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$1,25(OH)_2D_3$ and its analogue calcipotriol inhibit the migration of human synovial and mesenchymal stromal cells in a wound healing model – A comparison with glucocorticoids

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ABSTRACT

Vitamin D analogue calcipotriol is currently used in the local treatment of psoriasis. However, it also has anti-proliferative and anti-inflammatory effects in the cells of the joint – suggesting a possible benefit in local treatment of arthritis. In this study, calcipotriol was studied in different in vitro methods to find out its effect on synovial and mesenchymal stromal cells. Primary human cell lines of osteoarthritis or rheumatoid arthritis patients (five mesenchymal stromal cells, MSC, and four synovial stromal cells, SSC) were cultured to study migration and proliferation of the cells in a wound healing model. The media was supplemented with calcipotriol, $1,25(OH)_2D_3$, dexamethasone, betamethasone, methylprednisolone or control solution in 1–100 nM concentrations. To see possible toxic effects of calcipotriol, concentrations up to 10 μM in SSCs and MSCs were studied in apoptosis and necrosis assays in four cell lines. Calcipotriol and $1,25(OH)_2D_3$, as well as the three glucocorticoids, reduced the migration of both SSCs and MSCs. In SSCs, the effect of calcipotriol and $1,25(OH)_2D_3$ was at least as effective as with glucocorticoids, while with MSCs, the glucocorticoids were stronger inhibitors of migration. The antimigratory of calcipotriol and $1,25(OH)_2D_3$ was consistently maintained in 10 μM and 1 μM. Calcipotriol was not toxic to MSCs and SSCs up to concentrations of 10 μM. Calcipotriol, as well as $1,25(OH)_2D_3$, exerts antimigratory and antiproliferative effects on human SSCs and MSCs of the joint. These effects are not caused by apoptosis or necrosis. Both calcipotriol and $1,25(OH)_2D_3$ have similar effects as glucocorticoids without apparent toxicity, suggesting that calcipotriol might be an eligible candidate to the local treatment of arthritis with a broad therapeutic window.

1. Introduction

Vitamin D metabolites and particularly the most active form $1,25(OH)_2D_3$ (calcitriol) are well known for their effects in calcium and bone metabolism. The anti-inflammatory effect of vitamin D includes modulation of both innate immunity through e.g. stabilizing endothelial function and lowering vascular permeability [1] and the adaptive immunity through promoting Th2 immune response and B cell activation, immunoglobulin synthesis and plasma cell differentiation (Bikle, 2014; Charoenngam & Holick, 2020; El-Sharkawy & Malki, 2020).

The pharmacological possibilities of vitamin D in immunomodulation and calcium metabolism prompted the development of vitamin D analogues. These are currently clinically used in treatment of psoriasis, secondary hyperparathyroidism related to chronic kidney disease and osteoporosis [2,4,5]. Research on vitamin D and its analogues is being conducted in cancer and autoimmune diseases ([1–3,6–8]). We are interested in possible antiarthritic efficacy of a specific analogue, calcipotriol.

Abbreviations: $1,25OH_2D_3$, 1,25-dihydroxyvitamin D_3, calcitriol; BM, Betamethasone; CPT, Calcipotriol; DX, Dexamethasone; GC, Glucocoorticoid; MP, Methylprednisolone; MSC, Mesenchymal stromal cell; OA, Osteoarthritis; RA, Rheumatoid arthritis; SSC, Synovial stromal cell.

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Calcipotriol is a synthetic vitamin D analogue of $1,25(OH)_2D_3$ with slight modifications in its side chain [9]. In in vitro studies, it has been proven to have similar capabilities in receptor binding, cell proliferation inhibition and differentiation induction as its physiological counterpart [10], but its calcaemic effect is significantly lower than that of $1,25(OH)_2D_3$ due to weaker binding to vitamin D binding protein and thus faster elimination [11] which might decrease the probability of hypercalcemia as an adverse effect compared to $1,25(OH)_2D_3$.

Glucocorticoids have been a frequently used local treatment of rheumatoid arthritis (RA) and osteoarthritis (OA) for decades. However, their benefit is usually short-term with potential adverse effects [12]. In addition to negative effects on glucose and cortisol metabolism [13,14], glucocorticoids have catabolic adverse effects on bone and growth. As another example of these, intra-articular triamcinolone (a commonly used glucocorticoid for large joints) injections cause reduction in cartilage volume in osteoarthritis [15].

Previously, we have found that a single dose of calcipotriol ameliorates histological synovitis in arthritic rats without apparent systemic or local adverse effects [16,17]. From clinical experience in psoriasis, it is known that topically administered calcipotriol, as well as $1,25(OH)_2D_3$ are safe in repetitive and long-term dosing [18,19].

The inhibitory effect of $1,25(OH)_2D_3$ as well as calcipotriol for synovial stromal cell (SSC) proliferation and proinflammatory cytokine expression was proven previously [20], laying the foundation to research calcipotriol as an antiarthritic drug. In SSCs, $1,25(OH)_2D_3$ has previously been observed to inhibit invasion in human RA cells and murine pristane-induced arthritis fibroblast-like synoviocytes [21]. The inflammatory activation of synovial fibroblasts in arthritis has been suggested to be alleviated by $1,25(OH)_2D_3$ [22].

Mesenchymal stromal cells (MSCs) have been studied extensively, and they are known to interact with the immune system through anti-inflammatory cytokine secretion and surface molecule expression, suppressing the local immune response [23]. MSCs are nowadays known to reside in multiple tissues, including synovium, where they seem to differentiate towards chondrocytes more than other MSCs. [24,25] The role of MSCs particularly in RA, is ambivalent as MSCs might become detrimental to cartilage in inflammatory environment when forming pannus with SSCs, but their anti-inflammatory properties might also be beneficial [26–28]. MSC-based therapy relying on their anti-inflammatory properties has been tried for RA, giving promising results in preclinical studies and being safe in human studies [29].

We have previously shown the anti-proliferative effect of calcipotriol on SSCs [20]. Considering synovitis and pannus, the migration and invasion of the synovial cells are also relevant factors. For instance, enhanced migratory capabilities of fibroblast-like synoviocytes (a subset of SSCs [30]) were associated with worse synovitis in OA patients [31]. The migration and invasion of fibroblast-like synoviocytes are also key inflammatory mechanisms in RA synovitis, mimicking a tumour-like phenotype in pannus formation [32,33]. The inflammatory activation of these cells causes joint destruction in RA [34,35], but it is also recognized as a part of OA inflammation [36,37].

We hypothesized that calcipotriol should inhibit the migration and proliferation of SSCs at least as strongly as glucocorticoids considering the anti-inflammatory effect in rat synovium in vivo [16]. With MSCs, the effects were presumed to be similar even though similar comparison of different steroid hormones has not been done before. Based on the earlier findings we expected calcipotriol to be safe on the studied concentrations and an effective antiarthritic medication.

