,
II cell multiplication,
III metabolism of maintenance,
↓ removal and replacement of the foil.
Preincubation time of the foil: 10 hours.

Treatment with anthralin and some of its derivatives: The quantitative relationship between the decrease in heat evolution and the concentration of anthralin is given in fig. 2. Addition of anthralin to the monolayer culture resulted in a reduction of

the cellular power, when the anthralin concentration exceeded 2 μM. Concentrations less than 2 μM showed no effect.

A reduced heat production could already be seen two hours

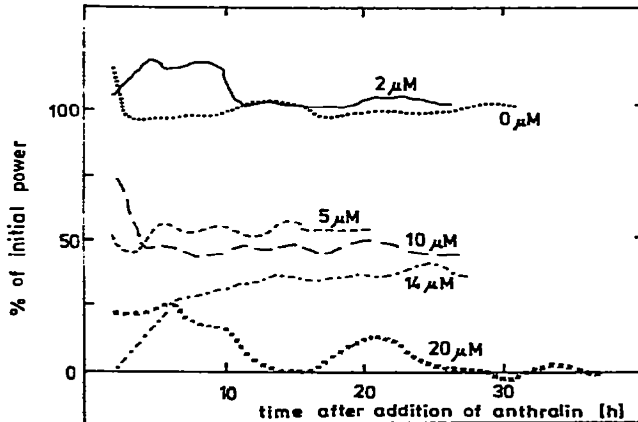


Fig. 2. Inhibition of heat production after addition of different concentrations of anthralin to the monolayer culture.

after drug treatment and was constant over the subsequent 20–40 hours.

In fig. 3 the effect of anthralin is compared with the effect of four derivatives of anthralin. Anthraquinone showed no reduction of the heat evolution at concentrations up to 15 μM, whereas anthralin dimer and 10-acetyl-anthralin are stronger inhibitors than anthralin.

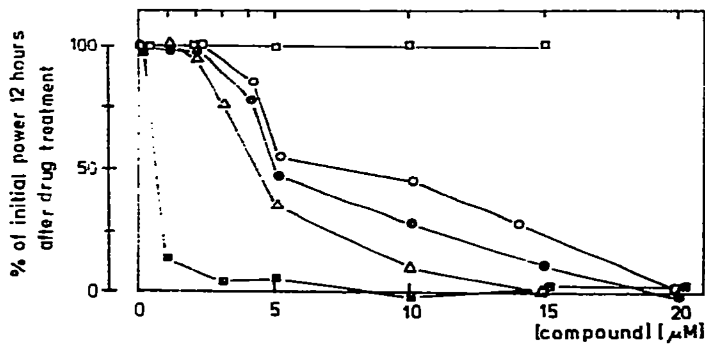


Fig. 3. Inhibition of heat production after addition of
□——□ anthraquinone,
○——○ anthralin,
●——● acetyl- anthralin,
△——△ anthralin dimer,
■——■ DT 86
at different concentrations to the fibroblast monolayer.

Cell treatment with DT 56 always resulted in a strong reduction of the heat output.

Discussion

The different phases of the thermogram of growing cells have been attributed by Pätel et al (6) to the following processes:

phase I  spreading and growth of the cells
phase II  cell multiplication
phase III  metabolism of maintenance

Though the attachment period in fig. 1 exceeds 5 hours, phase I could be observed. From microscopic observations it is known that cell spreading and cell growth are finished in a couple of hours. Obviously other metabolic processes than spreading and growth must be involved, perhabs due to a change from aerobic to anaerobic conditions. In fact, Warburg measurements on monolayer cultures have shown, that human skin fibroblasts are able to respirate under aerobic conditions (7) and most of the heat originates from respiration. When cultures from the tenter-frame are placed into the calorimetric vessel respiration stops and the energy metabolism becomes restricted to fermentation.

This assumption is supported by the fact, that cultures which are already adapted to vessel conditions do not exhibit any metabolic change (removal of the foil in fig. 1). But if the monolayer culture has grown up outside the calorimeter in the tenter-frame and was then exposed to the calorimeter, an initial maximum in the thermogram profile occurs. Thus the first maximum should be interpretated as following:

phase I  change from aerobic to anaerobic metabolism

For screening tests this artificial adaptation phase lasting over five hours should be avoided by adapting the culture prior to drug treatment.

