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Design, synthesis and identification of novel colchicine-derived immunosuppressant

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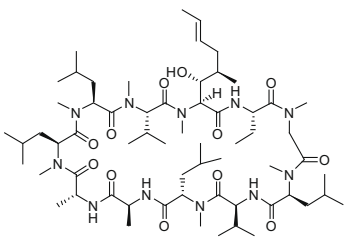
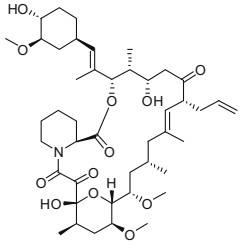
ABSTRACT

Synthesis and biological evaluation of various colchicine analogues through the mixed-lymphocyte reaction (MLR), lymphoproliferation, and inhibitory effects on the inflammatory genes are described. In addition, a new series of immunosuppressive agents developed on the structural basis of colchicine, as well as their structure–activity relationships is reported. The most potent analogue **20a** exhibited an excellent immunosuppressive activity on in vivo skin-allograft model, which is comparable to that of cyclosporin A.

Despite intensive efforts to overcome rejection following organ transplantation or autoimmune disease, the strong demand for effective and safe immunosuppressants still remains.[1] Cyclosporin A (CsA),[2] which is one of the best immunosuppressants for organ transplantation, is known to inhibit calcineurin, which subsequently induce block of T cell proliferation.[3] CsA also provides suppressive effects on nitric oxide (NO) production and expression of inflammatory genes such as *i*NOS, IL-1β and TNF-α associated with graft rejection.[4] Most of the currently available drugs for transplantation including corticosteroids, mycophenolate mofetil, CsA, FK506 and rapamycin are known to be accompanied with serious side effects[1,5] such as nephrotoxicity, neurotoxicity, hyperlipidaemia and hypertension.[6,7] The drugs for treatment of autoimmune disease also revealed side effects such as bone loss and cataracts.[6] In addition, many of the effective immunosuppressants including CsA and FK506 are not orally available mainly due to their high molecular weight and liphophilicity. Thus, development of potent and safe immunosuppressants with low molecular weight has been desired.



Cyclosporin A



FK506

Colchicine (**1**), isolated from *Colchicum autumnale*,[8] inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules, which results in effective mitotic poisoning.[9] This cytotoxic natural product is also known to have immunomodulatory effect, which increases IL-1 production of macrophages[10] while it suppresses TNF-α and IL-2 expression on mononuclear cells.[11] Moreover, potential immunosuppressive activity of colchicine on allograft transplantation has also been reported.[12] In this connection, we have recently worked on development of colchicine-based immunosuppressive agent, which is of low toxicity and low molecular weight.

To achieve our goal, we initially screened colchicine-derived analogues on the basis of allogenic mixed-lymphocyte reaction

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(MLR)[13] using splenocytes harvested from C57BL/6 and BALB/c mice. The analogues, which exhibited potent activities in MLR were also evaluated by lymphoproliferation assay.[14] Inhibition of both NO production and expression of inflammatory genes related to graft rejection[4] was also examined for the analogs, which showed good activity in lymphoproliferation assay. Finally, immunosuppressive activities of the selected analogues were validated on skin-allograft model.[15]

Acute allograft rejection is usually concerned in production of Th1 related cytokines such as IL-1β, IL-2 and TNF-α.[15b] In contrast, allograft tolerance is provided by an increase in expression of Th2 cytokines such as IL-3, IL-4 and IL-10 and a decrease in expression of Th1 cytokines.[15b,16] For elevation of immunosuppressive effect by regulating Th2 cytokines as well as down-regulating Th1 cytokines related to allograft tolerance, we designed the analogues possessing nitrate group, which could generate exogenous NO[17] (Scheme 1). As summarized in Table 1, immunosuppressive effects of the synthesized analogs were compared to those of the analogs without nitrate group.