2. Methods

In this article, synovial stromal cells (SSCs) refer to the plastic-adherent fibroblast-type cells originating from human synovial tissue. This includes the type B synovial cells and subintimal fibroblasts, together named synovial fibroblasts [30]. The mesenchymal stromal cells (MSCs) in the experiment were plastic-adherent bone-marrow derived stromal cells with confirmed differentiation capabilities into adipogenic and osteogenic differentiation. However, the common term 'mesenchymal stem cell' is not used in this article, as these cells have not been tested for the necessary surface markers mentioned in the literature [38].

2.1. Materials

The five drugs used in 1 nM, 10 nM and 100 nM concentrations were calcipotriol (CPT, Santa Cruz Biotechnology, Dallas, TX, USA), $1,25(OH)_2D_3$ (Sigma-Aldrich, St. Louis, MO, USA), dexamethasone (DX, Sigma-Aldrich), methylprednisolone acetate (MP, Solomet, Orion Pharma, Espoo, Finland) and betamethasone as a 50–50 mixture of betamethasone acetate and betamethasone sodium phosphate (BM, Celeston Chronodose, Schering-Plough, Kenilworth, NJ, USA). MP and BM were clinically used drugs, while DX, CPT and $1,25(OH)_2D_3$ were compound for laboratory purposes. The drugs were dissolved in 96% ethanol; and 10 μl of the ethanol solution of the drug was added to complete media. The control group was complete media with equal amount (0.1%) of added ethanol. In the cell culture, Minimum Essential Medium (MEM) with Earle's Salts and L-glutamine (Corning Inc., Corning, NY, USA) was used. Collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and DNase (Sigma-Aldrich) were used to process the synovia containing the SSCs. For the apoptosis and necrosis assay, the phosphatidylserine-binding Annexin V (Sartorius UK Ltd., Epsom, Surrey, United Kingdom) was used as apoptosis stain and DNA-binding Cytotox green (Sartorius) as necrosis stain.

2.2. Collection of synovial and mesenchymal stromal cells

Synovial and mesenchymal stromal cells were collected from total knee arthroplasty surgeries. Pieces of synovial tissue and bone marrow aspirates were collected. All patients gave their informed consent for the use of sample material. The protocol for collecting and using MSCs and SSCs has been approved by the ethical board of Northern Ostrobothnia Hospital District. For the MSCs, the aspirates were suspended in complete medium (MEM with 10% fetal bovine serum, 2% HEPES buffer, 100 U/ml penicillin and 0.1 mg/ml of streptomycin). For SSCs, the pieces of synovium were digested in 100 U/ml collagenase and 50 U/ml DNase, and the plastic-adherent cells were divided to the same complete media. During cell culture, half of the media was changed twice a week. 10% trypsin was used to detach the cells from cell culture flasks. All cells were stored in liquid nitrogen before use.

2.3. Wound healing model

Altogether 9 cell lines, 4 fibroblast (2 RA + 2 OA) and 5 MSC (2 RA + 3 OA) lines were defrosted and cultured in complete media. After obtaining enough cells, the cells were seeded on 96-well ImageLock plates (6000 cells/well, passages 2–4) and allowed to adhere and form a confluent monolayer. After 3 days the scratch wounds were introduced with Incucyte Woundmaker Tool (Sartorius) simultaneously to all wells. Manual scratching with sterile pipette tips was attempted at first, but the wounds were inconsistent. After wounding, the supplemented media were administered, and the plate was imaged using Incucyte S3 instrument (Sartorius) every 2–3 h for at least two days. All wells were checked prior to analysis, and unfeasible ones were excluded. Unfeasibility was defined to be absence of confluent monolayer or uniform cell front, clear apoptosis or cell death, failure to introduce the wound, problems with the wound area (full of cells/debris) or error in camera focus, resulting in images that could not be analysed. The cells were seeded in two to six technical replicates, depending on the amount of cells that could be grown. The values are averages of the replicates.

Image analysis was done using Incucyte 2021 C software (Sartorius). Approximately a dozen, random images from different time-points and supplementations were selected, and analysis parameters (e.g. object

size range, recognition threshold, small object removal) for the recognition of the cells from the background were manually adjusted for SSCs and MSCs separately. The contrast of MSCs in grayscale images was lower, which complicated wound recognition. Adjusting the recognition parameters was balancing between too much and too little cell recognition.

Three variables were measured over time for the wound. The primary variable was relative wound density, being the difference of density (=confluence) of the wound area in each time-point and t = 0 h, divided by the difference of density of the area outside the wound and the wound area at t = 0 h. This takes into account that the density outside the wound is not always 100%, and there might be some cell density in the wound area at the beginning. This variable will be named wound closure (%) from now on. Density was 0% at 0 h, and approached 100% when the wound became as confluent as its surroundings. The secondary variable was wound confluence: a simpler variable between 0% and 100%, showing the confluence of the original (0 h) wound area over time. This variable was independent of wound recognition beyond t = 0 h. The third variable, wound width in micrometres, proved to be unfeasible as it did not accurately describe healing when the wound fronts were uneven.

2.4. Cell apoptosis and necrosis

Apoptosis and necrosis were studied in MSCs and SSCs to find out, whether calcipotriol causes cell death, which could affect wound healing. Two MSC and SSC cell lines (passages 2–5) from two patients, (one RA, the other OA) were defrosted and cultured as mentioned above. 3500 cells per well were seeded in 100 μl of the complete medium on a 96-well plate in (technical) triplicates and the cells were allowed to adhere overnight.

On the following day, 100 μl of complete medium supplemented with $1,25(OH)_2D_3$ (10 nM), calcipotriol (10 nM, 100 nM, 1 μM or 10 μM), DX (10 nM or 100 nM) or control (medium with 0.1% ethanol) was administered on the cells with 1:400 Annexin V red and 1:800 Cytotox green dyes. The drugs were diluted in 96% ethanol. No higher concentrations for $1,25(OH)_2D_3$ and DX were used for technical reasons (limited number of cells). The main goal was to observe the effect of calcipotriol. Half of the medium (100 μl) was added at the first division, and the supplemented media (100 μl) after six hours incubation. The cells were imaged using Incucyte S3 instrument every 2 h for six days on phase, green and red channel. First images were taken in approximately one hour after supplementation of dyes and drugs. Four images (each 1.3 mm × 1.75 mm) as technical quadruplicates were taken per well using 10x objective.

The images were analyzed using Incucyte S3 2020 C Rev1 software. 12 images from different supplementations and time-points were chosen to represent the image set for parameter adjustment. For the red and green channels, object count was used as the primary variable, and area as well as total integrated intensity as secondary variables in the 1.3 mm × 1.75 mm area. For the red+green channel, object count was the primary and area the secondary variable. The value of each well was the mean value of four images taken at each time point. As the cells were seeded in triplicates, there were twelve images in total for each supplement and cell line.

