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BRIEF COMMUNICATION

Delay in resumption of the activity of tetracycline-regulatable promoter following removal of tetracycline analogues

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The tetracycline-regulatable system (TRS) has become a widely adopted tool for modification of gene expression and analysis of gene function in mammalian cells, plants and transgenic animals. We have studied the potential application of the TRS in gene therapy, using a single vector containing both the tetracycline-controlled transactivator (tTA) and the tTA-responsive promoter (tRP) transcribing mouse GM-CSF. Stable 293 cells established using this vector were used to study the kinetics of the TRS in response to various tetracycline analogues. Dose–response studies show that doxycycline is the most potent-analogue in abolishing tTA activity. Kinetic studies indicate that, at 1000 ng/ml, all the analogues have similar efficiencies in down-regulating the system in a given time. In contrast, following the removal of the analogues, there is a temporal, dose-dependent delay in resumption of the tRP activity. The time taken for resumption of near-optimal tRP activity is approximately 48 h for tetracycline, 144 h for anhydrotetracycline, 192 h for minocycline and 216 h for doxycycline when cells were pretreated with 1000 ng/ml of these antibiotics. This property of the analogues can be employed in planning a desired course of transgene regulation.

Keywords: *tetracycline; doxycycline; minocycline; anhydrotetracycline; oxytetracycline; tTA-responsive promoter*

Controlling the level and timing of transgene expression is clearly desirable in many gene therapy applications. Successful treatment of certain disease states requires a specific dose of the therapeutic gene product[1,2] and excessive production of some proteins may prove to be toxic.[3] A regulatable system that can offer tight control in response to pharmacological agents that can be safely and repetitively administered is clearly of value in modifying specific therapies. Although a variety of regulatable promoters have been developed, most require inducible agents that are associated with adverse effects on mammalian cells.[4] The TRS[5] avoids the problems related to many of the other systems by offering substantial regulation of transgene expression in response to concentrations of tetracycline that cause little cytotoxicity in mammalian cells. Since its original description, it has emerged as an invaluable tool for tight and reversible control of transgene expression, both *in vitro* and *in vivo*.[6] The popularity of the system for *in vivo* gene regulation in transgenic mice[7–11] and its potential applications in gene therapy,[12–14] necessitates a better understanding of this system in response to tetracycline derivatives available for clinical use. Despite the existence of over 1000 tetracycline derivatives, only seven of these are commonly used in clinical or veterinary settings.[15] The favourable pharmacokinetics and pharmacodynamics of doxycycline have encouraged the use of this agent for most of the clinical conditions requiring tetracycline.[16] Moreover, doxycycline has recently been used to achieve quantitative regulation of both tTA and reverse tTA in transgenic mice.[17] Here, we study the kinetics of repression/induction of the TRS in response to the clinically used minocycline and doxycycline, and to tetracycline and anhydrotetracycline.

The kinetics of TRS repression in response to tetracycline analogues were compared to that of tetracycline at 1000 ng/ml. As can be seen from Figure 2, this repression is very similar for doxycycline, minocycline, anhydrotetracycline and tetracycline. Oxytetracycline, also, follows the same kinetics of TRS repression as seen in Figure 2 (data not shown). All the agents are capable of repressing the tRP to the same degree within 4 h of treatment when the mGM-CSF level is reduced to about 9% of its control. Furthermore, the degrees of repression remain comparable for all the later times. The system reaches its basal expression within 18–24 h when the mGM-CSF level is about 0.22% of its control. This rapid repression to basal expression may reflect the short half-life of mGM-CSF mRNA (1.5 h)[18] rather than degradation of mGM-CSF protein which is extremely stable in culture medium (data not shown). Hence, despite having different lipophilicity and cellular/tissue penetration,[19,20] for practical applications of the system (where the above con-

