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# Anti-epileptic drugs inhibit viability of synoviocytes *in vitro*

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## Abstract

**Introduction and objective**: The hyperplasia of synovial fibroblasts is considered to be essential for the evolution of joint destruction in rheumatoid arthritis (RA). Previously, we reported that anti-rheumatic drugs, both COX inhibitors and disease-modifying anti-rheumatic drugs inhibit proliferation of synoviocytes *in vitro*. The presented study investigates the effect of anti-epileptic drugs on the viability and proliferation of synovial fibroblasts *in vitro*.

**Methods**: Experiments were conducted on human synoviocytes derived from an RA patient and rabbit synoviocytes cell line HIG-82. Cell proliferation and viability were assessed by means of BrdU assay and MTT assay, respectively. The $IC_{50}$ value (the concentration of drug necessary to induce 50% inhibition) together with confidence limits was calculated.

**Results**: Carbamazepine inhibited proliferation of human fibroblasts and viability of HIG-82 with $IC_{50}$ values of 86 μM and 82 μM, respectively. Diphenylhydantoin, valproate and phenobarbital inhibited viability of HIG-82 cells with the $IC_{50}$ values of 110, 500 and 1031 μM, respectively.

**Conclusion**: Based on these findings, it can be suggested that anti-epileptic drugs may have a disease-modifying effect on rheumatoid synovial proliferation.

## Key words

anti-epileptic drugs, synoviocytes, proliferation, *in vitro*

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease affecting approximately 1% of the population. Recently, it was indicated that urbanization is associated with an increased prevalence of RA[1]. This finding suggests that environmental factors may affect RA development. The pathogenesis of RA is still not well understood. However, it is widely accepted that the inflammatory process in the synovial tissue is dominated by proliferation of activated synovial fibroblasts. They are considered to play an important role in both the initiation and progression of joint destruction in RA [2, 3, 4]. The hyperplasia of synovial fibroblasts is one of the most striking features of RA and is considered to be essential for the evolution of joint destruction in RA. Thus, it seems that control of synovial hyperplasia represents a target for novel therapeutic approaches for the inhibition of joint destruction.

Previously, we found that glutamate antagonists inhibit proliferation of HIG-82 synovial fibroblast *in vitro* with potency comparable to anti-rheumatic drugs, COX inhibitors: celecoxib, diclofenac, nimesulide, naproxen and disease-modifying anti-rheumatic drugs, methotrexate and sulfasalazine, and suggested that glutamate receptor antagonists may have a disease modifying effect on rheumatoid synovial proliferation [5].

Recently, anti-epileptic drugs, valproic acid and carbamazepine were found to inhibit histone deacetylases [6] which regulate expression of tumour suppressor genes and activities of transcriptional factors involved in cancer cells proliferation. The therapeutic anti-cancer potential of valproic acid in monotherapy or combined with other anti-tumour drugs is currently being tested in several clinical trials (see for review: [7]).

Therefore, the aim of the presented study was to investigate the effects of anti-convulsant drugs commonly used in the treatment of epilepsy on the proliferation of synovial fibroblasts *in vitro*. Diphenylhydantoin, phenobarbital, and two histone deacetylase inhibitors: valproate and carbamazepine were chosen.

## MATERIALS AND METHODS

### Drugs

Carbamazepine, diphenylhydantoin, valproate were purchased from Sigma-Aldrich (St. Louis, USA). Phenobarbital was obtained from Polfa (Cracow, Poland). All other chemicals were purchased from commercial suppliers and were of the highest available purity.

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### Human synoviocytes

Synovial tissue was obtained from the knee of a patient who fulfilled the American College of Rheumatology 1987 criteria for diagnosis of RA [8] at the time of synovectomy during total joint replacement as a standard clinical procedure. Primary synoviocyte cell culture was prepared as previously described [8]. Briefly, synovial tissue was processed within 2 h after harvesting from the patient. After discarding fat and fibrous tissue, the synovium was mechanically dispersed, cut into small pieces, and plated on 75 $cm^2$ plastic culture flasks (Nunc) in a culture medium consisting of Ham F-12 (Sigma), supplemented with 10% of FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% of antibiotic-antimycotic solution (Life Technologies). Cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. Culture medium was changed every 3 days. After reaching confluency, cells were subcultured by means of 0.25% Trypsin-ethylene diamine tetraacetic acid solution and subjected to experiments.