2.5. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics Data Editor (version 27).

The effect of different drugs was analysed by linear mixed models. Cell lines were considered as subjects, drug, cell type (MSC/SSC) and disease (RA/OA) as fixed effects, and time was considered both a fixed and random effect as a covariate. The models were adjusted with two-way interaction terms of fixed effects. The model fit was estimated stepwise using −2 log likelihood and Akaike's information criterion. The analysis was done separately for dependent variables in 1 nM, 10 nM and 100 nM concentrations. Pairwise comparisons between the drugs were done using Sidak correction for multiple testing.

For the wound healing model, the mixed model analysis was run several times. Fixed effect estimates and pairwise comparisons between groups (Sidak correction for multiple testing) were considered the main variables. It was noticed that the MSC and SSC cell lines differed significantly over time ($p < 0.001$) on initial analysis, which led to separate mixed models with SSCs and MSCs in the end. Methodologically, this allowed separate analysis for the cell types. The time period of analysis was set between 0 and 45 h to equally include all cell lines, as analysis did not go further than 45 h for some cell lines. Originally, the experiment was planned to last two days to eliminate the need of changing media.

For the wound closure values, the model was improved upon introducing the RA/OA variable and its pairwise interactions in all cell types and concentrations. All the results are presented according to the final form of the model.

In the apoptosis and necrosis assay, cell line (n = 4) was used as a subject, drugs (each concentration separately) as fixed effects and time as a fixed and random effect. Cell type or RA/OA factor was not used as a variable, as there were only four cell lines – the effect of these two variables could not be distinguished from variation between cell lines.

3. Results

3.1. Wound healing model

The wound healing model proved to be technically difficult. Of all 728 wells studied, 238 were excluded prior to analysis due to exclusion criteria (defined in the methods), leaving 490 wells in the analysis. The most usual reasons for exclusion were failures in wound recognition (29%) by the software due to low contrast or bubbles, problems with camera focus (27%), failure to introduce wound (15%) and other reasons (28%), including e.g. cell death or insufficient confluence. Of all the wells included, a substantial number (195 of 490) were suboptimal, usually (approximately 87% of suboptimal wells) due to only other side of the wound visible at the beginning. However, these wounds seemed to heal as other feasible wounds, and were included. It should be noted that the technical difficulties were mostly due to mesenchymal cell morphology, reflecting the technical difficulty of wound healing model in nonepithelial cells. Up to six technical replicates (if possible considering amount of cells) were used to tackle this issue beforehand. Thenumber of cell lines the experiment were for SSCs, n = 4 and for MSCs, n = 5, apart from individual cases where all replicates failed (100 nM DX in one RA SSC line and 1 nM MP in one RA SSC line).

3.1.1. 100 nM concentrations

The effect of 100 nM calcipotriol is demonstrated in Fig. 1. In SSCs, all drugs inhibited wound healing compared to control ($p < 0.001$). The effect of calcipotriol was more noticeable. In fact, calcipotriol inhibited wound healing significantly more (27.1% compared to control) than DX or BM, and even $1,25(OH)_2D_3$ at the same concentrations, but the difference to MP remained nonsignificant (Table 1, Fig. 2).

In MSCs, all drug groups inhibited wound healing at 100 nM concentration compared to control ($p < 0.001$), showing delayed healing. However, the extent of this was different. Calcipotriol and $1,25(OH)_2D_3$ reduced the mean closure values less (Table 1, Fig. 2), while the glucocorticoids (DX, MP, BM) showed greater inhibition in comparison to control ($p < 0.001$). These glucocorticoids groups differed significantly from calcipotriol and $1,25(OH)_2D_3$ in all pairwise comparisons. The effect of the strongest inhibiting glucocorticoid (DX) is demonstrated in Fig. 3.

3.1.2. 10 nM concentrations

In SSCs, the effect of calcipotriol and $1,25(OH)_2D_3$ was stronger than

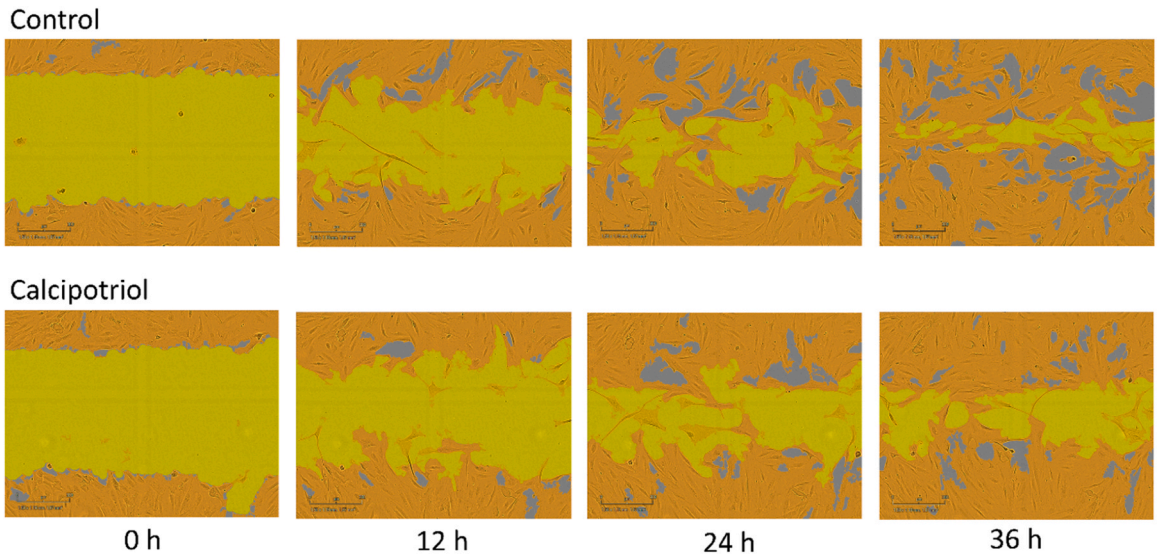


Fig. 1. The antimigratory effect of 100 nM calcipotriol on OA SSCs. The wound area (yellow) is filled by fibroblasts migrating from two fronts over time. The confluence of the cells is shown in orange. At 36 h, the delayed healing due to calcipotriol is clearly seen.

Table 1
The pairwise comparisons of a mixed model of wound healing in 100 nM concentrations. All drugs inhibit the wound healing significantly in both SSCs and MSCs. In SSCs, calcipotriol and 1,25$(OH)_2D_3$ are at least as effective as glucocorticoids – calcipotriol was more effective than DX and BM and equally effective as MP. In MSCs, calcipotriol and 1,25$(OH)_2D_3$ have a weaker effect, whereas all glucocorticoids (BM, MP, DX) more clearly inhibit the wound healing with significant differences to calcipotriol and 1,25$(OH)_2D_3$.