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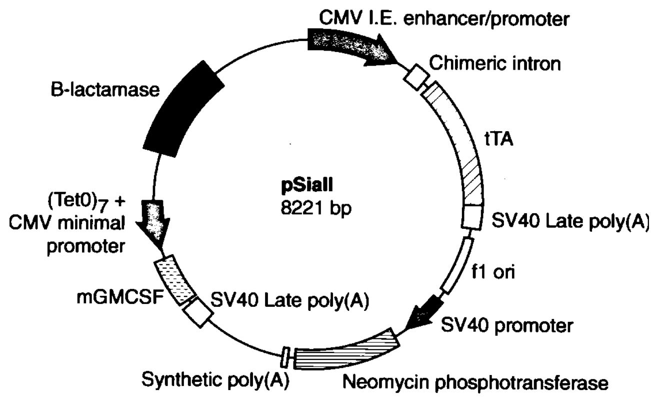

Figure 1 *Schematic map of pSiaII plasmid used in establishing stable 293 cells.*

centrations of these analogues are administered and maintained), any of these agents is capable of rapidly suppressing tRP to its fully repressed level.

Comparing the degree of repression of tRP in response to tetracycline derivatives indicates that the level of regulation depends on both the concentration and intrinsic properties of a given analogue (Figure 3a–d, 0.0 h). For tetracycline, maximum repression occurs at 100–1000 ng/ml, with partial activity of the tRP occurring at 100 pg/ml to 100 ng/ml. For anhydrotetracycline, the tRP is fully repressed at both 100 and 1000 ng/ml with partial activity of the promoter occurring at 10 pg/ml to 100 ng/ml. Minocycline also fully represses the promoter at 100 and 1000 ng/ml with partial activity of the promoter seen at 100 pg/ml to 100 ng/ml. As is evident at lower concentrations, minocycline is less efficient in inactivating tTA than anhydrotetracycline and more efficient than tetracycline. Doxycycline causes maximum repression of the tRP at 10, 100 and 1000 ng/ml and partial activity of the promoter occurs at 10 pg/ml to 10 ng/ml. Finally, oxytetracycline causes maximum repression of tRP at 1000 ng/ml and partial activity at concentrations below this value (data not shown). Our set-up appears to be slightly less sensitive than the observations previously made using anhydrotetracycline[21] and doxycycline.[22] This probably reflects differences in the cell lines, the reporter genes, the nature of collection and analysis of the reporter genes and/or the order of regulation of the two approaches.

The behaviour of the system in response to the withdrawal of tetracycline and its derivatives is more distinctive. When pretreated with 1000 ng/ml of tetracycline or its analogues, there is a characteristic delay in resumption of tRP activity depending on the analogue used. For tetracycline, promoter activity is resumed during the first 24 h of tetracycline removal and it reaches near-optimal activity at 48 h (Figure 3a), consistent with previous observations.[5] Pretreatment with oxytetracycline results