### HIG-82 cell culture

*In vitro* experiments were conducted on rabbit synoviocytes cell line HIG-82 obtained from ATCC (American Type Culture Collection, Menassas, VA, USA). Culture medium consisted of Nutrient Mixture F-12 Ham (Sigma Chemicals, St. Louis, MO, USA) supplemented with 10% of FBS (Life Technologies, Karlsruhe, Germany), 100 U/ml penicillin (Sigma) and 100 µg/ml streptomycin (Sigma). Cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$.

### Cell proliferation assessment

5-bromo-2'-deoxy-uridine (BrdU) assay is an immunoassay for the quantification of BrdU incorporation into newly synthesized DNA of actively proliferating cells. Human rheumatoid arthritis fibroblasts-like synoviocytes were plated on 96-well microplates (Nunc, Roskilde, Denmark) at a density of $2 \times 10^4$ cells/ml. Next day, the culture medium was removed and the cells exposed to fresh medium (control) or serial dilutions carbamazepine (10–500 µM) in fresh medium. Cell proliferation was quantified after 48 h by measurement of BrdU incorporation during DNA synthesis, according to the manufacturer's procedure (Cell Proliferation ELISA BrdU, Roche Diagnostics GmbH, Penzberg, Germany).

### Cell viability assessment

HIG-82 cells were plated on 96 well microplates (NUNC, Roskilde, Denmark) at a density of $1 \times 10^4$ cells/ml. Next day, the culture medium was changed and cells exposed to serial dilutions of tested compounds. Cell viability was assessed after 96 h by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell proliferation kit I, Roche Diagnostics GmbH, Penzberg, Germany). HIG-82 cells were incubated for 3 h with MTT solution (5 mg/ml). Formazan crystals were solubilized overnight in SDS buffer, and the product quantified spectrophotometrically by measuring absorbance at 570 nm wavelength using E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

### Data analysis

Results are expressed as a percentage of the control. The mean value and standard deviation of (SD) was calculated from 6–7 independent experiments.

The $IC_{50}$ value (the concentration of drug necessary to induce 50% inhibition), together with confidence limits, was calculated using computerised linear regression analysis of quantal log dose-probit functions, according to the method of Litchfield and Wilcoxon [9].

## RESULTS

The proliferation of human synovial fibroblasts determined by means of the quantification of BrdU incorporation into newly synthesized DNA of proliferating cells was inhibited by carbamazepine in a dose-dependent fashion (Fig. 1) with $IC_{50}$ values of 86 (65–115) µM.

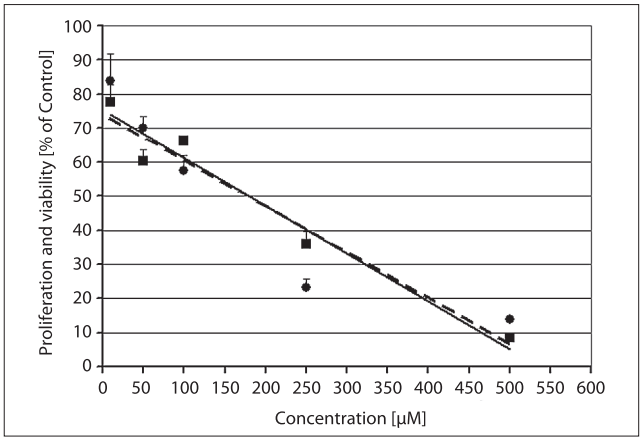


**Figure 1.** Effect of carbamazepine on the proliferation and viability of human rheumatoid arthritis fibroblasts-like synoviocytes *in vitro* (BrdU assay) and rabbit synoviocytes cell line HIG-82 *in vitro* (MTT assay). Results are expressed as percentage of control; mean ± SD; n = at least 7 independent experiments. Circles and continuous line represent human rheumatoid arthritis fibroblasts-like synoviocytes; squares and dashed line represent rabbit synoviocytes cell line HIG-82. Regression curves were calculated using GraphPAD software. Human rheumatoid arthritis fibroblasts-like synoviocytes (solid line): $y=-0.1402x+75.237$, $r=-0.87$; rabbit synoviocytes cell line HIG-82 (dashed line): $y=-0.1353x+74.408$, $r=-0.96$.

The viability of rabbit synovial fibroblasts HIG-82 determined by means of tetrazolium salt reduction in living cells was inhibited by antiepileptic drugs, carbamazepine (Fig. 1), diphenylhydantoin, valproate and phenobarbital dose-dependently with the $IC_{50}$ values of 82, 110, 500 and 1031 µM, respectively (Tab. 1).