Cell type	Drug	Mean wound closure estimate (%)	Difference of average confluence (%-units)					
SSC			CTRL	CPT	1.25D3	DX	MP	BM
	CTRL	45.409	0	-12.294	-8.154	-8.398	-11.259	-4.464
	CPT	33.115		0	4.14	3.896	1.035	7.83
	1.25D3	37.255			0	-0.244	-3.105	3.69
	DX	37.011				0	-2.861	3.934
	MP	34.15					0	6.795
	BM	40.945						0
MSC								
	CTRL	30.451	0	-3.942	-5.738	-13.921	-11.766	-8.075
	CPT	26.509		0	-1.796	-9.979	-7.824	-4.133
	1.25D3	24.713			0	-8.183	-6.028	-2.337
	DX	16.53				0	2.155	5.846
	MP	18.685					0	3.691
	BM	22.376		p<0.05	p<0.01	p<0.001		0

that of any glucocorticoid ($p < 0.001$ in all pairwise comparisons). In fact, BM was no longer effective in this concentration compared to control (p = ns). The average wound closure was 20.3%− 21.0% lower with calcipotriol/1,25$(OH)_2D_3$ than in control group (Table 2, Fig. 4A).

In MSCs, the glucocorticoids were still potent inhibitors of wound healing. The average closure estimate in DX group was 40.9% lower than in the control group. Meanwhile, the effect of calcipotriol and 1,25$(OH)_2D_3$ was much lower (7.8%−8.1%), albeit statistically significant compared to control. In comparison to DX, MP and BM, calcipotriol and 1,25$(OH)_2D_3$ were weaker (Fig. 4B).

3.1.3. 1 nM concentrations

In SSCs, the effects of calcipotriol and 1,25$(OH)_2D_3$ were still seen in 1 nM concentrations. Calcipotriol reduced wound healing by an average of 22.7% ($p < 0.001$) compared to control (Table 3, Fig. 5A). For 1,25$(OH)_2D_3$, the effect was smaller (14.0%), but significant. DX and BM did not slow down wound healing anymore in 1 nM concentration. However, MP was able to reduce wound healing significantly (16.1%).

In MSCs, BM and MP were no longer able to inhibit wound healing (p = ns, Table 3). In contrast, calcipotriol, 1,25$(OH)_2D_3$ and DX reduced wound healing by 16.2%; 8.8%; and 9.0%, respectively ($p < 0.001$ in all cases, Table 3, Fig. 5B). The effect of calcipotriol was significantly stronger than other drugs, but again, this should be interpreted cautiously.

Similar mixed model analyses were done with the confluence parameter (data not shown). The main results were the same: calcipotriol inhibited wound healing at least as strongly in SSCs as all glucocorticoids; and the effect in MSCs was weaker. The effect was observable in 1 nM concentrations.

3.2. Cell apoptosis and necrosis

Firstly, some clear observations could be made from the apoptosis and necrosis data. The object counts of neither marker, nor their simultaneous expression were increasing over time, indicating that calcipotriol did not cause a continuous toxic effect (see Fig. 6A-C). In the

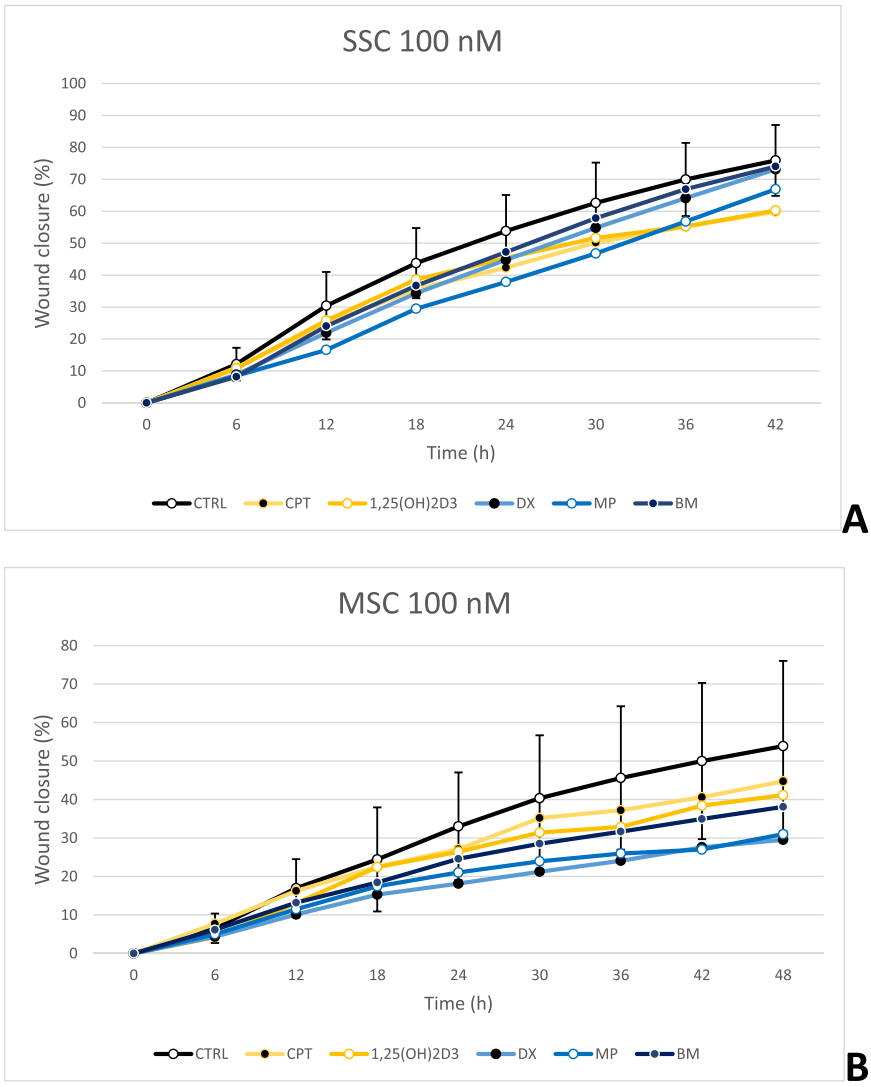


Fig. 2. The average wound closure values over time in SSCs (A) and MSCs (B) using 100 nM concentration of the drugs. The values are averages between the cell lines. In SSCs, calcipotriol and $1,25(OH)_2D_3$ are approximately as strong as glucocorticoids, while in MSCs, the effect of glucocorticoids is higher. The error bar is represented to the control group as ± SD, showing the extent of variation.

control group, a rise in apoptosis is seen in the end, probably caused by to too much proliferation and depletion of nutrients (media was not changed during the observation). The antiproliferative effects of the drugs seem to prevent this in other groups.

