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# Dissecting the Pharmacophore of Curcumin. Which Structural Element Is Critical for Which Action?

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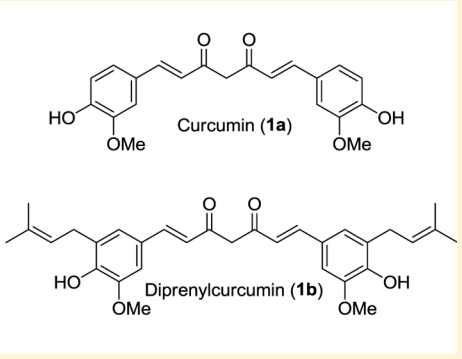
Supporting Information




**ABSTRACT:** The dietary phenolic curcumin (**1a**) is the archetypal network pharmacological agent, but is characterized by an ill-defined pharmacophore. Nevertheless, structure−activity studies of **1a** have mainly focused on a single biological end-point and on a single structural element, the aliphatic bis-enoyl moiety. The comparative investigation of more than one end-point of curcumin and the modification of its aromatic region have been largely overlooked. To address these issues, we have investigated the effect of aromatic *C*-prenylation in the three archetypal structural types of curcuminoids, namely, curcumin itself (**1a**), its truncated analogue **2a** ($C_5$-curcumin), and (as the reduced isoamyl version) the tetrahydro derivative **3a**, comparatively evaluating reactivity with thiols and activity in biochemical (inhibition of NF-κB, HIV-1-Tat transactivation, Nrf2 activation) and phenotypic (anti-HIV action) assays sensitive, to a various extent, to thia-Michael addition. Prenylation, a validated maneuver for bioactivity modulation in plant phenolics, had no effect on Michael reactivity, but was detrimental for all biological end-points investigated, dissecting thiol trapping from activity, while hydrogenation attenuated, but did not completely abrogate, the activity of **1a**. The $C_5$-curcuminoid **2a** outperformed the natural product in all end-points investigated and was identified as a novel high-potency anti-HIV lead in a cellular model of HIV infection. Taken together, these observations show that Michael reactivity is a critical element of the curcumin pharmacophore, but also reveal a surprising sensitivity of bioactivity to *C*-prenylation of the vanillyl moiety.

With almost 3000 preclinical investigations and potential beneficial effects that even include genetic diseases, the diarylheptanoid curcumin (**1a**) is one of the most thoroughly investigated and most promising dietary natural products.[1] Due to a dismally low oral bioavailability[2] and to the lack of financial incentives in the development of a nonpatentable natural product, the clinical documentation on curcumin is inconsistent with its pharmacological potential, and most of its beneficial effects in humans lack validation, being only suggested by epidemiological correlations, supported by studies on animal models, or simply extrapolated from in vitro findings.[3] At a molecular level, curcumin is believed to act as a master switch of inflammation by inhibiting pro-inflammatory transcription factors (NF-κB, STAT-3) and the function and expression of inflammatory enzymes (COXs and LOs).[1,3] This broad-spectrum anti-inflammatory profile is of interest for the prevention/treatment of pathological conditions sustained or triggered by chronic inflammation (cancer, diabetes, Alzheimer's disease),[1,3] but curcumin has also been reported to interact with over one hundred further biological targets,[4] including some of great relevance for HIV research, such as the proteins integrase and protease, as well as Tat-mediated transactivation of the HIV-LTR.[5]

A systematic study of the biological profile of curcumin (**1a**) is impossible under the reductionist mantra of one target−one disease−one drug, but several of its actions might be related, sharing a common mechanistic basis. Thus, the NF-κB pathway

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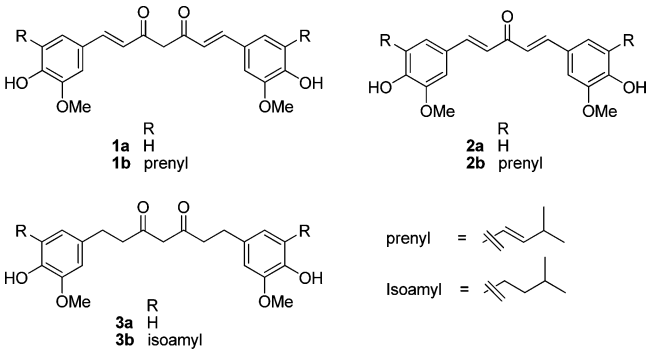
might be involved not only in the anti-inflammatory action of curcumin but also in its antiviral action, since this compound reverses Tat-induced dissociation of HDAC1 from LTR by preventing the binding of p65/NFκB to the LTR promoter.[5] From a medicinal chemistry standpoint, the pharmacophore of curcumin is a unique blend of Michael acceptor, metal-chelating, and antioxidant properties, and its dissection has so far been elusive.[6] Most structure−activity studies on curcumin have aimed at the modification of its diacryloylmethane moiety, which can be simplified to a dienone to afford the so-called $C_5$-curcuminoids, replaced by a 1,3-distyryl-substituted heterocyclic core, or alkylenated (alkylated) at its central methylene carbon with substantial retention, and sometimes even improvement, of activity against a single specific biochemical or phenotypic end-point.[6] Conversely, less attention has been devoted to the two aromatic moieties of the natural product, and most studies in the area refer to simplified $C_5$-curcuminoids or to analogues of the natural product that bear additional oxygenated functions.[6] Surprisingly, the introduction of carbon substituents on the aromatic moieties has received scarce attention. Nature seems to have followed suit, since none of the over three hundred diarylheptanoids reported so far from various Zingiberaceous plants is *C*-prenylated,[7] despite the relevance of this group for the biological profile of phenolics (flavonoids, stilbenoids), where it can induce novel bioactivity, modulate a pre-existing one, and markedly improve bioavailability.[8] Apparently, Nature seems to have overlooked an important possibility to generate biologically interesting chemical diversity. Intrigued by these considerations, we have investigated the prenylation of curcumin (**1a**) and its $C_5$-analogue (**2a**), evaluating the effects of this maneuver on the reactivity with thiols, an important feature of the curcuminoid pharmacophore,[3,9] and on the activity toward three thiol-trapping-sensitive biochemical targets of curcuminoids (NF-κB, Tat-induced activation of the HIV-LTR promoter, Nrf2),[3] as well as on the phenotypic translation of the NF-κB and Tat inhibition into HIV inhibition. Tetrahydrocurcumin (**3a**) and its dihydroprenyl (=isoamyl) analogue (**3b**) were also prepared and evaluated as nonelectrophilic versions of curcumin (**1a**) and its diprenyl derivative (**1b**), respectively.