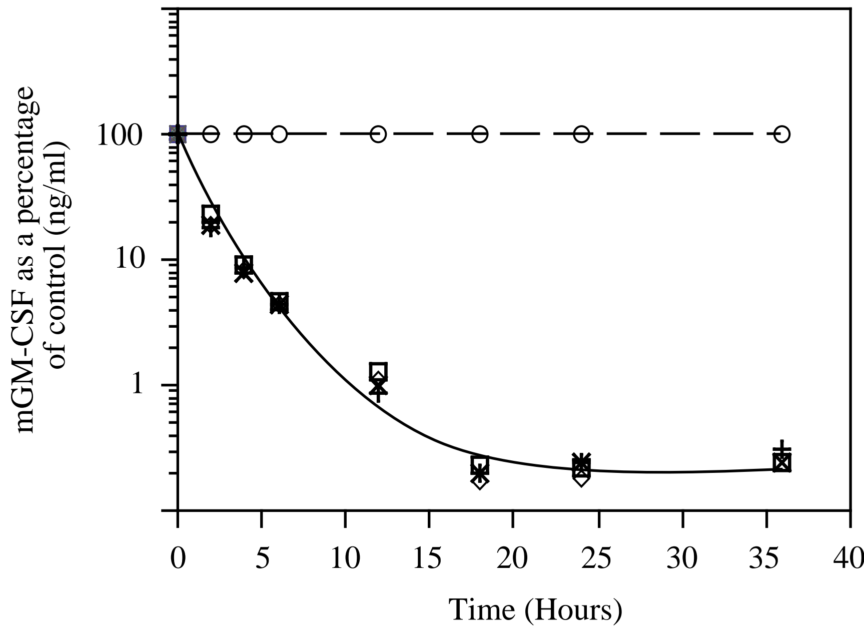

Figure 2 *Kinetics of mGM-CSF repression in the presence of 1000 ng/ml of tetracycline derivatives. 293 Cells were transfected with 10 μg of pSiaII using the calcium phosphate transfection procedure. 293-pSiaII cells were selected and maintained in the presence of 750 μg/ml of G418 and 5 μg/ml of tetracycline in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) for more than 3 months. Tetracycline hydrochloride (Sigma, Poole, UK) and minocycline (Sigma) were prepared fresh as a 0.5 mg/ml stock in DMEM for each experiment. Doxycycline hydrochloride (Sigma) was prepared as a 0.02 N solution of 0.5 mg/ml in millipore water and anhydrotetracycline (Janssen Chimica, Hyde, UK) was prepared as above in 20% dimethyl sulphoxide (DMSO; Fisons, Loughborough, UK)/DMEM. Mouse granulocyte–macrophage colony-stimulating factor (mGM-CSF) assays were done using ELISA kits (Endogen, Leicestershire, UK) according to the manufacturer's protocol. All experiments were repeated more than once. Pooled 293–pSiaII cells in tetracycline-free culture medium were seeded at 5×10^4 cells per well of six-well plates (Corning, New York, NY, USA) 1 day before manipulation. The cells were maintained in 2 ml of culture medium only (control cells) or culture medium containing tetracycline, doxycycline, minocycline and anhydrotetracycline at concentrations of 1000 ng/ml. A sample of the supernatant was taken at 2, 4, 6, 12, 18, 24 and 36 h for mGM-CSF ELISA analysis when the medium was replaced for fresh culture medium only or medium containing the tetracycline analogues at 1000 ng/ml. For each point, the mean mGM-CSF for five samples and its standard error are expressed as a percentage of the mean of control. (○) Medium only, (□) + tetracycline, (◇) + doxycycline, (×) + minocycline, (+) + anhydrotetracycline.*