A slight increase in the apoptotic and necrotic objects both together and separately was seen between the first and the second measurements in all groups, but after that, the number of apoptotic and necrotic objects remained approximately constant. The increase is probably due to rapid influx of the dyes after administration to already dead cells and due to external stress from e.g. adding medium, changing incubator etc. Representative images of the experiment are shown in Fig. 7, where the vast majority of cells remained unstained.

The number of apoptotic and necrotic objects was studied using a mixed model analysis where all cell lines were involved. For the apoptosis stain (Annexin V), an average of 19.6 apoptotic objects were seen per image over time in control group, with higher values only in 10 μM calcipotriol group (21.6). This difference was significant in pairwise comparisons with Sidak multiple adjustment ($p < 0.001$, 95% CI = [−2.46; −1.52]). For the necrotic objects (Cytotox Green), the result was similar; with an average of 22.1 objects over time in the control group, and as the only higher values 23.0 and 26.4 objects in 1 μM and 10 μM calcipotriol groups, respectively. Both these differences were significant in pairwise comparisons ($p < 0.001$). Finally, for the objects that were stained with both red and green, the average number over time was 15.9 in control group, with higher values only in 1 μM (16.5) and 10 μM (19.1) calcipotriol groups. These differences were also significant compared to the control group. However, the absolute difference is minimal.

4. Discussion

In this study, calcipotriol, $1,25(OH)_2D_3$, DX, MP and BM were proven to inhibit wound healing in both SSCs and MSCs. This finding supports our previous results where $1,25(OH)_2D_3$ and calcipotriol were shown to inhibit proliferation of SSCs in a similar manner, and 10 nM calcipotriol had no proapoptotic or pronecrotic effects, as studied with annexin V and propidium iodide staining followed by flow cytometry [20]. The wound healing model analyses the migration of cells [39], and in our experiment, it also analyses proliferation to a lesser extent, as no traditionally used serum starvation or antiproliferative agent [40] was used. Using only calcipotriol as an active pharmaceutical ingredient allowed us to see possible apparent adverse effects on the cells – however, there proved to be none. It seemed that within two days, calcipotriol and $1,25(OH)_2D_3$ were at least as effective as glucocorticoids on

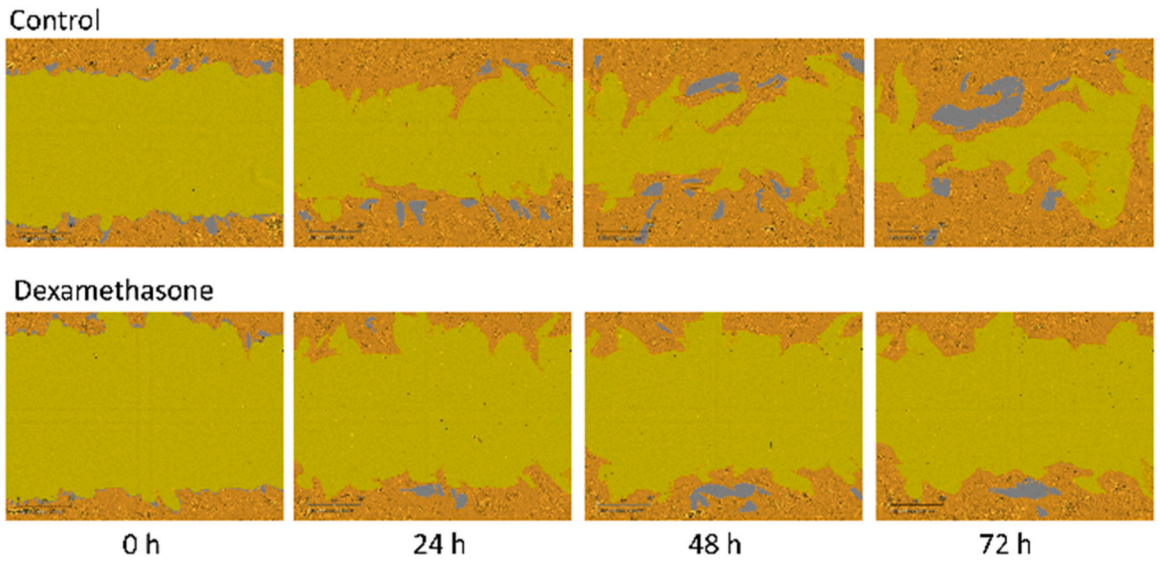


Fig. 3. The antimigratory effect of 100 nM DX on OA MSCs. The wound area (yellow) is filled by MSCs migrating from two wound fronts over time. The confluence of the cells is shown in orange. At 48 h and 72 h, the delayed healing due to DX is clearly seen.

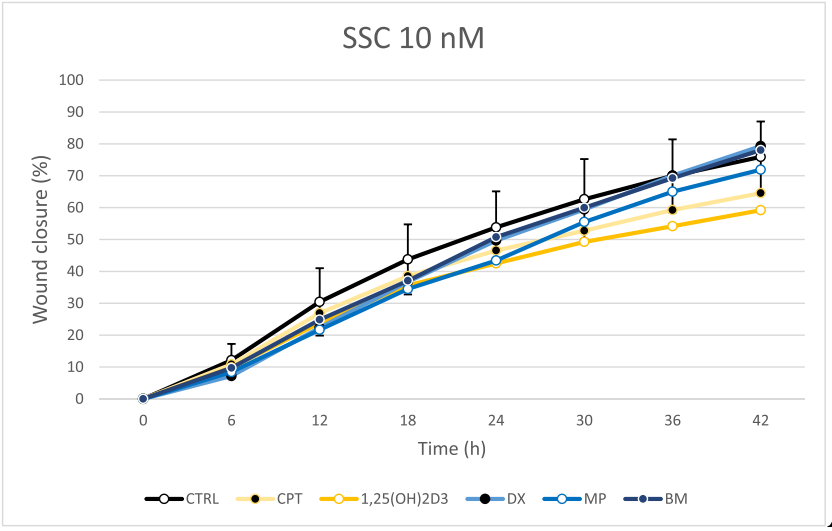
Table 2
The pairwise comparisons of a mixed model of wound healing in 10 nM concentrations. All drugs inhibit the wound healing significantly in MSCs, but in SSCs, BM no longer had a significant effect. In SSCs, calcipotriol and 1,25$(OH)_2D_3$ are stronger inhibitors of wound healing than glucocorticoids. In MSCs, calcipotriol and 1,25 $(OH)_2D_3$ have a weaker effect, whereas all glucocorticoids more clearly inhibit the wound healing with significant differences to calcipotriol and 1,25$(OH)_2D_3$.