## RESULTS AND DISCUSSION

Curcumin (**1a**) is the classic network pharmacological agent, since its cellular mechanism of action is not dependent on the targeting of a single molecular target, but is, apparently, the result of a biochemical synergism that involves the weak targeting of different proteins within related signaling networks.[1,4] This biochemical pleiotropism is paralleled by the vagueness of the curcumin pharmacophore.[6] Aromatic *C*-prenylation could, in principle, improve absorption[8] while leaving the biological profile largely unaffected. Owing to the inherent propensity of phenols to undergo *O*- rather than *C*-prenylation, and the high reactivity of the enolized 1,3-dicarbonyl system of curcumin with electrophiles,[10] the direct prenylation of the natural product was not possible. 5′,5″-Diprenylcurcumin (**1b**) was therefore prepared by condensing the boric complex of 2,4-pentandione (acetylacetone) with 5-prenylvanillin (**6**). By stabilizing its enol tautomer, formation of a boric complex prevents alkylation of the central methylene of acetylacetone, quenching the methylene-centered Knoevenagel reactivity at the expense of the methyl-centered aldol-type reactivity (Pabon reaction).[12] 5-Prenylvanillin (**6**) could, in principle, be obtained from vanillin by α,α-dimethyl propargylation, semireduction, and Claisen rearrangement (Scheme 1).[11] Unexpectedly, this seemingly straightforward strategy could not be implemented without a redox detour, since the semireduction of the triple bond of **5** could not be affected at the aldehyde stage, but only with the corresponding benzylic alcohol, which next had to be reoxidized (Scheme 1). Apparently, the palladium catalyst was inactive in the presence of the aldehyde carbonyl, a somewhat surprising observation. For the synthesis of the corresponding $C_5$-curcuminoids (**2b**), acetylacetone was replaced by acetone, a change that, for unclear reasons, required protection as a Mem (methoxyethoxymethyl) ether of the phenolic hydroxyl group of 5-prenylvanillin (**6**) to effect the bidirectional crotonic condensation. The protecting group could then be removed with the THF complex of $SnCl_4$[13] without affecting the acid-sensitive prenyl group. The saturated analogues **3a** and **3b** were prepared from the corresponding curcuminoids (**1a** and **2a**) by catalytic hydrogenation, an uneventful reaction.

Curcumin (**1a**), $C_5$-curcumin (**2a**), and their prenyl derivatives (**1b** and **2b**, respectively) all gave a positive assay for thiol trapping, reacting instantaneously and irreversibly with cysteamine in DMSO to afford the corresponding 1,7-bis Michael adduct.[14] The positivity of the assay was evidenced by the disappearance of the AB olefin system in the spectra, and the irreversible nature of the addition by the failure of the AB olefin system to reappear upon dilution with $CDCl_3$.[14] Competitive experiments were carried out by reacting equimolecular binary mixtures of these curcuminoids with substoichiometric amounts of cysteamine. Under these conditions, no difference in reactivity was observed between curcumin (**1a**) and its prenyl derivative (**1b**) or between the monocarbonyl analogue of the natural product **2a** and its prenyl derivative **2b**. Conversely, **2a** showed a higher reactivity than curcumin in the comparative cysteamine assay. Thus, when an equimolecular mixture of **1a** and **2a** was treated with half of the stoichiometric amount of cysteamine required for the formation of the bis-adducts, a ca. 4:1 mixture of the bis-Michael adduct of **2a** and that of curcumin was obtained (Figure 1). As expected, no reaction occurred with the tetrahydro derivative **3a** or its isoamyl derivative **3b** and cysteamine.

Having assessed the reactivity of the curcuminoids **1a**−**3b** with cysteamine, their NF-κB inhibitory activity was investigated in a stably transfected cell lines 5.1, a lymphoid T cell line in which the HIV-1 LTR is activated by TNF-α through an NF-κB-dependent mechanism.[15] The HIV-1 LTR promoter contains two κB sites that are critically required to respond to TNFα, and it is well known that deletion of the two κB sites in the LTR promoter abolishes completely the response to TNFα. Thus, 5.1 cells represent an excellent cellular model for the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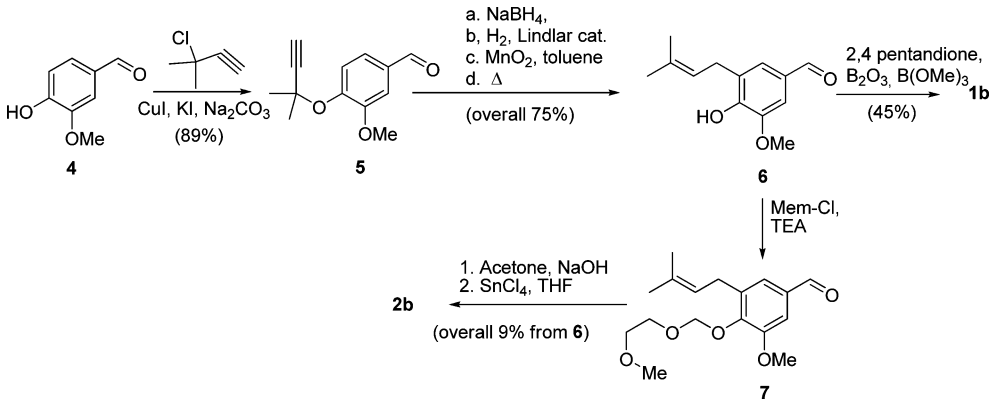
**Scheme 1. Synthesis of 5,5′-Diprenylcurcumin (1b) and 5,5′-Diprenyl-$C_5$-curcumin (2b)**



