Identification of UV-protective Activators of Nuclear Factor Erythroid-derived 2-Related Factor 2 (Nrf2) by Combining a Chemical Library Screen with Computer-based Virtual Screening*$	extsuperscript{a}$

Franziska Lieder$	extsuperscript{1,4}$, Felix Reisen$	extsuperscript{2,4}$, Tim Geppert$	extsuperscript{2,4}$, Gabriel Sollberger$	extsuperscript{4}$, Hans-Dietmar Beer$	extsuperscript{4}$, Ulrich auf dem Keller$	extsuperscript{4}$, Matthias Schäfer$	extsuperscript{3,4}$, Michael Detmar$	extsuperscript{2,4}$, Gisbert Schneider$	extsuperscript{5}$, and Sabine Werner$	extsuperscript{2,4}$

From the$	extsuperscript{4}$Institute of Molecular Health Sciences, Department of Biology, and the$	extsuperscript{5}$Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology (ETH) Zurich, 8093 Zurich and the$	extsuperscript{6}$Department of Dermatology, University Hospital of Zurich, 8006 Zurich, Switzerland

Background: The Nrf2 transcription factor is a master regulator of cellular antioxidant defense systems.

Results: We identified novel Nrf2 activators in keratinocytes with low toxicity and strong UV-protective potential.

Conclusion: Chemical library screening combined with virtual screening is a potent strategy to identify optimized Nrf2 activators.

Significance: Our new Nrf2 activators are potential lead compounds for the development of drugs for skin protection under stress conditions.

Nuclear factor erythroid-derived 2-related factor 2 (Nrf2) is a master regulator of cellular antioxidant defense systems, and activation of this transcription factor is a promising strategy for protection of skin and other organs from environmental insults. To identify efficient Nrf2 activators in keratinocytes, we combined a chemical library screen with computer-based virtual screening. Among 14 novel Nrf2 activators, the most potent compound, a nitrophenyl derivative of 2-chloro-5-nitro-N-phenyl-benzamide, was characterized with regard to its molecular mechanism of action. This compound induced the expression of cytoprotective genes in keratinocytes isolated from wild-type but not from Nrf2-deficient mice. Most importantly, it showed low toxicity and protected primary human keratinocytes from UVB-induced cell death. Therefore, it represents a potential lead compound for the development of drugs for skin protection under stress conditions. Our study demonstrates that chemical library screening combined with advanced computational similarity searching is a powerful strategy for identification of bioactive compounds, and it points toward an innovative therapeutic approach against UVB-induced skin damage.

Ultraviolet (UV) irradiation, γ-irradiation, or various toxic or irritant chemicals challenge the skin, the outermost surface of our body. Many of these insults cause oxidative stress through enhanced production of reactive oxygen species (ROS). This results in cell damage and inflammation and also contributes to skin aging and even neoplastic transformation (1, 2). One of the most important regulators in the defense against oxidative stress is the transcription factor nuclear factor erythroid-derived 2-related factor 2 (Nrf2). It is ubiquitously expressed, and particularly high expression levels are seen in epithelial cells, including keratinocytes of the skin (3). Nrf2 belongs to the Cap’n’Collar family of transcription factors, which also includes the related Nrf1 and Nrf3 proteins, as well as p45NF-E2, Bach1, and Bach2 (4, 5). Under basal conditions, Nrf2 is predominantly present in the cytoplasm where it is anchored to the actin-binding protein Keap1 that mediates its degradation through the ubiquitin-proteasome pathway. Electrophilic substances can activate Nrf2 through modification of Keap1 cysteine residues (6). This results in the stabilization of Nrf2 and its accumulation in the nucleus (7). In addition, it has been suggested that ROS can activate certain kinases, which in turn phosphorylate Nrf2, resulting in its stabilization and activation (8). However, we could not verify the latter mechanism in keratinocytes, and it may therefore be cell type-specific (9). Upon nuclear translocation, Nrf2 dimerizes with small Maf proteins or other leucine zipper proteins and binds as a heterodimer to cis-acting elements in the promoters of its target genes, designated as antioxidant-response elements (AREs). The core consensus sequence of an ARE was characterized by deletion analysis and is defined as 5’-TGACnnnTGAC-3’ (10).
Nrf2 Activators in Keratinocytes

