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CALCIPOTRIOL (MC 903): PHARMACOKINETICS IN RATS AND BIOLOGICAL ACTIVITIES OF METABOLITES

A COMPARATIVE STUDY WITH $1,25(OH)_2D_3$

ANNE-MARIE KISSMEYER* and LISE BINDERUP
Department of Biology and Toxicology, Leo Pharmaceutical Products Ltd, Denmark

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Abstract—Calcipotriol (MC 903) is a novel analogue of the physiologically active metabolite of vitamin D_3, 1α,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$]. MC 903 and $1,25(OH)_2D_3$ have similar effects on cell proliferation and cell differentiation *in vitro* using the human histiocytic lymphoma cell line U 937, but *in vivo* MC 903 has 100–200 times less effect on calcium metabolism. To elucidate this difference, the pharmacokinetic profiles after a single intravenous dose (50 μg/kg) of the two compounds to rats were compared. The area under the serum level/time curve (AUC) was more than 100 times higher for $1,25(OH)_2D_3$ than for MC 903 and the rate of clearance was more than 100 times higher for MC 903 than for $1,25(OH)D_3$. Serum from MC 903 or $1,25(OH)_2D_3$ dosed rats (i.v. 10 μg/kg) was investigated for biological activities by incubation of U 937 cells with serum collected 0–24 hr after drug administration. Serum from MC 903 dosed rats had an effect only when collected shortly after dosing, whereas serum from $1,25(OH)_2D_3$ dosed rats had an effect when collected up to 4 hr after dosing. The biological effects on the U 937 cells of the two major metabolites of MC 903 (MC 1046 and MC 1080) were investigated. The metabolites had effects that were more than 100 times weaker than those of the parent compound. The effect of MC 903 on proliferative disorders, its fast elimination and the formation of inactive metabolites makes MC 903 suitable for topical treatment of psoriasis.

Calcipotriol (MC 903) is a novel analogue of the physiologically active metabolite of vitamin D_3, 1α,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$). *In vitro*, MC 903 has been found to be a potent inducer of cell differentiation and to inhibit proliferation and DNA-synthesis of cells possessing the receptor for $1,25(OH)_2D_3$ [1–3]. These effects were similar to those exerted by $1,25(OH)_2D_3$. However, when administered *in vivo* to both normal and rachitic rats, MC 903 was found to be 100 to 200 times less active than $1,25(OH)_2D_3$ in its effect on calcium metabolism [1]. This reduced risk of inducing classical vitamin D associated side effects, such as hypercalciuria, hypercalcemia and induction of bone resorption, has greatly stimulated the interest in using new vitamin D analogues such as MC 903 in the treatment of various proliferative disorders. In this respect, topical treatment of psoriasis with MC 903 has recently been shown to constitute an effective and safe therapy for this disease [4, 5].

As one of the major advantages of MC 903, in relation to $1,25(OH)_2D_3$, lies in its low calcemic activity *in vivo*, it is of course of obvious importance to elucidate the reasons for this difference in the mode of action of the two compounds. It has previously been shown that MC 903 has a lower ability than $1,25(OH)_2D_3$ to bind to serum proteins, including the vitamin D binding protein, thus suggesting a decreased capacity for the transport of MC 903 by the vitamin D binding protein in blood [2, 6]. In addition, investigations on the metabolism of MC 903 *in vitro* using liver homogenates from rats, mini-pigs and humans suggested that MC 903 was rapidly metabolized. Two major metabolites of MC 903 were identified and synthetized [7]. This paper describes the pharmacokinetic profile of MC 903, in comparison to $1,25(OH)_2D_3$, after administration of a single intravenous (i.v.) dose of each compound to rats. In addition, sera from dosed rats were assayed for their ability to induce cell differentiation and to inhibit cell proliferation *in vitro* in a human cell line responsive to both MC 903 and $1,25(OH)_2D_3$. This type of cellular bioassay was also used to test the biological activity *in vitro* of the two synthetically prepared metabolites of MC 903.