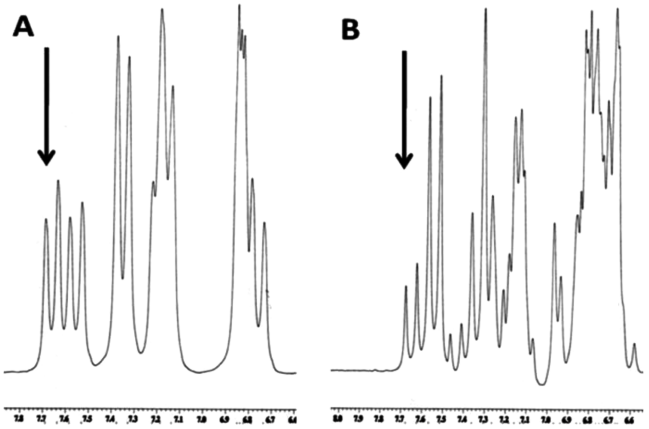




**Figure 1.** Comparative thiol trapping assay of an equimolecular mixture of curcumin (**1a**) and $C_5$-curcumin (**2a**) treated with two molar equivalents of cysteamine in DMSO-$d_6$. The arrows indicate the signals of the two equivalent enone $\beta$-protons (H-1 and H-7) of $C_5$ curcumin (**2a**, lower field doublet, $\delta$ 7.66) and curcumin (**1a**, higher field doublet, $\delta$ 7.55). After the addition of two equivalents of cysteamine, the ratio between the two signals became 4:1, showing the preferential reaction of $C_5$-curcumin (**2a**) compared to curcumin (**1a**).

screening of anti-NF-κB compounds. Curcumin (**1a**) inhibited NF-κB with an $IC_{50}$ of 16.4 μM, a value consistent with those reported previously for this compound (Figure 2).[1] This activity was completely lost for its prenylated derivative (**1b**) and was markedly attenuated in compounds **2b**, **3a**, and **3b**. Conversely, and in accordance with previous findings,[6] activity was higher for the $C_5$ analogue **2a**, which showed an $IC_{50}$ value of 4.92 μM. The effect of compound **2a**, the most potent compound toward cysteamine and the most powerful inhibitor of NF-κB transcriptional activity, was investigated on IκBα degradation in Jurkat cells. Stimulation with PMA (phorbol miristate acetate) induced a rapid phosphorylation and degradation of the cytoplasmic IκBα protein, coupled with phosphorylation of the MAPKs JNK 1+2 and NF-κB subunit p65.[16] All these activities were inhibited by **2a** in a concentration-dependent manner (Figure 3). PMA-induced ERK 1+2 activation by phosphorylation was not affected by preincubation with **2a**, indicating the selectivity of action on NF-κB and JNK inhibition (Figure 3). These observations validated the mechanism of NF-κB inhibition by curcuminoids in a functional assay.

To evaluate the functionality of these observations, inhibition of Tat-induced HIV LTR transactivation by **1a**–**3b** was investigated in HeLa Tat-Luc cells in which the HIV-1 LTR is directly activated by the HIV-1 Tat protein. In accordance with the results observed in the NF-κB assays, prenylation abolished the anti-Tat activity of curcumin (**1a**) and was detrimental in all cases, while the most potent compound was the C5-analogue **2a** ($IC_{50}$ = 4.7 μM) (Figure 4). These results provided a rationale for investigating the HIV-1 inhibitory activity of curcuminoids. The antiviral activity was evaluated in Jurkat cells infected with the pNL4-3 HIV-1 clone pseudotyped with the VSV envelope, bypassing the natural mode of HIV-1 entry into these cells and supporting a robust HIV-1 replication. Upon integration into host chromosomes, this recombinant virus expresses the firefly luciferase gene, and consequently luciferase activity in infected cells correlates with the rate of viral replication. High luciferase activity levels were detected 12 h after cellular infection with the VSV-pseudotyped HIV-1 clone, and pretreatment of the cells 30 min prior to infection with increasing doses of **1a** and **2a** revealed that the latter outperforms curcumin by 4–5-fold (Figure 5). In our experiments, the time-points used to study the activities of the compounds were 6 h (Figures 2 and 6) and 12 h (Figures 4 and 5), respectively, and only marginal cytotoxicity was found with compound **2a** at these time-points.