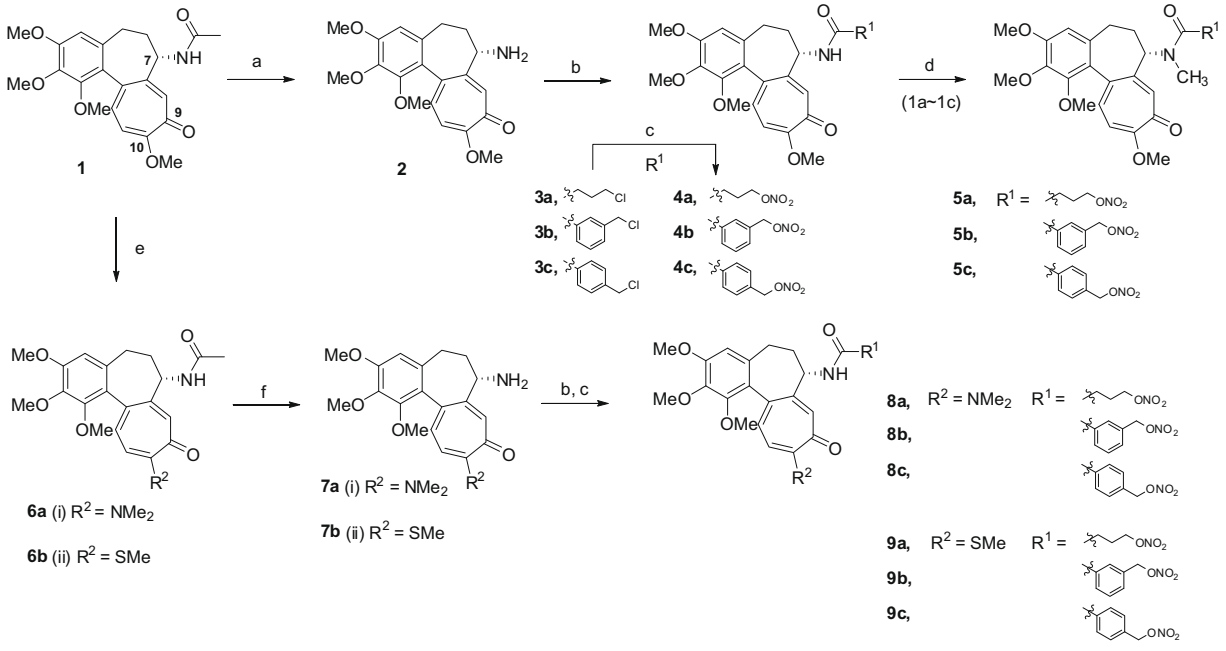
We next synthesized the *C*-10 and *C*-7 modified analogues in an existence of terminal nitrate substituent because our preliminary work revealed that ring-modification did not provided improved activities. First, 7-deacetylcolchicine (**2**) and 7-deacetylthiocolchicine (**7b**) were prepared according to the reported method.[18] 7-Deacetyl-10-*N*,*N*-dimethylamino colchicine (**7a**) was prepared by substitution of 10-methoxy group with dimethylamine followed by acid-catalyzed hydrolysis. Analogs **3a**–**3c** were prepared by amidation of **2** with appropriate acyl chlorides. Subsequent displacement of chloride with nitrate afforded the analogs **4a**–**4c**. To confirm the substitution effect of the amides, analogues **4a**–**4c** were methylated to provide **5a**–**5c**. The 10-*N*,*N*-dimethylamino analogues (**8a**–**8c**) and the 10-thiomethoxy analogues (**9a**–**9c**) were prepared from **7a** and **7b**, respectively by the same procedure for **4a**–**4c**.