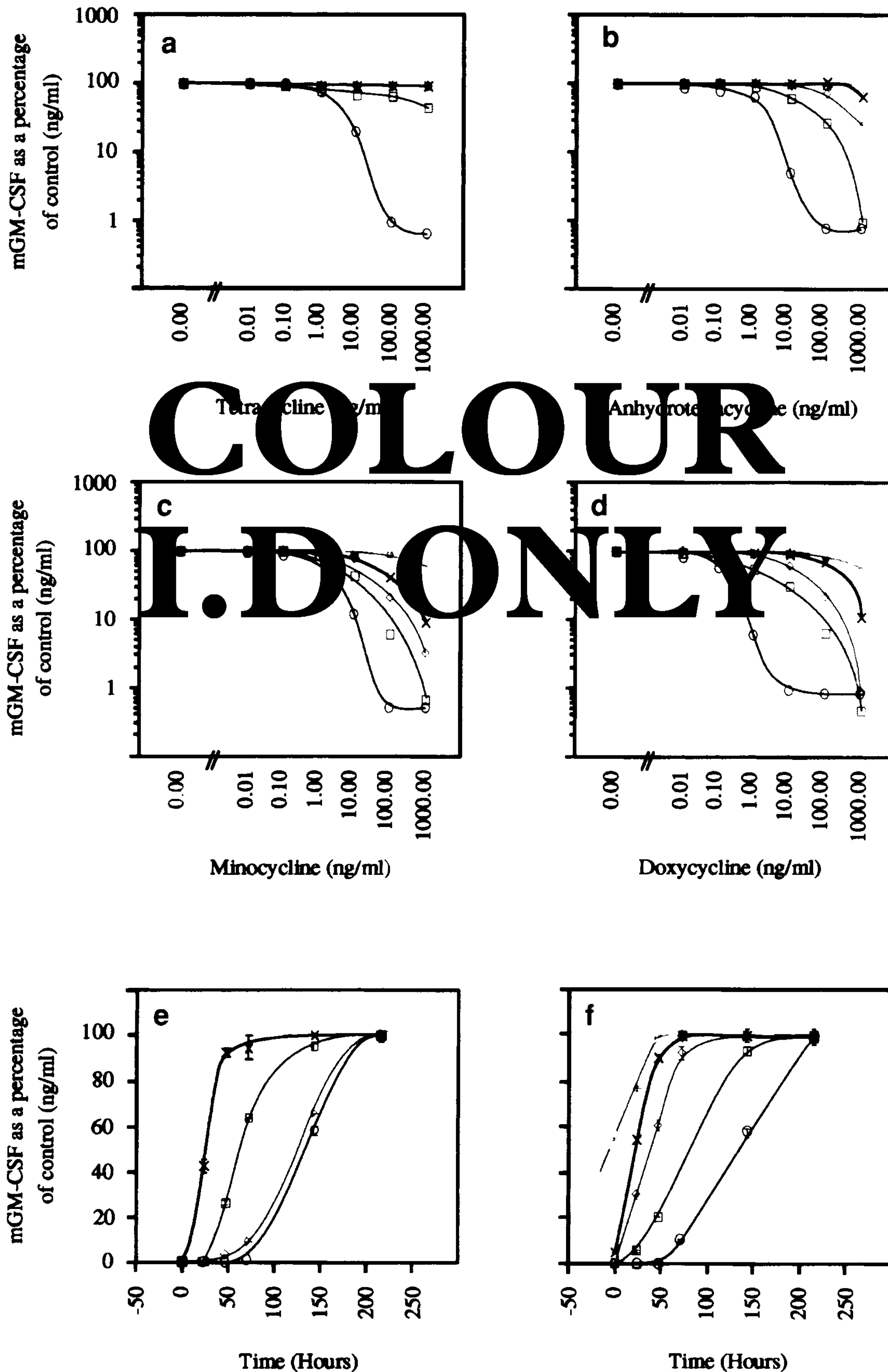

Figure 3 (a–d). Degree of regulation and the kinetics of resumption of tRP activity in response to tetracycline analogues. Pooled 293-pSiaII cells in tetracycline-free culture medium were seeded at 5×10^4 cells per well of six-well plates 1 day before manipulation. The cells were maintained in 2 ml of culture medium only (control cells) or culture medium containing tetracycline, doxycycline, minocycline and anhydrotetracycline at concentrations of 0.01, 0.1, 1.0, 10, 100 and 1000 ng/ml. The medium was replaced every 24 h for fresh culture medium only or medium containing the tetracycline analogues at the appropriate concentrations. After 3 days of treatment with tetracycline analogues, a sample of the supernatant was taken to compare the degree of mGM-CSF regulation in response to different analogues. This represents the time-point 0.0 h and the mGM-CSF produced within –24 to 0.0 h (mGM-CSF protein is extremely stable in culture medium, with no change in its concentration for over 72 h at 37°C (data not shown)). To follow the time taken for resumption of tRP activity, the medium was removed at this time-point, the cells were washed twice with 10 ml of PBS, and tetracycline-free culture medium was added to the cells. The medium was replaced every 24 h for fresh culture medium only. The mGM-CSF level was analysed at the indicated times. For each point, both mean of five samples and the standard error of the mean is expressed as a percentage of the mean of mGM-CSF produced by control cells. (○) 0.0 h, (□) 24 h, (◇) 48 h, (×) 72 h, (+) 144 h, (△) 216 h. (e) Summarises the above results for 1000 ng/ml of the tetracycline derivatives only. (×) Tetracycline, (□) anhydrotetracycline, (◇) minocycline, (○) doxycycline. (f) Summarises the dose-dependence delay in resumption of tRP activity for doxycycline and shows that resumption occurs linearly over time. (△) 0.01, (+) 0.1, (×) 1, (◇) 10, (□) 100, (○) 1000 ng/ml of doxycycline.