MATERIALS AND METHODS

Compounds. 1α, 25 - Dihydroxycholecalciferol - ($1,25(OH)_2D_3$), calcipotriol (MC 903), MC 1046 and MC 1080 were synthetized in the Department of Chemical Research, Leo Pharmaceutical Products [7, 8]. All chemicals and reagents were commercially available.

Pharmacokinetics of $1,25(OH)_2D_3$ *and MC* 903. Groups of three male Sprague–Dawley rats (180–220 g) with access to food and water were dosed i.v. with $1,25(OH)_2D_3$ or MC 903, at doses of 10 and 50 μg/kg. The compounds were dissolved in isopropanol and diluted prior to administration with a vehicle suitable for i.v. administration (citric acid 0.16 mg, trisodium citrate 6.8 mg, ethanol 80 mg, propylene glycol 415 mg and water up to 1.0 mL). Blood samples were collected from one group per time at 0, 5, 10 and 30 min, and at 1, 2, 4, 6

* Correspondence to: Anne-Marie Kissmeyer, Department of Biology and Toxicology, Leo Pharmaceutical Products Ltd., 55 Industriparken, DK-2750 Ballerup, Denmark.

and 24 hr after dosing. The concentrations of $1,25(OH)_2D_3$ and MC 903 were determined by high performance liquid chromatography (HPLC) [7] with the following sample preparation: serum was precipitated with acetonitrile before the supernatant was diluted and passed through a BondElut LRC C18/OH cartridge [9]. After washing with water–methanol (30:70 v/v) and with plain water, the analytes were eluated with acetonitrile, which was then evaporated. The residue was reconstituted with water–methanol (30:70 v/v) before injection into the column. The limits of detection for both compounds were 10 ng per mL serum. [The serum half-life ($t_{1/2} = \ln 2/K_e$) was calculated from the elimination rate constant (K_e), which was determined from linear regression of ln(conc) for 1–6 hr. The area under the serum level/time curve (AUC) was calculated by the trapezoidal rule and the clearance (Cl) was calculated by dividing the dose by the AUC.]

Cell line and propagation. The human histiocytic lymphoma cell line U 937 (10) was propagated *in vitro*, with twice-weekly passages, in medium RPMI 1640 (25 mM HEPES, Gibco, Uxbridge, U.K.), supplemented with 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum (Gibco).

The cellular effects of sera from rats dosed with $1,25(OH)_2D_3$ *or MC* 903. Groups of three rats (150–180 g) were treated i.v. with $1,25(OH)_2D_3$ or MC 903 at a dose of 10 μg/kg. The compounds were dissolved in propylene glycol and diluted prior to administration with the vehicle as described above. Blood was collected from one group per time at 0, 10, and 30 min and at 1, 2, 4, 6 and 24 hr after administration. To test the effects of the serum on cell proliferation and cell differentiation, the U 937 cells were adjusted to 1×10^5 cells/mL in the supplemented RPMI 1640 media containing 10% of rat serum instead of the fetal calf serum. The cells were plated at 2 mL per well in large multi-dishes with six wells per plate (Nunc, Cat. No. 152795, Denmark) and incubated for 96 hr at 37° in 5% CO_2 in air. At the end of incubation non-adherent cells were collected and counted to evaluate cell proliferation. A sample from each culture was fixed with formalin and assayed for the presence of membrane-associated non-specific esterase [11] to evaluate cell differentiation.

Receptor binding and cellular effects of the MC 903 *metabolites MC* 1046 *and MC* 1080. To study the binding of the major metabolites of MC 903 (MC 1046 and MC 1080) to the $1,25(OH)_2D_3$ receptor from the intestinal epithelium of rachitic chickens, a $1,25(OH)_2D_3$ assay reagent system (TRK.870) was purchased from Amersham (Little Chalfont, Bucks, U.K.). Five hundred microlitres of receptor protein were incubated with approximately 10,000 dpm [^{3}H]$1,25(OH)_2D_3$ and increasing concentrations of test compounds were added. After incubation for 60 min at 22°, bound and free [^{3}H]$1,25(OH)_2D_3$ were separated on dextran-coated charcoal. The samples were centrifuged and the supernatants containing the receptor-bound [^{3}H]$1,25(OH)_2D_3$ were counted in a liquid scintillation counter. The concentration of the test compound resulting in 50% displacement of bound [^{3}H]$1,25(OH)_2D_3$ was calculated.