Finally, the set of curcuminoids was evaluated for another bioactivity sensitive to thiol trapping, namely, the activation of antioxidant response element (ARE)-mediated transcriptional induction.[17] This activity was investigated in a luciferase reporter assay in SK-N-SH cells. In this cellular model, curcumin (**1a**) was a weak activator of the Nrf2 pathway, and this activity was clearly enhanced in its $C_5$ analogue (**2a**), in accordance with all the previous structure–reactivity and structure–activity observations. Interestingly, it was found that low doses of **2a** activated ARE, while higher concentrations were less potent and even reduced the basal level of ARE-Luc activity in SK-N-SH cells. Hormesic models of activity have been desscribed for curcumin also in other cellular models,[18] and the activity of **2a** at 1 μM concentration may be physiologically relevant. Saturated curcumin (**3a**) did not activate the ARE pathway, and prenylated curcumin (**1b**) and the $C_5$ analogue (**2b**) were also inactive in this assay. In nonstimulated cells, NF-κB is normally sequestered in the cytoplasm by a family of inhibitory proteins, which bind NF-κB, inhibit its DNA-binding, and prevent its nuclear accumulation, namely, IκB proteins.[19] Specific extracellular stimuli such as cytokines and phorbol esters lead to the rapid phosphorylation, ubiquitination, and ultimately proteolytic degradation of IκBα, which frees NF-κB to translocate to the nucleus, where it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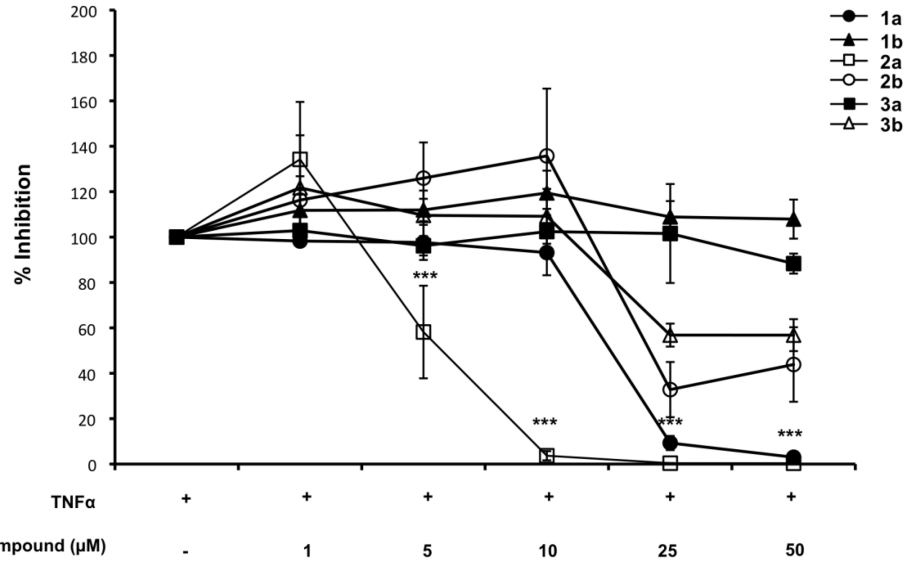


**Figure 2.** Effects of curcumin (**1a**) and derivatives on TNF$\alpha$-induced HIV-1-LTR activation. 5.1 cells were preincubated with the compounds at the indicated doses and stimulated with TNF$\alpha$ (2 ng/mL) for 6 h. The luciferase activity was measured, and results are presented as the percentage of inhibition compared to TNF$\alpha$ alone. Five independent experiments were performed, and data are presented as mean ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$ in Student's $t$ test.

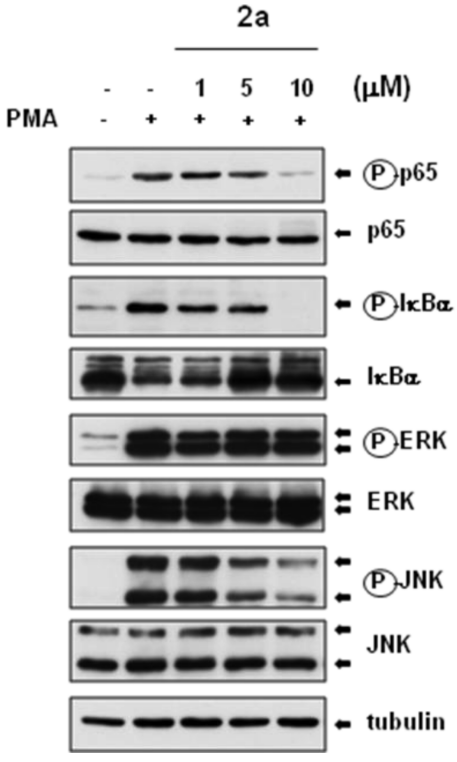


**Figure 3.** $C_5$-Curcumin (**2a**) inhibits NF-$\kappa$B and JNK signaling pathways. Jurkat cells were incubated with **2a** at the indicated concentrations and treated with PMA for 30 min, and total protein was extracted. The detection of I$\kappa$B$\alpha$ phosphorylation and degradation, total and phosphorylated p65, total and phosphorylated ERK, and total and phosphorylated JNK was performed by Western blots. The results are representative of three independent experiments.

regulates gene transcription. I$\kappa$B$\alpha$ degradation is triggered by its phosphorylation from the IKK complex (IKC) at Ser-32 and Ser-36 and is considered the major step in NF-$\kappa$B regulation.[20] In addition to this pathway, a second level of NF-$\kappa$B activation involves the phosphorylation of the p65 and the subsequent stimulation of NF-$\kappa$B transactivation.[19] Several reports have shown that curcumin inhibits NF-$\kappa$B by targeting the IKC complex and also the MAPK JNK pathway.[21,22] Taken together, these observations suggest that the NF-$\kappa$B inhibitory mechanism of curcumin (**1a**) and its C-5 analogue (**2a**) is remarkably similar, despite the truncation of the pharmacophore. Owing to the lower potency,[21] details on the action of the tetrahydrocurcuminoids could not be elucidated in this system.