Immunosuppressive activities for the synthesized analogs were evaluated by MLR assay. Shown in Table 1, analogs **3a** with 4-chlorobutanoyl group and **3b** with *m*-chloromethyl benzoyl group exhibited slightly lower activity than colchicine, whereas **3c** with *p*-chloromethylbenzoyl group showed sevenfold potent activity than colchicine. However, it showed 32-fold lower potency than CsA. Analogs **4a**–**4c** possessing the exogenous NO donor exhibited 470-fold (**4a**), 25-fold (**4b**) and fivefold (**4c**) higher activities respectively, compared to the parent analogs **3a**–**3c**. Increased activities of the analogues with the terminal nitrate substituent implies an important role of nitrate possibly as exogenous NO source. Analogs **5a**–**5c** void of amide hydrogen, which could function as hydrogen bonding donor, exhibited significant decrease (**5a**) or loss of activity (**5b, 5c**). This partly supports the essential role of free amide group as a hydrogen-bonding donor. Regarding replacement of methoxy group with dimethylamino or thiomethoxy group, thiomethoxy group seems similar to methoxy group

Table 1
Inhibition of allogenic MLR by compounds **1**–**9c**

Compound[a]	R^1	R^2	N[b]	IC_{50}[c] (μM)
CsA	—	—	—	0.01
Colchicine	Methyl	OMe	NH	2.30
3a	3-Chloropropyl	OMe	NH	4.70
3b	*m*-Benzyl chloride	OMe	NH	6.30
3c	*p*-Benzyl chloride	OMe	NH	0.32
4a	3-Propyl nitrate	OMe	NH	0.01
4b	*m*-Benzyl nitrate	OMe	NH	0.25
4c	*p*-Benzyl nitrate	OMe	NH	0.07
5a	3-Propyl nitrate	OMe	NCH_3	3.50
5b	*m*-Benzyl nitrate	OMe	NCH_3	>100
5c	*p*-Benzyl nitrate	OMe	NCH_3	>100
8a	3-Propyl nitrate	NMe_2	NH	6.60
8b	*m*-Benzyl nitrate	NMe_2	NH	10.0
8c	*p*-Benzyl nitrate	NMe_2	NH	2.50
9a	3-Propyl nitrate	SMe	NH	0.21
9b	*m*-Benzyl nitrate	SMe	NH	0.07
9c	*p*-Benzyl nitrate	SMe	NH	0.02

[a] All compounds were purified by column chromatography and then recrystallization (>95%).
[b] Nitrogen in amide side chain at C-7.
[c] IC_{50} values are mean of three experiments, standard deviation below ±20%.



Scheme 1. Reaction conditions and reagents: (a) (i) di-*t*-butyl carbonate, DMAP, Et_3N, THF; (ii) NaOMe, MeOH; (iii) TFA, DCM; (b) proper acyl chlorides, Et_3N, THF, −78 to −10 °C; (c) (i) KI, acetone, reflux; (ii) $AgNO_3$, acetonitrile, 40–50 °C; (d) NaH, iodomethane, THF, 35–40 °C; (e) $R^2 = NMe_2$ (**6a**): dimethylamine in THF (1.0 M), MeOH, reflux; R^2 = SMe (**6b**): NaSMe, THF/water (1:1); (f) 2 N-HCl, MeOH, reflux.

Table 2
Inhibition of lymphoproliferation by compounds **9a**–**93**

Compound	LPS (B cell, nM) IC_{50}[a]	ConA (T cell, nM) IC_{50}[a]
CsA	>100,000	550
Colchicine	7.3	548
9a	1.0	582
9b	1.0	1.0
9c	349	392

[a] IC_{50} values are mean of three experiments, standard deviation below ±20%.

in terms of steric and electronic effects. However, the dimethylamino-substituted analogs exhibited poor activity, which seems to reflect the size effect of the substituent. Interestingly, the thiomethoxy-substituted **9a** showed a 20-fold lower activity compared to the methoxy-substituted analog **4a**, whereas thiomethoxy-substituted **9b** and **9c** exhibited 3–4-fold potent activities than the methoxy-substituted **4b** and **4c**.