in a shorter delay in resumption of tRP activity, with optimal expression being reached at about 36 h (data not shown). In contrast, the TRS takes much longer to regain activity after removal of the other three analogues. The TRS is completely inactive at 48 h after removal of both anhydrotetracycline and minocycline, and at 72 h after the removal of doxycycline. When pretreated with anhydrotetracycline, it takes the tRP 144 h to resume near-optimal activity (Figure 3b). Both minocycline and doxycycline behave similarly in this respect, with TRS taking about 192 h (estimated from the data) (Figure 3c) and 216 h to recover near-optimal activity (Figure 3d) when pretreated with these agents, respectively. The above results for 1000 ng/ml of the tetracycline, anhydrotetracycline, minocycline and doxycycline are summarised in Figure 3e. For all the tetracyclines used, promoter activity is regained earlier when pretreated with lower concentrations. Thus, there is a temporal delay in resumption of promoter activity based on the type and the dose of the analogue used for repression. Furthermore, at any given concentration, the activity of tRP is resumed linearly over time (Figure 3f).

To ensure that the above observation is not due to any residual analogue remaining in the wells, cells pretreated for 72 h with 100 ng/ml of doxycycline were detached, washed extensively and re-plated into new culture plates. These cells produced exactly the same results as seen in Figure 3 (data not shown). Our results are further substantiated by noting a similar observation when using doxycycline to manipulate the surface expression of a chimeric molecule in Jurkat cells[23] and to regulate a modified TRS in AM12 cells.[24]

Both repression and induction of the TRS depend on the concentration of the tetracycline analogue within a cell and the efficiency of the analogue in reducing tetR–tet operator interaction. The efficiency of an analogue, given as the concentration of the analogue resulting in 50% reduction in tetR–tet operator interaction, is 2.2×10^{-6} M for tetracycline, 0.09×10^{-6} M for anhydrotetracycline, and 0.14×10^{-6} M for doxycycline. For most tetracycline analogues, this efficiency has been shown to be correlated with the association equilibrium constant (K_A) of the analogue for tetR.[25] K_A of tetracycline, doxycycline, anhydrotetracycline and oxytetracycline is approximately 3×10^9 M^{-1},[26] 15×10^9 M^{-1} [26]/23×10^9 M^{-1},[25] 100×10^9 M^{-1},[26] and 3×10^9 M^{-1},[26] respectively. K_A for minocycline is lower than that of 4-epi-7-chloro-tetracycline which is about 0.4×10^9 M^{-1}.[26] Our observed order of analogue potency (doxycycline > anhydrotetracycline > minocycline > tetracycline > oxytetracycline) does not directly correlate with the order of efficiency or the association equilibrium constants for tetR. Despite having a higher efficiency and K_A than doxycycline, anhydrotetracycline is less effective than this agent in repressing tRP. Also, minocycline has a significantly lower K_A than tetracycline but is more efficient in repressing the system. In addition, the pattern of resumption of tRP activity (earliest for oxytetracycline, followed by tetracycline, anhydrotetracycline, minocycline and doxycycline) does not correlate with the association equilibrium constants or our observed order of potency. In this respect, it is interesting to realise that anhydrotetracycline results in a shorter delay in resumption of tRP activity than minocycline or doxycycline, despite having much higher K_A for tetR compared with these derivatives. Moreover, tRP activity is resumed similarly after the removal of both minocycline and doxycycline, even though minocycline has a far lower K_A for tetR than doxycycline. These observations suggest the importance of other factors, especially concentration of the analogue within the cell. The *in vitro* and *in vivo* intracellular concentration of a tetracycline may be influenced by the duration of treatment, concentration of the analogue administered, lipophilicity, protein binding characteristics of the analogue and transport mechanisms across the cell membrane. Other processes that will affect this are pharmacokinetics and pharmacodynamics of the analogue in animals. Minocycline and doxycycline are 10-fold and five-fold[19] more lipophilic than tetracycline and can achieve higher concentrations in many tissues than in serum *in vivo*.[27] Furthermore, doxycycline has been shown to reach higher intracellular concentrations in red blood cells and in neutrophils than that of the surrounding culture medium.[28] Also, anhydrotetracycline[20] is more lipophilic and thus has a higher level of cellular/tissue penetration than tetracycline. Thus, the lipophilic property of the agents may explain why lower concentrations of these analogues can achieve the same degree of repression as higher concentrations of tetracycline. This property may also explain why tetracycline is more efficient in repressing tRP than the less lipophilic oxytetracycline.[29] Both the order of potency and the pattern of resumption of tRP activity resemble the pattern of binding of these agents to serum proteins (20–35% for oxytetracycline,[29] 25–65% for tetracycline, 75–80% for minocycline[27] and 80–90% for doxycycline[29]). Considering this, the affinity of these analogues for intracellular proteins may be at least partly responsible for the differing concentrations of these agents within the cell and thus for the pattern of resumption of tRP activity.