The downstream biochemical implications of the NF-$\kappa$B inhibition data were investigated by evaluating the HIV-1 Tat- (transactivator or transcription) inhibitory activity of compounds **1a**,**b**−**3a**,**b**. Tat is a protein essential for virus replication that interacts with the transactivation response element (TAR) RNA stem-loop and recruits the positive transcriptional elongation factor (p-TEFb), which, in turn, phosphorylates the C-terminal domain of the RNA polymerase II and activates transcription elongation from the HIV long terminal repeat promoter (LTR).[23] Curcumin (**1a**) inhibits HIV-1 Tat-mediated transactivation of the of the HIV-LTR seemingly by a mechanism dependent on the HDAC1-NF-$\kappa$B pathway, since this substance reverses Tat-induced dissociation of HDAC1 from LTR, preventing the binding of p65/NF-$\kappa$B to LTR promoters stimulated in Tat-stimulated cells.[24] It remains to be investigated if compound **2a** inhibits Tat-induced HIV-1 LTR activation by the same mechanims described for curcumin (**1a**).

The inhibition of HIV-gene regulation by targeting both Tat and NF-$\kappa$B signaling pathways cripples crucial stages in the cycle of viral replication and has therefore great clinical potential to prevent drug resistance, the scourge of AIDS treatment. Curcumin has also been reported to target the viral proteins integrase and protease with $IC_{50}$ values of 40 and 100 $\mu$M, respectively.[25] To investigate the phenotypical translation of the biochemical assays, the anti-HIV activities of curcumin (**1a**) and **2a** were evaluated comparatively in a model of HIV-1 infection. Jurkat cells infected with the pNL4-3 HIV-1 clone pseudotyped with the VSV envelope were employed. As shown


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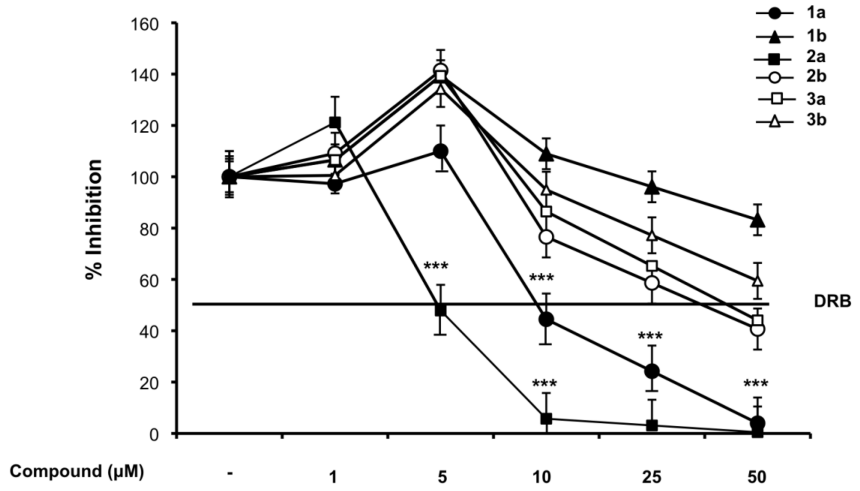


**Figure 4.** Effects of curcumin (**1a**) and its derivatives **1b**, **2a**,**b**, and **3a**,**b** on Tat-induced HIV-1-LTR activation. HeLa Tat-Luc cells were incubated with either DRB (50 $\mu$M) or the test compounds at the indicated doses, and after 12 h luciferase activity was measured. Results are presented as the percentage of activation relative to untreated cells. Four independent experiments were performed, and data are presented as mean ± SD; *$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.005 in Student's $t$ test.

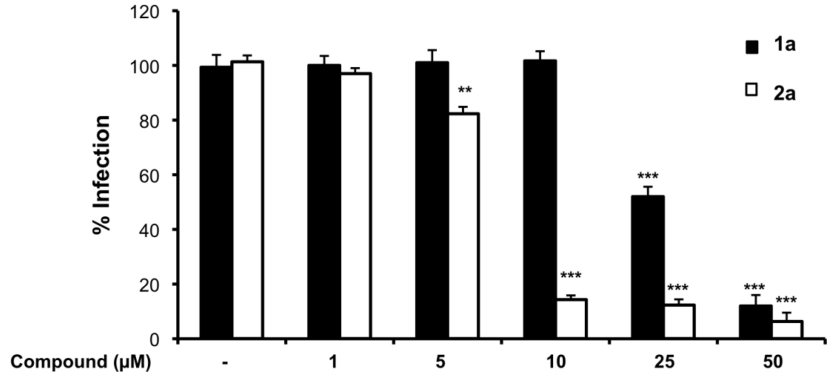


**Figure 5.** Effects of curcumin (**1a**) and $C_5$-curcumin (**2a**) on HIV-1 replication. Jurkat cells ($10^6$/mL) were pretreated with the compounds for 30 min and then infected with VSV-pseudotyped-pNL4-3.Luc.R$^-$E$^-$ (200 ng p24) for 12 h. Luciferase activity in cell extracts was determined, and results are represented as percentage of activation ± SD compared to nontreated infected cells (100% activation). Three independent experiments were performed, and data are presented as mean ± SD; *$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.005 in Student's $t$ test.