Cell type	Drug	Mean wound closure estimate (%)	Difference of average confluence (%-units)					
SSC			CTRL	CPT	1.25D3	DX	MP	BM
	CTRL	45.413	0	-9.213	-9.542	-3.551	-5.641	-2.368
	CPT	36.2		0	-0.329	5.662	3.572	6.845
	1.25D3	35.871			0	5.991	3.901	7.174
	DX	41.862				0	-2.09	1.183
	MP	39.772					0	3.273
	BM	43.045						0
MSC								
	CTRL	30.459	0	-2.375	-2.467	-12.37	-6.587	-4.543
	CPT	28.084		0	-0.092	-9.995	-4.212	-2.168
	1.25D3	27.992			0	-9.903	-4.12	-2.076
	DX	18.089				0	5.783	7.827
	MP	23.872					0	2.044
	BM	25.916		p<0.05	p<0.01	p<0.001		0

SSCs, but less effective on MSCs. The SSC results are in line with our earlier findings, but the MSC results are surprising, and their relevance is unclear.

It is noteworthy that the inhibitory effect of 1,25$(OH)_2D_3$ and calcipotriol was clearly observable even in 1 nM concentrations, while the effect of glucocorticoids seemed to diminish at the same concentration. Calcipotriol and 1,25$(OH)_2D_3$ have similar affinity to vitamin D receptor (VDR), but the similar antiproliferative effect was measured in histiocytic lymphoma cell line by calcipotriol at half the concentration compared to 1,25$(OH)_2D_3$, indicating a stronger effect by calcipotriol [10,41]. Therefore, it was expected that their antimigratory effect would be approximately equal, which was true for 10 nM, but not for 1 nM and 100 nM (SSC) concentrations, which showed stronger inhibition by calcipotriol. It is possible that the small difference is pure chance or variation, as the lack of difference in 10 nM concentrations alone seems illogical – the small sample size might explain this. However, it was proven that calcipotriol is effective at inhibiting wound closure, which was previously proven for proliferation of SSCs with at the same 1 nM, 10 nM and 100 nM concentrations [20]. Even the smallest 1 nM concentration is clearly supraphysiological, as the concentration of 1,25 $(OH)_2D_3$ is at the picomolar range (59–159 pmol/l [42]). It is evident that calcipotriol is effective at multiple concentrations, but whether its effect is dose-dependent needs to be proven in larger sample size.

Multiple glucocorticoids were used to verify the antimigratory effects. DX seemed to be the most effective in MSCs, followed by MP, whereas in SSCs, MP was the strongest inhibitor. BM proved to be the least effective. Considering that DX and BM are nearly equipotent, and MP is generally weaker corticosteroid in similar doses [43], the finding seems surprising but may be partly explained by the drug formulations, which affect the solubility of the drug. It is noteworthy that a crystal size and dissolution rate of the steroid hormone in aqueous buffer affects the speed and potency of receptor binding and biological effect on cells. Another explanation may be the difference in non-genomic and genomic potencies of corticosteroids. The non-genomic potency of BM is extremely low compared to that of DX or MP [44], while the genomic potency favours DX and BM as stated above. Looking closely at Figs. 2, 4 and 5, one can see that the effect of DX seems to clearly deviate from control already after 6 h, suggesting a strong non-genomic effect.



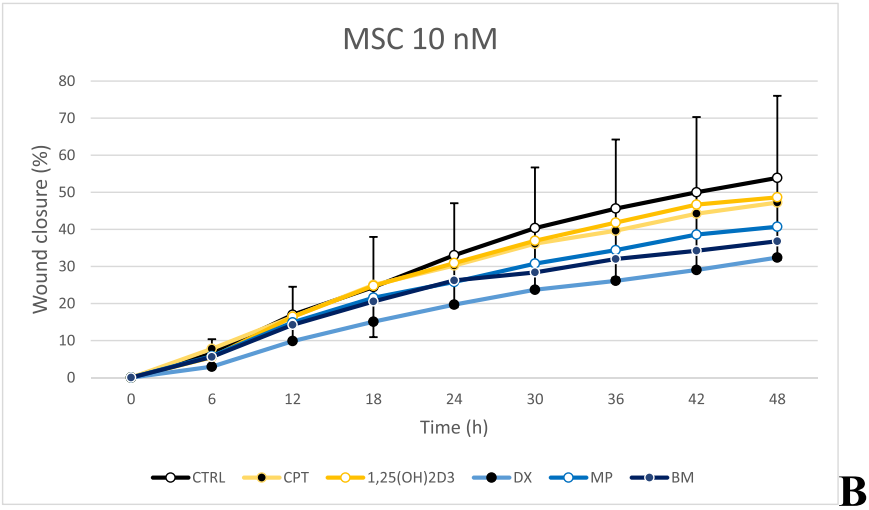


Fig. 4. The average wound closure values over time in SSCs (A) and MSCs (B) using 10 nM concentration of the drugs. The values are averages between the cell lines. In SSCs, calcipotriol and $1,25(OH)_2D_3$ are stronger than glucocorticoids, while in MSCs, the effect of glucocorticoids is higher. The error bar is represented to the control group as ± SD, showing the extent of variation.

Table 3
The pairwise comparisons of a mixed model of wound healing in 10 nM concentrations. In SSCs, calcipotriol, $1,25(OH)_2D_3$ and MP inhibited wound healing, while in MSCs, calcipotriol, $1,25(OH)_2D_3$ and DX were able to inhibit healing. In both cell types, calcipotriol was the strongest inhibitor.

Cell type	Drug	Mean wound closure estimate (%)	Difference of average confluence (%-units)					
SSC			CTRL	CPT	1.25D3	DX	MP	BM
	CTRL	45.473	0	-10.333	-6.359	-1.471	-7.336	-1.891
	CPT	35.14		0	3.974	8.862	2.997	8.442
	1.25D3	39.114			0	4.888	-0.977	4.468
	DX	44.002				0	-5.865	-0.42
	MP	38.137					0	5.445
	BM	43.582						0
MSC								
	CTRL	30.444	0	-4.93	-2.673	-2.749	-1.202	0.51
	CPT	25.514		0	2.257	2.181	3.728	5.44
	1.25D3	27.771			0	-0.076	1.471	3.183
	DX	27.695				0	1.547	3.259
	MP	29.242					0	1.712
	BM	30.954		p<0.05	p<0.01	p<0.001		0