We selected **9a**–**9c**, which showed potent activities in MLR. However, the methoxy-substituted **4a** was not selected because demethylation of 10-methoxy group in rats has recently been reported.[19] The result of lymphoproliferation assay for **9a**–**9c** on B lymphocyte-activated cells using lipopolysaccharide (LPS) as B cell activator and on T lymphocyte-activated cells using concanvalin A (ConA) as T cell activator[14] is shown in Table 2. CsA exhibited T cell selective immunosuppressive activity, whereas colchicine exhibited B cell selective activity. The inhibitory activity of analog **9a** on T cells was similar to that of CsA. However, its antiproliferative activity on B cells was superior to those of colchicine and CsA. The analog **9c**, which was the most potent analogue among three thiomethoxy analogs in MLR, exhibited an equipotent inhibitory activity compared to CsA on T cells while it showed significantly lower activity than colchicine on B cells. Surprisingly, lymphoproliferation assay revealed that **9b** was much more potent than CsA and colchicine on both B and T cells.

The analogs **9a** and **9b** were also tested for cytotoxicity, inhibition of NO production and expression of TNF-α and *i*NOS on Raw 264.7 macrophages in the same concentration range (Fig. 1). Both **9a** and **9b** did not show cytotoxicity at 0.01–10 μM as shown in Figure 1a. Analog **9b** showed the similar inhibitory activities in NO production which is known as one of the factors associated with graft rejection[20] and *i*NOS expression compared to CsA in a dose-dependent manner whereas **9a** lost inhibitory activity. In respect of TNF-α expression as inflammation mediator related to graft rejection and immune disease, **9b** equipotent to CsA seems more attractive than **9a**. Figure 1 supports that **9b** would be more effective than **9a** in immunosuppressive activity.

We next turned our attention to further optimization of amide side chain of **9b**. Thus, we introduced a variety of substituents including –Ph, –OMe, –F, –Cl, –Br, –I, –CN and $–NO_2$ at 2, 4, 6-position of phenyl ring (Scheme 2) to investigate electronic and steric effects of the phenyl ring attached to amide side chain. All analogues of **9b** were prepared by standard amide coupling of **7b** and the corresponding benzoic acids. Benzoic acids **16** having methoxy or nitro substituent were synthesized according to method I, which involved esterification, bromination and subsequent hydrolysis. Synthesis of benzoic acids possessing halogen and nitrile substituent were prepared by method II, which included hydrogenation of nitro group and subsequent Sandmeyer reaction.[21] The sterically hindered phenyl group was introduced by treatment of methyl iodobenzoate with phenyl boronic acid in the presence of tetrakistriphenylphosphine palladium(0) (method III).

As shown in Table 3, 24 analogues of **9b** were prepared and evaluated by MLR. Among the 4-substituted analogues, the 4-methoxy analogue **18b** with an electron donating substituent exhibited enhanced and most potent activity (15 nM). However, other analogues including **19b**–**25b** with electron withdrawing groups retained similar activity regardless of electronic or steric ef-