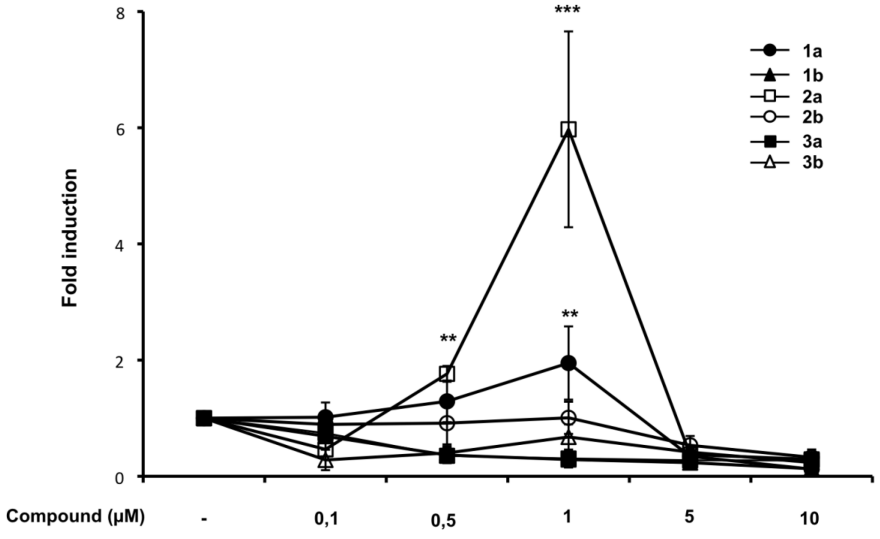


**Figure 6.** Effects of curcumin (**1a**) and derivatives on ARE-Luc activation. SK-N-SH cells were transfected with the ARE-Luc plasmid and then stimulated with increasing concentrations of the compounds for 6 h. The luciferase activity was measured, and results are presented as the fold induction over untreated cells. Three independent experiments were performed, and data are presented as mean ± SD; *$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.005 in Student's $t$ test.


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in Figure 4 and in accordance with the results of the biochemical assays, the truncated analogue **2a** outperformed curcumin (**1a**) by 4−5-fold, while prenylcurcumin was devoid of any significant action.

Taken together, our observations show that a close relationship exists between reactivity and bioactivity in curcumin, C5-curcumin, and tetrahydrocurcumin, the archetypal curcuminoids from the $C_7$-, $C_5$-, and tetrahydro series. $C_5$-Curcumin (**2a**), the most electrophilic compound and most potent ligand for the transcription factors, was also validated in a phenotypic assay for anti-IV activity. Remarkably, insertion of an *ortho*-prenyl group on the phenyl moiety was detrimental for activity but not relevant for thiol trapping, suggesting that a specific curcuminoid recognition site, unable to accommodate relatively bulky aromatic alkyl substituents such as a prenyl group, should exist on these targets.[26] The discovery that a single point mutation unrelated to the pharmacophoric signature of curcumin can abolish its biological action against specific targets is a remarkable observation for a group of substances otherwise dominated by an overall modest sensitivity to chemical modification and somewhat flat structure−activity relationships.[6,26] Therefore, prenylcurcumin (**1b**) may well serve as a negative control agent for studies aimed at unraveling the biological profile of curcumin (**1a**), dissecting actions mediated by thiol trapping from those differently modulated. The observation that prenylation was detrimental in compounds from all three series also suggests that the pharmacophore of curcuminoids is complex and not exclusively reactivity-based.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** IR spectra were taken on a FT-IR Thermo Nicolet equipment. $^1$H (300 MHz) and $^{13}$C (75 MHz) NMR spectra were measured on a JEOL Eclipse 300 instrument. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: $\delta_H$ = 7.26, $\delta_C$ = 77.0). High-resolution ESIMS were obtained on a LTQ OrbitrapXL (Thermo Scientific) mass spectrometer. Silica gel 60 (70−230 mesh) and RP-18 used for gravity column chromatography (GCC) were purchased from Macherey-Nagel. Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates, which were visualized by UV inspection and/or staining with 5% $H_2SO_4$ in ethanol and heating. Organic phases were dried with $Na_2SO_4$ before evaporation. Synthetic curcumin (**1a**), prepared using the Pabon reaction,[12] was used in all biological assays, while tetrahydrocurcumin (**3a**) was obstained by catalytic hydrogenation of synthetic curcumin on Pd/C.[27] Chemical reagents and solvents were from Aldrich. All final compounds were at least 95% pure as evaluated by HPLC.[28]

*3-Methoxy-4-(2-methylbut-3-in-2-yloxy)benzaldehyde (**5**).* To a stirred solution of vanillin (1 g, 6.56 mmol) in dry DMF (12 mL) were added sequentially $K_2CO_3$ (1.78 g, 13.13 mmol, 2 molar equiv), KI (1.85 g, 11.15 mmol, 1.7 molar equiv), CuI (25 mg, 0.13 mmol, 0.02 molar equiv), and 3-chloro-3-methyl-1-butine (1.7 mL, 15.08 mmol, 2.3 molar equiv). The reaction was stirred at 65 °C, with its progress monitored by TLC (petroleum ether−EtOAc, 8:2, as eluant, $R_f$ = 0.33). After 1.5 h, the reaction was quenched by the addition of water and extraction with ether. The combined organic layers were dried, filtered, and evaporated, and the residue purified over silica gel (petroleum ether−EtOAc, 9:1, as eluant) to afford **5**[29] (1.29 g, 89%) as a pale yellow solid.