**Table 1.** Effect of anti-epileptic drugs on the viability of rabbit synoviocytes cell line HIG-82 *in vitro*

| Drug | $IC_{50}$ [µM] | 95% confidence limits |
|---|---|---|
| Carbamazepine | 82 | 47–142 |
| Diphenylohydantoin | 110 | 64–189 |
| Valproate | 500 | 435–576 |
| Phenobarbital | 1031 | 815–1303 |

$IC_{50}$ – the concentration of drug necessary to induce 50% inhibition

## DISCUSSION

It was found that carbamazepine in micromolar concentrations inhibited proliferation of human rheumatoid arthritis fibroblasts-like synoviocytes *in vitro*. It decreased viability of rabbit synovial fibroblast HIG-82 which shares

many of the characteristics of activated human rheumatoid synovium [10] in similar concentrations. Diphenylhydantoin, valproate and phenobarbital affected the viability of HIG-82 less effectively. It should be emphasised that the viability of these cells was decreased also by anti-rheumatic drugs, COX inhibitors: celecoxib, diclofenac, nimesulide, naproxen and disease-modifying anti-rheumatic drugs, methotrexate and sulfasalazine. Interestingly, the antiproliferative potential of antiepileptic drugs is comparable to that of anti-rheumatic drugs [5].

Publications dealing with the effect of carbamazepine on cell proliferation are sparse. It has been reported that carbamazepine induced mitotic arrest and inhibited proliferation in mammalian Vero cells (with $IC_{50}$ value of 406 μM) [11], human choriocarcinoma cell line (BeWo) [12] and inhibited the proliferation of breast cancer cell lines MCF-7 and T47D stimulated by estradiol (in concentration 10–1000 μM) [13]. On the other hand, no effect of carbamazepine on the proliferation of human malignant glioma cells [14], human neuroblastoma cells SH-SY5Y [15] and mouse splenocytes [16] was found. The effect of valproate on fibroblast proliferation has never been reported before. However, its anti-proliferative properties were found in numerous cell lines, e.g. rat glomerulal mesangial cells [17], human hepatoblastoma cells HepG2 [18], human hepatocellular cancer cells HuH7 [19], human gastric carcinoma cells BGC-823 [20] and human coronary vascular cells [21].

On the contrary, diphenylhydantoin is known to stimulate proliferation of gingival fibroblast [22, 23] and skin fibroblasts [24, 25]. This effect is attributed to low nanomolar concentrations [22, 23, 25]. At higher concentrations it was reported to inhibit proliferation of human skin fibroblasts [25], mouse muscle cells [26] and mouse embryonic palatal mesenchymal cells [27]. These results point to the biphasic, dose- and time-dependent effect of diphenylhydantoin on cell proliferation *in vitro*. No report on the effect of phenobarbital on cell proliferation has been found. The molecular mechanism of anti-proliferative action of anti-epileptic drugs has to be elucidated. It seems that it is not directly linked to histone diacetylase inhibition.

The comparison of anti-proliferative activity of anti-epileptic drugs expressed as $IC_{50}$ with their plasma concentrations recommended in the treatment of epilepsy suggests their potential clinical application in RA prevention. The plasma concentration of valproate is 300–600 μM [28] or 347–833 μM [29] and its anti-proliferative $IC_{50}$ value was 500 μM. Similarly, the anti-proliferative concentrations of carbamazepine (82 μM) and diphenylhydantoin (110 μM) are close to their anti-epileptic plasma concentrations 16–48 μM [28] or 17–51 μM [29] and 40–80 μM [28, 29], respectively. On the contrary, anti-proliferative concentration of phenobarbital *in vitro* (1031 μM) is distinctly higher than its anti-epileptic range *in vivo* (15–40 μM or 64–172 μM) [28, 29]. Thus, considering the therapeutic concentrations of anti-epileptic drugs recommended for the treatment of epilepsy the most effective and promising one among the studied drugs is valproate. Both, carbamazepine and phenytoin exerted inhibitory action at concentrations nominally higher than their respective therapeutic ranges against epilepsy. However, the low cost of drugs and acceptable toxicity of these drugs do not rule out their use as disease-modifying anti-rheumatic drugs. In this context, phenobarbital can be excluded. Unexpectedly, to the best of our knowledge, there is no analysis in the literature of the efficacy of anti-epileptics in RA patients.

Summing up, the presented study reports for the first time that anti-epileptic drugs inhibit the viability and proliferation of synovial fibroblasts *in vitro*. Based on these findings, it can be suggested that anti-epileptic drugs may have a disease-modifying effect on rheumatoid synovial proliferation. Since the most effective drugs in the presented study were an experimental paradigm, whereas carbamazepine, diphenylhydantoin and valproate, are widely used in clinical practice and it should be possible to establish a meta-analysis or clinical proof of the concept.

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