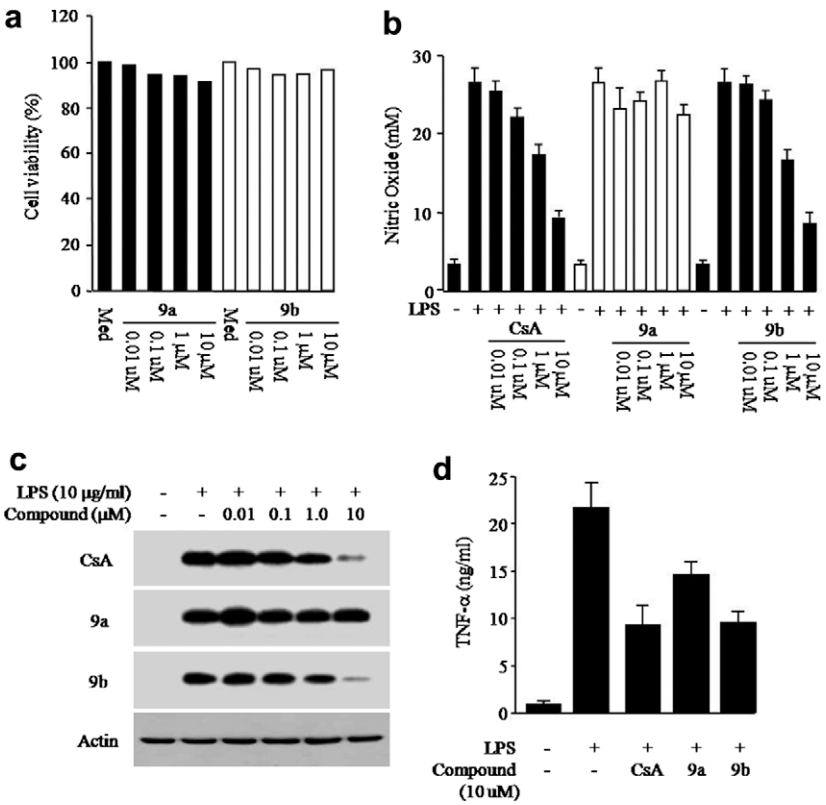
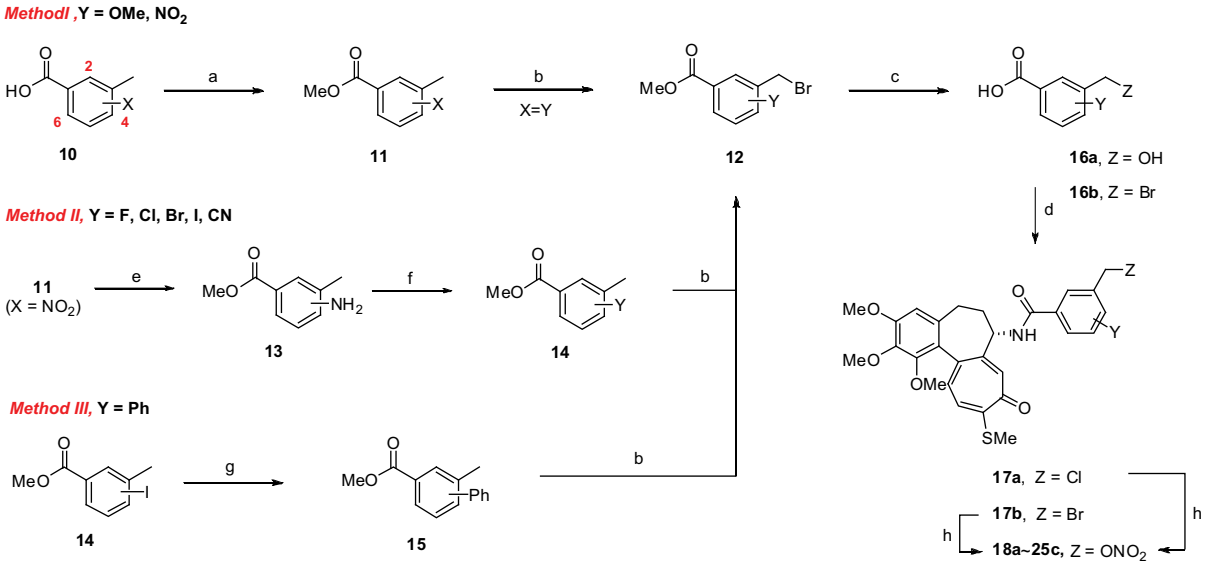


Figure 1. (a) Cytotoxic effects, (b) inhibitory effects of NO production, (c) *i*NOS expression levels, (d) TNF-α expression levels in the presence of **9a** and **9b** in Raw 264.7 macrophages.