*5-Prenylvanillin (**6**).* To a stirred solution of **5** (1.29 g, 5.88 mmol) in EtOH (11 mL) and $CH_2Cl_2$ (1.1 mL) was added $NaBH_4$ (199 mg, 5.29 mmol, 0.9 mol.ar equiv). The reaction was stirred at room temperature for 1 h and then quenched by the addition of 2 N $H_2SO_4$ and extraction with $CH_2Cl_2$. The combined organic layers were dried, filtered, and evaporated. The residue was dissolved in MeOH (10 mL) and stirred overnight at room temperature in the presence of Lindlar catalyst (ca. 80 mg) under a hydrogen atmosphere. The suspension was filtered on a Celite pad and washed with $CH_2Cl_2$. The combined organic layers were evaporated, and the crude product was dissolved in toluene (10 mL) and next stirred at room temperature with activated $MnO_2$ (5 g) for 3 h. The suspension was worked up by filtration on Celite, and the filtrate was evaporated, dissolved in *N,N*-diethylaniline (20 mL), and heated with stirring at 165 °C (oil bath temperature) for 1.5 h. After cooling to RT, the reaction was quenched by the addition of 2 N $H_2SO_4$ and extraction with EtOAc. After drying and evaporation, the residue was purified over silica gel (petroleum ether−EtOAc, 8:2, as eluant) to afford **6** as a pale yellow solid (967 mg; 75% over the four steps): mp 66 °C; IR (KBr) $\nu_{max}$ 3141, 3001, 2912, 2852, 2728, 1666, 1592, 1464, 1310, 1149, 945, 851, 698 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 9.79 (1H, s), 7.28 (2H, bs), 6.26 (1H, s), 5.31 (1H, t, $J$ = 7.35 Hz), 3.93 (3H, s), 3.38 (1H, d, $J$ = 7.35 Hz), 1.75 (3H, s), 1.72 (3H, s); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$ 191.4, 149.5, 147.0, 133.7, 129.0, 127.8, 127.66, 121.3, 107.0, 56.3, 27.8, 25.8, 17.9; HREIMS $m/z$ 220.1084 (calcd for $C_{13}H_{16}O_3$, 220.1099)

*5′,5″-Diprenylcurcumin (**1b**).* To a stirred solution of 5-prenylvanillin (**5**, 1 g, 4.53 mmol) in dry DMF (1 mL) were added sequentially $B_2O_3$ (317 mg, 2.13 mmol, 0.47 molar equiv), 2,4-pentandione (232 $\mu$L, 2.26 mmol, 0.5 molar equiv), and $B(OCH_3)_3$ (504 $\mu$L, 0.73 mmol, 3.3 molar equiv). The reaction was stirred at 90 °C, and *n*-butylamine (100 $\mu$L) was added over a period of 2.5 h. After 1 h, the reaction was cooled to 60 °C, and 5% AcOH (10 mL) was added. The mixture was stirred at 60 °C for 3 h, during which a precipitate was formed. After filtration and washing with water, the crude product was purified over silica gel (petroleum ether−EtOAc, 8:2, as eluant) to afford 5′,5″-diprenylcurcumin (**1b**) as an orange solid (510 mg; 45%): mp 145 °C; IR (KBr) $\nu_{max}$ 3515, 2998, 2968, 2913, 2854, 1624, 1598, 1492, 1301, 1265, 1132, 1078, 1067, 967, 840, 550 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 7.55 (2H, d, $J$ = 16 Hz), 6.98 (2H, d, $J$ = 1.8 Hz), 6.92 (2H, d, $J$ = 1.8 Hz), 6.44 (2H, d, $J$ = 16 Hz), 5.92 (1H, s), 5.79 (1H, s), 5.30 (2H, t, $J$ = 7.0 Hz), 3.90 (6H, s), 3.34 (4H, d, $J$ = 7.0 Hz), 1.75 (6H, s), 1.70 (6H, s); $^{13}$C NMR ($CDCl_3$,75 MHz) $\delta$ 183.4, 146.6, 145.7, 141.0, 133.4, 127.9, 126.9, 123.6, 121.7, 121.6, 107.4, 101.0, 56.2, 28.0, 25.9, 17.9; HREIMS $m/z$ 504−2523 (calcd for $C_{31}H_{36}O_6$, 504.2512).

*5′,5″-Diprenyl $C_5$-Curcumin (**2b**).* To a stirred solution of 5-prenylvanillin (200 mg, 0.9 mmol) in dry $CH_2Cl_2$ (2.5 mL) were added sequentially TEA (252 $\mu$L, 1.8 mmol, 2 molar equiv) and MEM-Cl (132 $\mu$L, 1.17 mmol, 1.3 molar equiv). The reaction was stirred at room temperature overnight and was next quenched by the addition of 2 N $H_2SO_4$ and extraction with $CH_2Cl_2$. The combined organic layers were dried, filtered, and evaporated. The crude product dissolved in absolute EtOH (2.5 mL) was stirred for 3 h at room temperature in the presence of acetone (916 $\mu$L; 0.45 mmol, 0.5 equiv) and 20% NaOH (264 $\mu$L). The reaction was finally quenched by the addition of 2 N $H_2SO_4$ and extraction with EtOAc, and the combined organic phases were dried, filtered, and evaporated. The crude residue was purified over silica gel (petroleum ether−EtOAc, 7:3, as eluant) to afford 100 mg (17% over two steps) of Mem-protected **2b**, which was next dissolved in THF (2.5 mL). After cooling (0 °C), $SnCl_4$ (500 $\mu$L) was added, and the reaction was stirred at room temperature overnight. The reaction was quenched by the addition of saturated $NaHCO_3$ and extraction with EtOAc. The combined organic layers were dried, filtered, and evaporated, and the crude product was purified over silica gel (petroleum ether−EtOAc, 7:3, as eluant) to afford **3b** as a brownish powder (60 mg, overall 9% from **5**): mp 120 °C; IR (KBr) $\nu_{max}$ 3513, 3461, 3394, 2965, 2929, 2855, 1732, 1591, 1492, 1428, 1273, 1154, 1078, 984, 938, 847 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 7.64 (2H, d, $J$ = 15.9 Hz), 7.03 (2H, bs), 7.00 (2H, bs), 6.95 (2H, d, $J$ = 15.9 Hz), 5.32 (2H, t, $J$ = 1.2 Hz), 3.93 (6H, s), 3.36 (4H, d, $J$ = 7.3 Hz), 1.76 (6H, s), 1.73 (6H, s); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$ 189.0, 146.4, 146.0, 143.6, 133.5, 127.9, 126.7, 124.1, 123.2, 121.7, 107.4, 56.2, 28.0, 25.9, 17.9; HREIMS $m/z$ 462.2415 (calcd for $C_{291}H_{34}O_5$, 462.2406).