The effects of the metabolites MC 1046 and MC 1080 on cell proliferation and cell differentiation were also studied. The U 937 cells were adjusted to 1×10^5 cells/mL in the supplemented RPMI 1640 medium. The cells were plated at 5 mL in small tissue culture flasks (Nunc Cat. No. 163371, Denmark) and incubated for 96 hr at 37° in 5% CO_2 in air. MC 1046 or MC 1080 were present during the whole period of incubation. The concentration of the compound resulting in 50% inhibition of cell proliferation was assessed (IC_{50}) and the induction of cell differentiation was recorded as the lowest concentration of test compound giving a 3× increase in the number of esterase positive cells, compared to control cultures with vehicle alone. The cellular effects were compared to values of $1,25(OH)_2D_3$ and MC 903 as previously described [1, 2].

Calcium levels in the in vivo *studies.* The total serum calcium was determined in all samples by complex formation with *o*-cresolphthalein [12].

RESULTS

Pharmacokinetics of $1,25(OH)_2D_3$ *and MC* 903

In order to compare the pharmacokinetics of $1,25(OH)_2D_3$ and MC 903, the serum half-life ($t_{1/2}$), the area under the serum level/time curve ($AUC\infty$) and the serum clearance have been calculated from the serum concentrations determined by HPLC (Table 1). $1,25(OH)_2D_3$ was still detectable 6 hr following i.v. dosing with either 10 or 50 μg/kg, whereas MC 903 was detectable at 5 min only after i.v. dosing with 10 μg/kg and up to 10 min after i.v. dosing with 50 μg/kg. The elimination of $1,25(OH)_2D_3$ could be separated into an α-phase and a β-phase with half-lives of 15 min and 2.3–3.8 hr, respectively. For MC 903 only an α-phase was observed, with a half-life of 4 min. Furthermore, the calculation of the area under the serum level/time curve revealed that the value for $1,25(OH)_2D_3$ was 140 times higher than for MC 903. The rate of clearance was approximately 140 times higher for MC 903 than for $1,25(OH)_2D_3$.

Cellular effects of sera from rats dosed with $1,25(OH)_2D_3$ *or MC* 903

The pharmacokinetic analysis of MC 903 and $1,25(OH)_2D_3$ by HPLC permits the estimation of serum concentrations ≥ 10 ng/mL. Lower concentrations, which may be more physiologically relevant, can only be estimated by combining the HPLC assay with the use of radiolabelled compounds, by the use of immuno- or protein-binding assays, or by a bioassay measuring the biological activity of the compounds *in vitro*. The latter type of assay does not detect the presence of the compound under investigation alone, but includes the activity of any biologically active metabolite(s). To evaluate the biological activities of MC 903 and $1,25(OH)_2D_3$ in serum from dosed rats, the human histiocytic lymphoma U 937 cell line was used. This cell line possesses high affinity receptors for $1,25(OH)_2D_3$ [10] and has previously been shown to be

Table 1. Pharmacokinetic profile of $1,25(OH)_2D_3$ and MC 903 after i.v. administration to rats

Treatment	Dose (μg/kg)	ng unchanged compound per mL serum								$t_{1/2}$		AUC_∞	Cl
		0 min	5 min	10 min	30 min	1 hr	2 hr	4 hr	6 hr	24 hr	α-phase (min)	β-phase (hr)	(ng/mL × hr)	(mL/hr)
$1,25(OH)_2D_3$	10	—	130	99	83	40	38	23	17	—	13	3.8	309	6
$1,25(OH)_2D_3$	50	—	676	537	422	370	263	165	78	—	15	2.3	1683	5
MC 903	10	—	12	—	—	—	—	—	—	—	—	—	—	—
MC 903	50	—	62	27	—	—	—	—	—	—	4	—	12	679

The half-life is separated in an α-phase (0–10 min) and a β-phase (1–6 hr) after dosing.