Scheme 2. Synthesis of **9b** analogues (**18a**–**25c**). Reaction conditions and reagents: (a) methanol, HCl, reflux; (b) *N*-bromosuccinimide, benzoyl peroxide or *hv* (300 W tungsten lamp), CCl_4, reflux; (c) LiOH or KOH, MeOH/water; (d) Z = OH: (i) $SOCl_2$, benzene, reflux; (ii) **7b**, Et_3N, THF, 0 °C; Z = Br: **7b**, EDCI, Et_3N, DCM; (e) Pd/C, H_2, ethyl acetate; (f) Y = F: $NaNO_2$, HCl, HBF_4, water, 0–5 °C; Y = Cl: $NaNO_2$, HCl, CuCl, water, 0–5 °C; Y = Br: $NaNO_2$, HCl, CuBr, water, 0–5 °C; Y = I: $NaNO_2$, H_2SO_4, KI, water, 0–5 °C; Y = CN: $NaNO_2$, HCl, CuCN, water; 0–5 °C; (g) $Pd(PPh_3)_4$, $C_6H_5B(OH)_2$, Na_2CO_3, DME/water, reflux; (h) Z = Cl; (i) KI, acetone, reflux; (ii) $AgNO_3$, acetonitrile, 40–50 °C; Z = Br: $AgNO_3$, acetonitrile, 40–50 °C.

fects of the substituents. On the basis of inhibitory activities of the methoxy-substituted analog **18a** and the analogs **19a**–**25a** possessing electron withdrawing group at 2-position, the electron withdrawing substituent at 2-position seems essential to retain or increase the activity. The 2-chloro (**20a)** and 2-nitro (**24a**) analogues exhibited noticeably increased activities as compared to the analogues with the weak electron withdrawing groups (**21a**–**23a**), which implies activity enhancement by electron withdrawing substituents at 2-position. In case of the 6-substituted analogues, the electron withdrawing substituents (**19c** and **20c**) seem to enhance suppressive activity. However, increase of steric effect at 6-position (**21c**, **22c** and **25c**) decreased activity.

The analogs **19c**, **20a**, **20c** and **24a**, which showed potent activities in MLR were further evaluated by lymphoproliferation assay on T-lymphocyte-activated cells using concanvalin A (ConA).[22] As shown in Table 4, the 2-substituted analogues (**20a** and **24a**) exhibited more potent activity than the 6-substituted analogues (**19c** and **20c**). In particular, the analog **20a** exhibited the most potent antiproliferation activity (IC_{50} = 3 nM).

Finally, we performed in vivo skin-allograft using C57BL/6 and BALB/c mice in order to confirm the immunosuppressive efficacy of the most potent analogue **20a**.[15b] The analog **20a** was administrated to the skin recipients (C57BL/6) after skins were grafted from BALB/c to C57BL/6 mice, and then the survival of allograft skin was compared to that of the control allograft. Shown in Figure 2, colchicine did not show the promotion of skin-allograft survival, whereas **20a** extended allograft survival to 11.8 ± 2.1 days (P <0.05), which was comparable to efficacy of CsA (12.3 ± 0.3, P <0.01).

We also inspected several key cellular events that can be used to assess toxicity of the analogs. This included loss of cell proliferation, membrane integrity, mitogenesis, and altered mitochondrial function. As shown in Table 5, the analog **20a** exhibited lower in vitro toxicity than CsA. The TC_{50}[23] values of **20a** for cell proliferation (cell number), the release of α-glutathione *S*-transferase (α-GST)[24] used to monitor membrane integrity or cell death, and altered mitochondrial function (MTT assay[25], intracellular ATP level)

Table 3
Inhibition of MLR by compounds **18a**–**25c**[a]

Compounds	Y[b]	Method[c]	IC_{50}[d] (nM)
9b	H	—	68
18a	2-OMe	I	700
18b	4-OMe	I	15
18c	6-OMe	I	78
19a	2-F	II	65
19b	4-F	II	31
19c	6-F	II	4
20a	2-Cl	II	8
20b	4-Cl	II	50
20c	6-Cl	II	4
21a	2-Br	II	61
21b	4-Br	II	89
21c	6-Br	II	89
22a	2-I	II	85
22b	4-I	II	68
22c	6-I	II	103
23a	2-CN	II	58
23b	4-CN	II	49
23c	6-CN	II	290
24a	2-NO_2	I	9
24b	4-NO_2	I	55
24c	6-NO_2	I	73
25a	2-Ph	III	434
25b	4-Ph	III	54
25c	6-Ph	III	NA

[a] All compounds were purified by column chromatography and then recrystallization (>95%).
[b] Substituent shown in Scheme 2.
[c] Synthetic method in Scheme 2.
[d] IC_{50} values are mean of three experiments, standard deviation below ±20%.