*5′,5″-Diisoamyltetrahydrocurcumin (**3b**).* To a stirred solution of prenylcurcumin (**1b**, 450 mg, 0.89 mmol) in EtOAc (4 mL) and MeOH (4 mL) was added Pd/C (10%, cat.). The reaction was stirred


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overnight at room temperature under an atmosphere of hydrogen and then worked up by filtration on Celite. The organic phases were evaporated, and the residue was purified over silica gel (petroleum ether−EtOAc, 9:1, as eluant) to afford **3b** as a yellowish solid (125 mg; 28%): mp 92 °C; IR (KBr) $\nu_{max}$ 3536, 3476, 2947, 2924, 2863, 1702, 1601, 1499, 1464, 1437, 1295, 1230, 1153, 1107, 1068, 915, 837, 795, 567 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) $\delta$ 6.54 (2H, bs), 6.52 (2H, bs), 5.50 (1H, s), 3.83 (6H, s), 2.89 (4H, m), 2.56 (4H, m), 1.53 (10H, m), 0.92 (12H, d, $J$ = 6.7 Hz); $^{13}C$ NMR ($CDCl_3$, 75 MHz) $\delta$ 193.4, 146.2, 141.8, 131.6, 128.8, 121.8, 108.3, 56.0, 40.7, 39.1, 31.6, 28.0, 27.6, 22.7; HREIMS $m/z$ 512.6766 (calcd for $C_{31}H_{44}O_6$, 512.6775).

**Cell Lines and Reagents.** Jurkat cells were grown at 37 °C and 5% $CO_2$ in supplemented RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (50 U/mL), and streptomycin (50 $\mu$g/mL). SK-N-SH, Hela-Tat-Luc, and 293T cells were grown in supplemented DMEM medium. The 5.1 clone line is a Jurkat-derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-LTR promoter and was maintained in complete RPMI medium supplemented with G418 (200 $\mu$g/mL).[15] The Hela-Tat-Luc is a stably transfected cell line with the plasmids pLTR-Luc and pcDNA3-Tat and was described previously.[15] The antibodies anti-I$\kappa$B$\alpha$ (C-21, sc-371), anti-NF-$\kappa$B-p65 (F-6, sc-8008), and antiphospho-ERK1+2 (sc-7383) were acquired from Santa Cruz Biotechnology (San Diego, CA, USA). The antibodies against antiphospho-p65 (Ser 536), antiphospho-I$\kappa$B$\alpha$ (Ser32/36), anti-JNK, and antiphospho-JNK 1+2 (9255S) were from Cell Signaling (Danvers, MA, USA). The mAb anti-tubulin and anti-ERK 1+2 (M5670) were purchased from Sigma Co. (St. Louis, MO, USA).

**Luciferase Assays.** For the anti-NF-$\kappa$B activity 5.1 cells were stimulated with TNF$\alpha$ (20 ng/mL) in the presence or the absence of the compounds for 6 h. For the activation of the antioxidant response element, SK-N-SH cells were transiently transfected with the pGL3-ARE-Luc plasmid and 24 h later stimulated with the compounds for 6 h. The cells were washed twice in PBS and lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol during 15 min at RT in a horizontal shaker. After centrifugation, the supernatant was used to measure luciferase activity using an Autolumat LB 9510 (Berthold) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA).

**VSV-Pseudotyped HIV-1 Infection Assa.** High-titer VSV-pseudotyped recombinant virus stocks were produced in 293T cells by co-transfection of pNL4-3.Luc.$R^-E^-$ together with either the $pcDNA_3$-VSV or the epNL3 plasmid encoding the vesicular stomatitis virus G-protein using the calcium phosphate transfection system as previously described.[30] Jurkat cells ($10^6$/mL) were plated on a 24-well plate and were pretreated with the compounds for 30 min. After pretreatment, cells were inoculated with virus stocks (200 ng of p24), and 24 h later cells were washed twice in PBS and lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol for 15 min at RT. Then, the lysates were spun down and the supernatant was used to measure luciferase activity using an Autolumat LB 9510 (Berthold, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega). The results are represented as the percent of activation (considering the infected and untreated cells 100% activation) or RLU. Results represent the mean of four different experiments.

**Western Blot.** Jurkat cells ($10^6$ cells/mL) were preincubated with the indicated compounds for 30 min and then stimulated with PMA (50 ng/mL) for 15 min. Cells were then washed with PBS and proteins extracted in 50 $\mu$L of lysis buffer (20 mM Hepes pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM $Na_3VO_4$, 5 mM NaF, 1 mM DTT, leupeptin 1 $\mu$g/mL, pepstatin 0.5 $\mu$g/mL, aprotinin 0.5 $\mu$g/mL, and 1 mM PMSF) containing 0.5% NP-40. Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA, USA), and 30 $\mu$g of proteins was boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V; 4 °C) for 1 h. Blots were blocked in TBS solution containing 0.1% Tween 20 and 5% nonfat dry milk overnight at 4 °C, and immunodetection of specific proteins was carried out with primary antibodies using an ECL system (GE Healthcare). The steady-state levels for $\beta$-tubulin, total p65, pan-ERK 1+2, and pan-JNK 1+2 served as a loading control for I$\kappa$B$\alpha$ (phospho and total), phospho-p65, phospho-ERK 1+2, and phospho JNK 1+2, respectively.

**Statistics.** Statistical analyses and $IC_{50}$ calculations were performed using GraphPad Prism (Graph Pad Software, San Diego, CA, USA). Sample population means were compared against control population means in an unpaired two-tailed Student's $t$ test. A $p$ value < 0.05 was considered statisitically significant.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

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### Notes

The authors declare no competing financial interest.

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