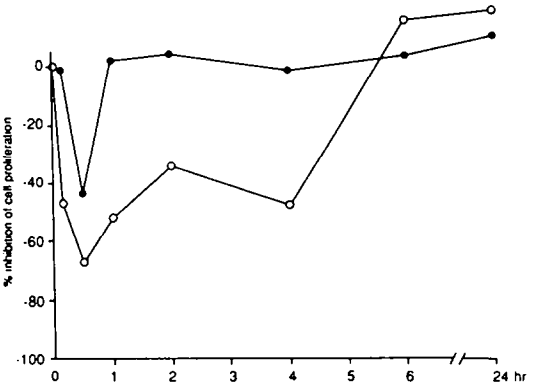


Fig. 1. Effects of MC 903 and $1,25(OH)_2D_3$ on cell proliferation. U 937 (1×10^5 cells/mL) were cultured at 37° for 96 hr in the presence of serum from $1,25(OH)_2D_3$ (○) or MC 903 (●) dosed rats (10 μg/kg). The sera were collected 0–24 hr following i.v. drug administration. At the end of the incubation, the cell proliferation was determined by cell counting.

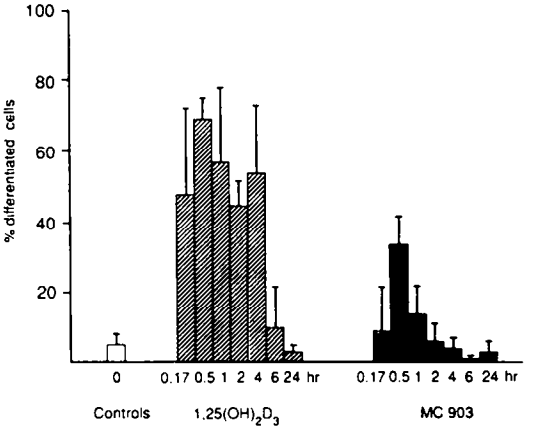
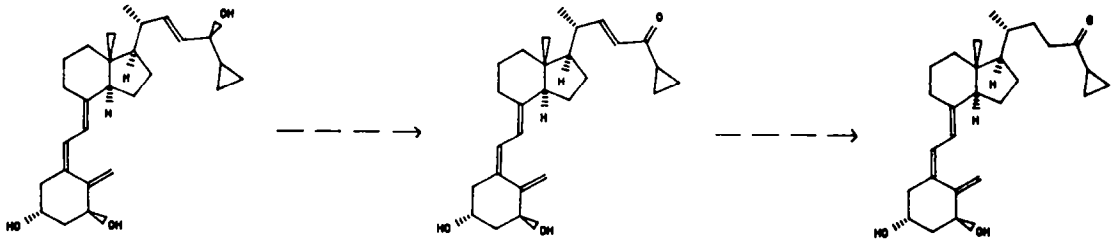


Fig. 2. Effects of MC 903 and $1,25(OH)_2D_3$ on cell differentiation. U 937 (1×10^5 cells/mL) were cultured at 37° for 96 hr in the presence of serum from $1,25(OH)_2D_3$ or MC 903 dosed rats (10 μg/kg). The sera were collected 0–24 hr following i.v. drug administration. At the end of the incubation, samples of cells were stained for esterase activity as a marker for cell differentiation.

equally responsive to $1,25(OH)_2D_3$ and MC 903 at concentrations from 1 nM (approx. 0.4 ng/mL) [1].

Serum was collected from rats at different times after dosing with either $1,25(OH)_2D_3$ or MC 903 (10 μg/kg i.v.). The cells were incubated with serum for 4 days. Figure 1 shows the effects on cell proliferation. The maximum inhibition of the cell proliferation was observed with serum collected 30 min after dosing with either $1,25(OH)_2D_3$ or MC 903. This inhibitory effect could be observed with serum collected up to 4 hr after dosing with $1,25(OH)_2D_3$, whereas no effect was observed with serum collected from rats dosed with MC 903 beyond the 30 min sample. Figure 2 shows the effects on the cell differentiation. Differentiation to cells with monocyte/macrophage characteristics was assessed



MC 903 MC 1046 MC 1080

Fig. 3. The chemical structure of MC 903 and its two major metabolites.