Overall our findings indicate that the different properties of tetracycline analogues can be employed in planning a desired course of transgene regulation. These are important for both the use of the TRS system in *in vitro* and *in vivo* applications. For example, in many *in vitro* or *in vivo* applications, a more rapid resumption of tRP activity may be achieved by using oxytetracycline rather than tetracycline. Conversely, for certain *in vitro* approaches, such as establishment of cell lines expressing toxic transgenes, it is beneficial to maintain a totally switched-off status of the promoter. In these cases, use of doxycycline is clearly more convenient. Both doxycycline and minocycline are used extensively in clinical settings. These tetracyclines, with their efficient oral absorption (93–100%),[29,30] long half-life (14–22 h for doxycycline and 11–13 h for minocycline),[30,31] excellent tissue penetration and hepatic excretion,[27,30] are ideal agents for *in vivo* control of TRS. The ability of doxycycline to switch off the promoter over the range of 10–1000 ng/ml could be of importance *in vivo* where the activity of the tRP can be maintained fully repressed so long as the tissue concentration of doxycycline remains above 10 ng/ml. Administration of 200 mg of doxycycline or minocycline orally to humans produces peak serum concentrations of 3–5 μg/ml after 2 h,[18] with a plateau-shaped plasma concentration curve[27] that falls below 1 μg/ml after approximately 24 h for minocycline[30] and 50 h for doxycycline.[32] This, together with the higher tissue to plasma concentration ratio of doxycycline and minocycline, ensures that the expression of a given gene can be kept switched off

despite continual changes in their concentrations due to distribution, metabolism and excretion. In conclusion, by understanding the effect of those tetracycline derivatives that have a greater efficiency in inactivating tTA and improved pharmacokinetics, we aim to highlight their application *in vivo*.

Our observations may help to explain the findings of a study comparing the ability of various tetracycline analogues in activating the reverse tTA, where doxycycline proved to be the most potent effector.[33] The longer presence of doxycycline within the cells and its higher potency may explain the superiority of this agent in activating reverse tTA.

In summary, we show that after an initial exposure of cells to 1000 ng/ml, oxytetracycline results in repression of tTA-controlled gene expression for less than 2 days, tetracycline up to 2 days, anhydrotetracycline up to 6 days, and minocycline and doxycycline up to 8 and 9 days, respectively. Thus, by selecting an appropriate analogue at a suitably administered dose, it is possible to plan the temporal resumption of the tRP activity following removal of the agent.

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