Genes that are regulated by Nrf2 encode, among others, proteins that help to control the cellular redox state and protect the cell against oxidative damage or toxic chemicals. These proteins include several ROS-detoxifying enzymes and other antioxidant proteins, including NAD(P)H dehydrogenase quinone 1 (NQO1), different glutathione S-transferases (GST), the regulatory and catalytic subunits of the glutathione biosynthesis enzyme γ-glutamylcysteine ligase (GCLM and GCLC), peroxiredoxins (PRDX) 1 and 6, and heme oxygenase-1 (HO-1). Studies with knock-out mice highlighted the important role of Nrf2 in the cellular stress response. Nrf2-deficient animals are more susceptible to diseases related to oxidative stress compared with their wild-type littermates (11). In the skin, loss of Nrf2 prolonged the inflammatory response after wounding (3). Most importantly, incidence and multiplicity of chemically induced skin tumors were strongly enhanced in transgenic mice expressing a dominant-negative mutant of Nrf2 in keratinocytes (12) as well as in Nrf2 knock-out mice (13). Because the basal activity was shown to mediate these protective effects (12), it was suggested that further activation of Nrf2 could be used for cancer prevention. Indeed, several preclinical and clinical studies with Nrf2-activating compounds support this hypothesis (14, 15). Furthermore, transgenic mice expressing a constitutively active (ca)Nrf2 mutant in keratinocytes were protected from UVB-induced apoptosis in the back skin epidermis. Expression of caNrf2 resulted in the activation of Nrf2 target genes and therefore reduced the levels of intracellular ROS in keratinocytes (16).

Because of these cytoprotective functions of Nrf2 in vivo, it is of major interest to identify Nrf2-activating compounds in keratinocytes that can be exploited therapeutically to impede skin-damaging effects, e.g. UV irradiation. One of the best characterized Nrf2 activators is the broccoli sprout component sulforaphane (SFN), which activates Nrf2 by chemical modification of highly reactive cysteine residues of Keap1 (17). Topical application of broccoli sprout extracts on SKH-1 hairless mice strongly reduced the tumor multiplicity and total tumor burden in a UV-induced skin carcinogenesis study (18). However, sulforaphane exerts Nrf2-independent effects, e.g. through direct regulation of glutathione levels (19, 20), and we recently showed that long term treatment of the skin with this compound causes an ichthyosis-like skin phenotype (21). In addition, a common feature of many cosmetic ingredients that cause skin sensitization is their capability to activate Nrf2 as shown in reporter gene assays with cultured cells (22). Therefore, it is desirable to identify and characterize novel Nrf2-activating compounds with high specificity and activity but low toxicity, which can be used for in vivo application. In this study, we used a novel strategy to identify Nrf2-activating compounds, combining a chemical library screen with computer-based virtual screening. We identified novel and highly potent Nrf2-activating compounds in keratinocytes with a remarkable cytoprotective function.

EXPERIMENTAL PROCEDURES

Chemicals—Rosiglitazone, T0070907, compound 1 (GW9662), and BADGE were from Enzo Life Sciences (Lausen, Switzerland). Compounds identified by the virtual screen were obtained from Asinex (Rijswijk, The Netherlands), InterBioScreen (Moscow, Russia), or Specs (Wakefield, RI). Recombinant human Keap1 protein was purchased from Origene (Rockville, MD). Forskolin was purchased from Sigma, and human tumor necrosis factor-α (TNF-α) was from PeproTech (Hamburg, Germany).

Cell Culture—Cells were cultured at 37 °C in a humid atmosphere containing 5% CO₂ (v/v). The immortalized non tolerant human HaCaT keratinocyte cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS; Invitrogen) and 1% penicillin/streptomycin.