(appearance of α-naphtyl acetate esterase activity). Cell cultures incubated with the 30 min samples had the highest effect on cell differentiation; 69% of the cells were differentiated by serum from the $1,25(OH)_2D_3$ dosed rats and 34% of the cells by serum from the MC 903 dosed rats. Furthermore, serum collected at 1, 2 and 4 hr following dosing with $1,25(OH)_2D_3$ were able to differentiate about 50% of the cells. Serum collected from rats dosed with MC 903 induced 14% differentiation of U 937 cells in the 1 hr sample, thereafter no effects could be detected. Serum samples collected shortly after the i.v. administration of MC 903 and $1,25(OH)_2D_3$ were difficult to evaluate. This was attributed to a slight hemolytic effect induced by the acute administration of the vehicle (results not shown).

Receptor binding and cellular effects of the metabolites MC 1046 and MC 1080

The results from the pharmacokinetic investigations and the bioassays performed with serum from rats dosed with MC 903 or $1,25(OH)_2D_3$ show that intact MC 903 is rapidly cleared from the circulation and that the biological effects of the serum on cell differentiation and proliferation are very short lived, in comparison to serum collected from rats treated with $1,25(OH)_2D_3$. These findings suggest that the metabolism of MC 903 may lead to the formation of metabolites with no or reduced biological activity.

This theory has been investigated by measuring the ability of the two major metabolites of MC 903 (MC 1046 and MC 1080, see Fig. 3) to bind to the $1,25(OH)_2D_3$ receptor isolated from the intestinal epithelium of rachitic chickens and to affect the cell proliferation and cell differentiation of U 937 cells. The receptor protein was incubated with [^{3}H]$1,25(OH)_2D_3$ and MC 1046 or MC 1080, and the concentration of the test compound resulting in 50% displacement of bound [^{3}H]$1,25(OH)_2D_3$ was calculated. Table 2 shows that half maximal displacement was obtained with MC 1046 at 9.5×10^{-9} M and with MC 1080 at 1.0×10^{-9} M. It demonstrates a much lower affinity of the two metabolites to the receptor than that of MC 903 and $1,25(OH)_2D_3$, where the half maximal displacement was obtained at 3.9×10^{-11} M and 3.0×10^{-11} M. To evaluate the biological effects of MC 1046 and MC 1080 on the cell proliferation and differentiation, cultures were incubated with MC 1046 and MC 1080 for 4 days (Table 2). MC 1046 and MC 1080 did not inhibit the cell proliferation at the highest concentration tested (1.0×10^{-7} M). The cell differentiation was induced with concentrations of both compounds at 1.0×10^{-7} M, which is 100 times higher than needed for MC 903 or $1,25(OH)_2D_3$. These results show that the two major metabolites of MC 903 have much weaker biological effects compared to the parent compound MC 903 and to $1,25(OH)_2D_3$.

Effects on calcium in serum

The serum calcium level of the samples collected at 24 hr after dosing with $1,25(OH)_2D_3$ (50 μg/kg i.v.) was 3.39 ± 0.03 mmol/L. This is significantly higher than in the control and MC 903 dosed rats (50 μg/kg i.v.), where the concentrations were 2.71 ± 0.10 mmol/L and 2.81 ± 0.11 mmol/L, respectively.

DISCUSSION

Previous studies [1, 2] have shown that the effects of MC 903 and $1,25(OH)_2D_3$ on cell proliferation, cell differentiation and binding to the intestinal $1,25(OH)_2D_3$ receptor are very similar. However, MC 903 has low vitamin D activity *in vivo* and therefore a lower risk of inducing calcium related side effects than $1,25(OH)_2D_3$. In order to investigate this difference, the pharmacokinetic profile of the two compounds was studied. Determination of MC 903 at low concentrations presents a number of difficulties. A sensitive protein-binding assay is under development, but until this assay is functional, a less sensitive HPLC method with a limit of detection of 10 ng/mL has been used. Therefore, rats had to be treated with relatively high doses. The study clearly demonstrated a great difference in the elimination of the compounds. The elimination, including distribution, of MC 903 could only be observed as monophasic with a half-life of 4 min. The elimination of $1,25(OH)_2D_3$, however, was biphasic, with a half-life for the α-phase of 15 min, which also included distribution, and a half-life for the β-phase of approx.