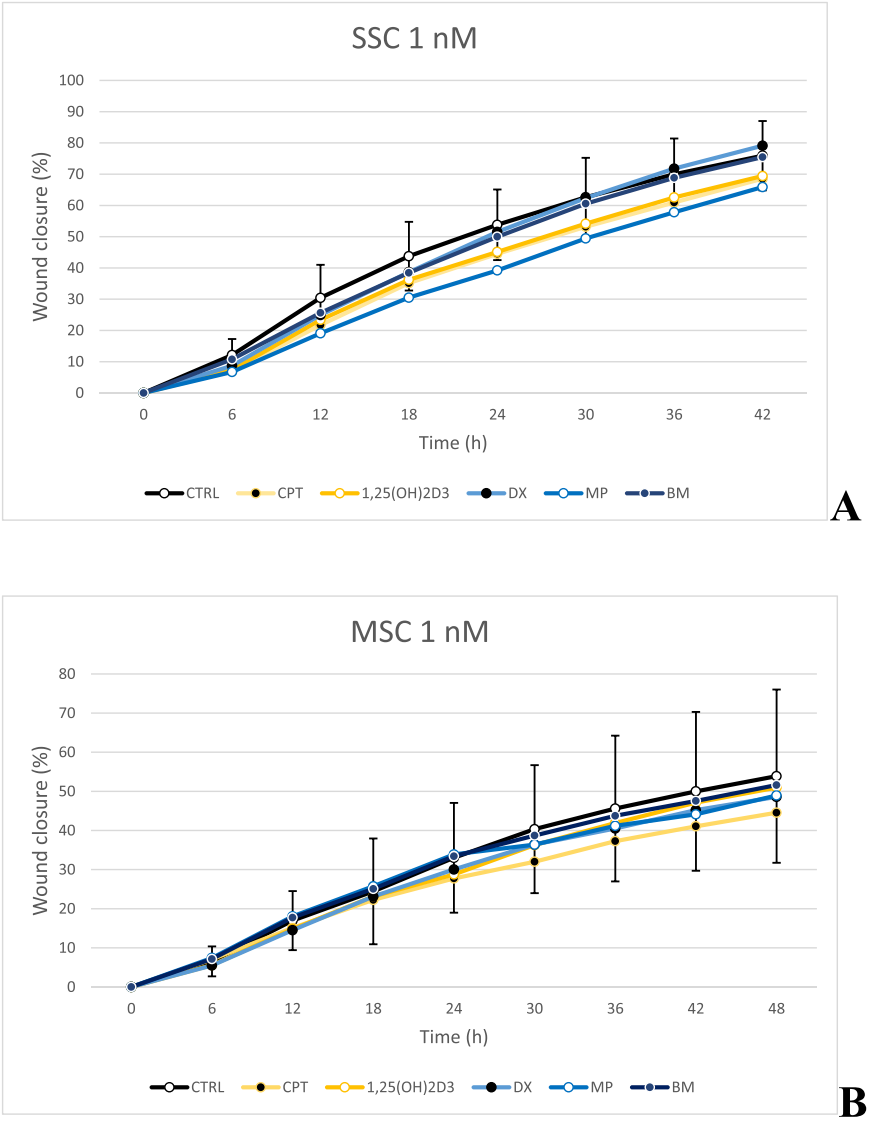


Fig. 5. The average wound closure values over time in SSCs (A) and MSCs (B) using 1 nM concentration of the drugs. The values are averages between the cell lines. In both cell types, calcipotriol and 1,25$(OH)_2D_3$ exert an inhibitory effect despite small concentration. The error bar is represented to the control group as ± SD, showing the extent of variation.

In contrast, nuclear receptor signalling and genomic effect of steroids happen in a timescale of hours [45], which is observable particularly in Fig. 2B for calcipotriol and 1,25$(OH)_2D_3$. 1,25$(OH)_2D_3$ also exerts some of its effects through non-genomic pathways, as regulating nitric oxide synthase, PI3K, phospholipases C and A2 as well as Ca-,Cl- and Pi-channels -these effects are mediated by a membrane receptor [46,47]. The antiproliferative effect of calcipotriol is described as VDR-mediated genomic effect in the literature [48,49], which might explain the inhibition of wound healing in a later stage rather than within the first hours – the antimigratory effect is likely mediated through the same mechanisms.

DX has an additive anti-inflammatory effect with 1,25$(OH)_2D_3$ on RA SSCs [50] and with calcipotriol in OA and RA SSCs [20], which suggests that corticosteroids and VDR agonists might have synergistic effects. The mechanisms of action of corticosteroids and calci(po)triol are complex, exerted through nuclear hormone receptors that act as either positive or negative transcription factors to a plethora of genes; for instance, the inhibition of NF-kB pathway is mediated through both compounds [43,51,52].

Considering MSCs, it is not certain which kind of alterations are beneficial, as MSCs can possibly have either beneficial or detrimental role in arthritis [27]. The immunomodulatory effects of MSCs are increased in inflammation [53], but the effects of drugs are less known. DX was detrimental to MSCs in high (100 nM-1 μM) concentrations, promoting apoptosis and decreasing cell activity in a previous study [54], however, in our study, no proapoptotic effect was seen in 100 nM concentrations. However, a low dose (1 nM) promotes expansion in umbilical cord-derived MSCs [55] and in BM-MSCs, a concentration of 10 nM maintains the stem cell phenotype and proliferation [56].

1,25$(OH)_2D_3$ and 25$(OH)D_3$ (a less active precursor of 1,25$(OH)_2D_3$) have been suggested to induce osteogenic differentiation of BM-MSCs [57,58]. In rats, the proliferation and migration of BM-MSCs was enhanced by 100 nM-1 μM 1,25$(OH)_2D_3$, promoting vasculogenesis, contrary to our results [59]. However, in chicken BM-MSCs undergoing osteogenic differentiation, 2,4 nM and 24 nM concentrations of 1,25$(OH)_2D_3$ had an antiproliferative effect [60]. More research on the matter is required – the effect on MSCs should optimally be observed in the inflammatory milieu of the joint.

In the apoptosis and necrosis assay, the number of fluorescent-positive objects remained constant. Within the first two hours, there tended to be a spike in dying cells, but this effect was visible in all wells and might represent the influx of the dyes into the cells and/or non-vital