Table 4
Inhibition of lymphoproliferation by potent **9b** analogues

Compounds	Y[a]	ConA[b] (IC_{50}, nM)
19c	6-F	259
20a	2-Cl	3
20c	6-Cl	392
24a	2-NO_2	7

[a] Substituent shown in Scheme 2.
[b] IC_{50} values are mean of three experiments, standard deviation below ±20%.

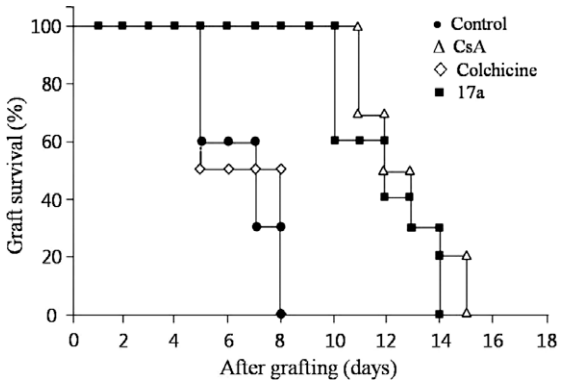


Figure 2. Effect of **20a** on skin-allograft survival: Full thickness skins of BALB/c mice were grafted to C57BL/6 mice and **20a**, colchicine and CsA were ip administrated with daily dose of 1 mg/kg, respectively.

Table 5
Non-GLP in vitro toxicity in a rat hepatoma (H4IIE) cell line

Compound	TC_{50}[a] (μM)			
	Cell number[b]	α-GST[c]	MTT[d]	ATP
CsA	48	75	42	42
20a	300	300	300	300

[a] TC_{50}: concentration that produced a half-maximal response.
[b] Cell number: cell proliferation assay.
[c] GST: α-glutathione *S*-transferase (membrane permeability).
[d] MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

were over 300 μM, whereas CsA showed much lower TC_{50} values than **20a**, supporting lower in vivo toxicity of **20a** than CsA.

In summary, we have synthesized 39 colchicine-derived analogues, which were evaluated by MLR, lymphoproliferation assay, inhibitory effects on inflammatory genes and in vivo skin-allograft model. The analog **9b** possessing nitrate substituent as an exogenous NO donor turned out to be equipotent to CsA. In addition, optimization of **9b** afforded more potent analogue **20a**, which was comparable to CsA on skin-allograft model and less toxic than CsA in in vitro toxicity. Considering low molecular weight of **20a**, its equipotent immunosuppressive activity implies that **20a** would be usable as orally available immunosuppressive agent. The structure–activity relationship of colchicine for immunosuppressive activity was also established. Currently, the immunosuppressant efficacy evaluation on **20a** for in vivo heart transplantation animal model is in progress and the successful results will be reported in due course.

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22. Because the parent compound (**9b**) of these analogues showed non-selective activity for B and T cells, we just performed lymphoproliferation assay on T-lymphocyte-activated cells as a general thing.
23. The TC_{50} values are the exposure concentrations (empirically estimated from a log dose versus response curve) that produce a half (50%) maximal response relative to controls. If a 50% response could not be determined, the TC_{50} value was expressed as greater than the highest exposure concentration. Each dose response curve is composed of a vehicle (DMSO) group and seven treatment groups spanning an exposure range of 0.1–300 μM. Each value in the curve represents the mean of 4–8 wells in a plate. The α-GST and MTT assays were run from one plate as were the GSH/8-isoprostane assays, while the cell proliferation and ATP assays were run from separate plates.
24. (a) Vickers, A. E. M. *Cell. Biol. Toxicol.* **1994**, *10*, 407; (b) Redi, H.; Schlag, G.; Paul, E. *Shock* **1995**, *3*, 395.
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