Table 2. MC 1046 and MC 1080: Effect on the receptor binding, cell proliferation and cell differentiation

Compound tested	Receptor binding 50% displacement of [^{3}H]$1,25(OH)_2D_3$ (M)	Inhibition of cell proliferation IC_{50} (M)	Induction of cell differentiation (M)
MC 1046	9.5×10^{-9}	$>1.0 \times 10^{-7}$	1.0×10^{-7}
MC 1080	1.0×10^{-9}	$>1.0 \times 10^{-7}$	1.0×10^{-7}
MC 903	3.9×10^{-11}	1.4×10^{-8}	1.0×10^{-9}
$1,25(OH)_2D_3$	3.0×10^{-11}	2.8×10^{-8}	1.0×10^{-9}

$1,25(OH)_2D_3$ receptor protein from the intestinal epithelium of rachitic chicken was incubated with [^{3}H]$1,25(OH)_2D_3$ and test compound. The concentration of the test compound resulting in 50% displacement of bound [^{3}H]$1,25(OH)_2D_3$ was calculated.

U 937 cells (1×10^5 cells/mL) were cultured at 37° for 96 hr in presence of test compound. At the end of incubation the cells were counted and the concentration of the compound resulting in 50% inhibition of cell proliferation was assessed (IC_{50}). The non-adherent cells were also collected and stained for the presence of non-specific esterase activity as a marker of cell differentiation.

3 hr. The biphasic elimination of $1,25(OH)_2D_3$ has been described previously [13, 14]. The area under the serum concentration time curve was more than 100 times higher for $1,25(OH)_2D_3$ than for MC 903. This correlates with the effect on calcium metabolism found *in vivo* in normal and rachitic rats [1], where it was demonstrated that the vitamin D activity of MC 903 was 100–200 times less than that of $1,25(OH)_2D_3$. The pharmacokinetic findings were confirmed in the experiment where the biological activities on human histiocytic lymphoma U 937 cells were evaluated after incubation for 4 days with serum from MC 903 or $1,25(OH)_2D_3$ dosed rats. The ability of serum from $1,25(OH)_2D_3$ and MC 903 dosed rats to affect cell proliferation and cell differentiation was in agreement with the concentration profiles obtained from the serum concentrations of the compound. Previous investigations using post-mitochondrial supernatants from the liver of rat, mini-pig and man have shown that MC 903 is rapidly metabolized *in vitro*. Two metabolites, MC 1046 and MC 1080, were identified and synthetized [7]. These metabolites were also detected in sera from rats and pigs dosed with MC 903.*

The metabolism of MC 903 to MC 1046 and 1080 involves oxidation at carbon 24 in the side chain, similar to the C-24 oxidation pathway of metabolism of $1,25(OH)_2D_3$ [15–17]. The present experiments indicate that the formation of these 24-oxidized metabolites constitute a deactivation pathway for MC 903, since the biological effects of MC 1046 and MC 1080 on cell proliferation and cell differentiation *in vitro* were much weaker than those exerted by the parent compound MC 903. This low biological activity of the two metabolites can be explained by their decreased ability to bind to the $1,25(OH)_2D_3$ receptor.

In conclusion, the results from the present studies indicate that the low vitamin D activity exerted *in vivo* by MC 903 is, at least in part, due to rapid elimination of MC 903, together with the formation of metabolites with low biological activity. The combination of potent, direct effects of MC 903 on cell proliferation and differentiation and a rapid rate of inactivation after systemic uptake makes MC 903 an ideally suited agent for topical treatment of proliferative skin disorders such as psoriasis.

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