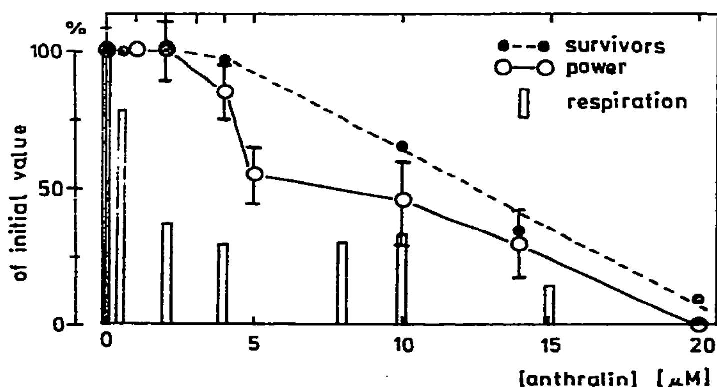


Fig. 4. Inhibition of cell surviving, power and respiration after addition of anthralin to human skin fibroblasts.

Usually the heat output was not measured before drug treatment. In this case the heat production of the treated culture was compared to the average heat evolution of 190 μW for non-treated monolayers. This procedure permits to perform at least three calorimetric runs per day. On the other hand the

calorimetric run allows to monitor the drug effect continously over long periods.

The heat evolution of an anthralin-treated culture does not change with time. This indicates cell damage just after drug addition. Further presence of the drug is of no influence.

When the cultures have been treated with concentrations of anthralin higher than 10 M, the heat output is no longer constant. This might be due to cell detachment, cell death, and lysis.

| concentration of anthralin | surviving cells | reduction of | |
|---|---|---|---|
| | | heat output | respiration[7] |
| 2 μM | 100% | 100% | 38% |
| 5 μM | 95% | 55% | 30% |
| 10 μM | 68% | 45% | 32% |
| 20 μM | 10% | 0% | 0% |

Table 1. Influence of anthralin on number of surviving cells, heat output, and respiration.

In fig. 4 and table 1 the effects of anthralin on cell death, heat production, and respiration are compared. With concentrations of 2 M anthralin no reduction in viability and heat production can be seen, whereas respiration is already inhibited to less than 40%. Up to concentrations of 5 M anthralin no cell death could be detected, but nevertheless the heat evolution is decreased to 55%. Up to concentrations of 10 M heat output and respiration are nearly unchanged, but cell death occurs. Further increases of the amount of anthralin reduce completely the number of surviving cells, the heat production and respiration.

These results compared with those reported by Jacques & Reichert (8) on multipliing cells indicate that the sensitivity of human skin fibroblasts to anthralin is of following order

$[^3H]$ thymidine incorporation > respiration > heat production > cell survival.

Anthralin, its 10-acetyl-analoque and the dimer at concentrations higher than 2 M inhibit heat production (fig. 3). Whereas anthraquinone shows no effect with concentrations up to 15 M. Con-

centrations, which reduce the heat evolution to 50% are listed in table 2.

The activity of anthralin, acetyl-anthralin and the dimer is in same order of magnitude. DT 86 is a ten times more active inhibitor.

Except for anthraquinone and the dimer, the other compounds cause cell death and lysis.

| Treatment with | $K_{0.5}$ ( $\mu$M) heat output | cell death |
| --- | --- | --- |
| anthralin | 7 | 13 |
| acetyl- anthralin | 5 | 10 |
| anthralin dimer | 4 | 100 |
| DT 86 | <1 | 2 |
| anthraquinone | 60 | 100 |

Table 2. Inhibitor concentrations, which reduce the heat production and the fraction of surviving dells to 50%.

Although anthralin dimer is a potent inhibitor of cellular heat evolution, it does not appear to kill cells up to concentrations of 20 $\mu$M dimer.

With these first experiments it could be demonstrated that different effects of drugs on the energy metabolism of fibroblasts may be monitored by microcalorimetry. Results from calorimetric screening experiments should be compared to those of conventional techniques, such as cell count, incorporation of radio-active compounds or manometric techniques to get further insight in the way of action of drugs.

I would like to thank Dr. B. Shroot, who kindly provided the investigated compounds.

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