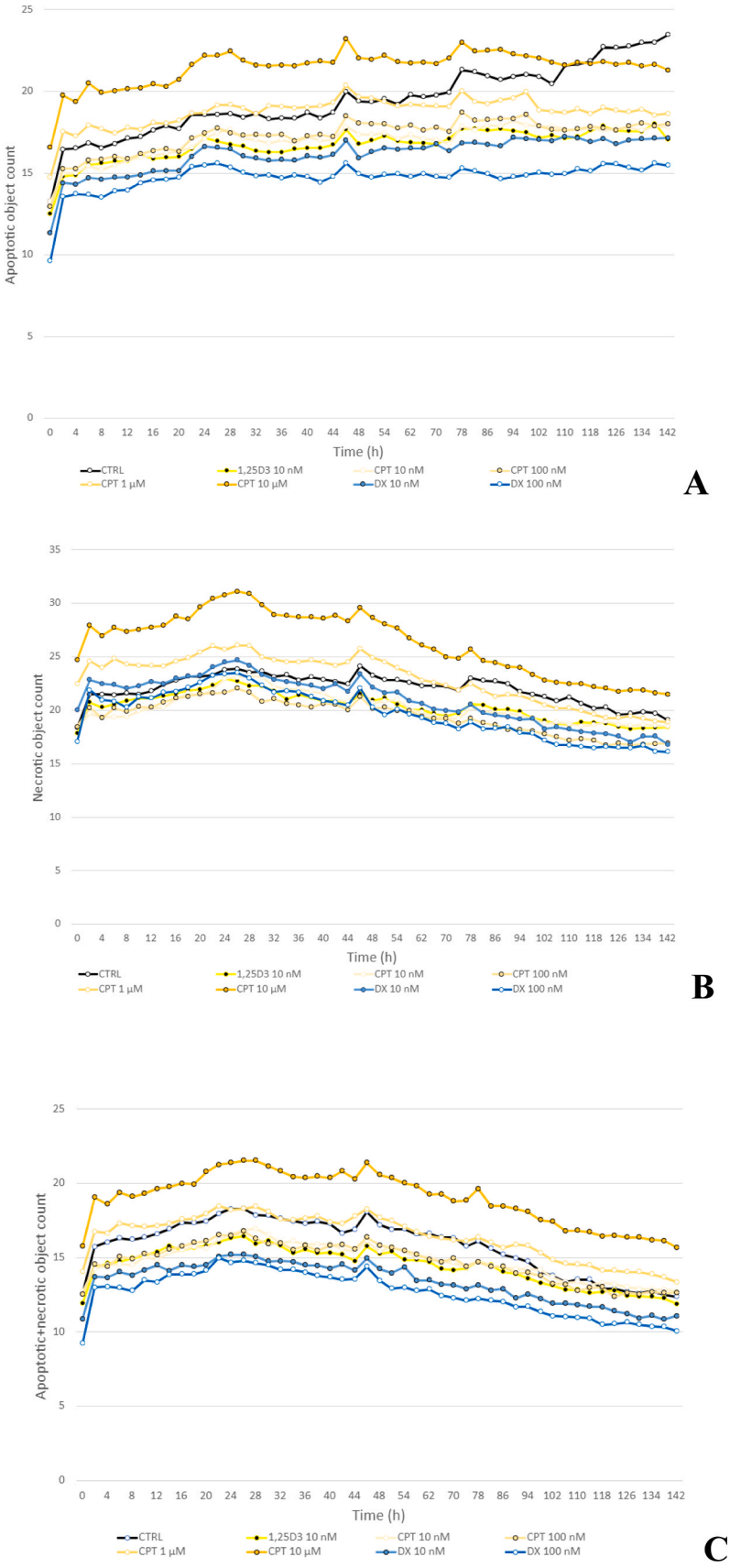


Fig. 6. The average (absolute) number of fluorescence-positive objects over time per 1.3 mm × 1.75 mm area. Apoptotic (A), necrotic (B) and objects that collected both dyes (C) were calculated separately. It is seen that the number of objects does not increase over time after the beginning.

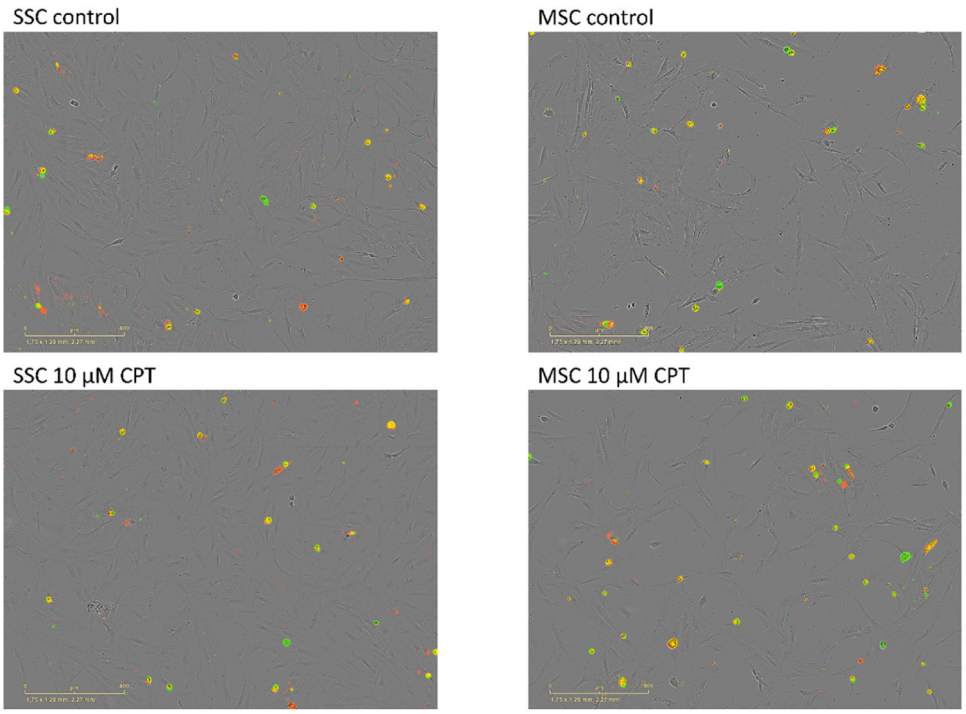


Fig. 7. Representative images of the apoptosis and necrosis assay. The apoptotic cells (Annexin V) are shown in red and necrotic (Cytotox) in green. Yellow color indicates the colocalization of the dyes. A slight difference in the number of positive cells is seen in the example images, but the number of apoptotic or necrotic cells was not increasing over time.

cells dying in response to external stress - adding medium and changing the incubator. We observed a slightly elevated number of apoptotic and/or necrotic objects particularly in the 10 μM calcipotriol group, and the difference compared to control was statistically significant. However, we argue that the result is irrelevant. Merely 2–4 additional stained objects were seen in the 10 μM group on average, while the image area included a few hundred cells. In addition, the number of stained cells was not increasing over time. Therefore, even 10 μM concentration of calcipotriol can still be considered relatively safe in MSCs and SSCs. For concentrations between 1 nM and 100 nM, the possibility of apoptosis and necrosis in wound healing inhibition can be excluded.

The cell culturing method does not take the individual in vivo environment of SSCs and MSCs into account. When RA SSCs have been cultured with their respective synovial fluid as their supplement, the proliferation increased considerably compared to control or stimulation with TNF-α alone [61]. This line of study could have yielded different results. Also, the exact effects of the drugs on MSCs (e.g. mRNA expression analysis of anti-inflammatory cytokines) were not studied, leaving the clinical significance of the observed phenomena unresolved in this cell type.

5. Conclusions

Calcipotriol and 1,25$(OH)_2D_3$ were proven to inhibit migration and proliferation in a wound healing model at least as strongly as GCs in SSCs, and slightly less than GCs in MSCs. Calcipotriol was safe in concentrations up to 10 μM, indicating a broad therapeutic window that includes several orders of magnitude down to at least 1 nM.

Author statement

The authors declare no conflicts of interest.

Declarations of interest

None.

Data Availability

Data will be made available on request.

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