Spontaneously immortalized keratinocytes from Nrf2 knock-out (23) mice and their wild-type littermates were obtained in our laboratory by serial passaging of primary cells. The latter were obtained from 3-day-old wild-type and Nrf2 knock-out mice (3). Although most cells became senescent upon multiple passaging, a few foci of immortalized cells appeared, which were further passaged. Cells were maintained in defined keratinocyte serum-free medium (Invitrogen) supplemented with 10 ng/ml epidermal growth factor, 10⁻¹⁰ M cholera toxin, and 1% penicillin/streptomycin (all from Sigma).

Primary human foreskin keratinocytes (HKF) were established from human foreskin obtained from the Department of Dermatology, University Hospital Zurich. They were cultured up to six passages in keratinocyte-SFM supplied with epidermal growth factor and bovine pituitary extract (Invitrogen).

Construction of ARE Luciferase Reporter Plasmids—ARE luciferase reporter plasmids were generated using the pGL3-promoter vector (Promega, Madison, WI), which includes an SV40 promoter upstream of the firefly luciferase gene. Double-stranded oligonucleotides were synthesized containing a part of the promoter of the respective Nrf2 target gene, including the ARE (underlined). The different inserts are 1XARE-mGclm (forward strand 5′-CTTGGGAAGAATGACTAAGCAGAAC-3′ and reverse strand 5′-TCGGATTTCTGCTTATGTCATGGGGTCATGACG-3′); 1XARE-ratNqo1 (forward strand 5′-CTGACTACATGAGAATGACTAAGCAGAAC-3′ and reverse strand 5′-TCGGATTTCTGCTTATGTCATGGGGTCATGACG-3′); and 8XARE (forward strand 5′-CATGACTACATGAGAATGACTAAGCAGAAC-3′ and reverse strand 5′-TCGGATTTCTGCTTATGTCATGGGGTCATGACG-3′). The sequence was extended (b) to generate 5′-terminal SacI and 3′-terminal XhoI restriction sites. After annealing of the complementary oligonucleotides, the double-stranded oligonucleotides were inserted into the pGL3 vector upstream of the SV40 promoter. In addition, a plasmid was generated with eight copies of the minimal functional ARE sequence present in the murine Gclm (ATGACTAAGCA) gene promoter. Each ARE copy was linked with the sequence 5′-CCC-3′ and 5′-GGG-3′ on the opposite strand to another copy. Oligonucleotide synthesis and sequencing of the final plasmids were performed by Microsynth (Bal gach, Switzerland). Additional reporter plasmids with a nuclear factor-κB (NF-κB)-binding site (pNF-κB-luc), a serum-re-
sponse element (pSRE-luc), or a cAMP-response element (pCRE-luc) were obtained from Clontech.

Transient Transfection and Luciferase Reporter Assays—Transfection of HaCaT cells and immortalized mouse keratinocytes was performed with linear polyethyleneimine (Polysciences, Warrington, PA). Cells were plated at a density of 2 × 10^5 cells/well in 12-well plates (Nunc, Roskilde, Denmark) and grown to 80% confluency (24 h). After two washing steps with PBS, 1 ml of Opti-MEM I (Invitrogen) was added, and the cells were co-transfected with 2 μg of the ARE luciferase reporter plasmid and 0.01 μg of the phRL-CMV Renilla luciferase vector plasmid (Promega) as an internal control for transfection efficiency. When different reporter plasmids were compared. 3.8 μl of polyethyleneimine solution (1 mg/ml) was then added; the mixture was vortexed, incubated at room temperature for 15 min to allow complex formation, and added to the cells. After 24 h, the cells were washed with PBS and incubated with fresh culture medium containing tBHQ (Merck), sulforaphane (Sigma), or DMSO as negative control. Cells were harvested 24 h later using passive lysis buffer (Promega). Firefly and Renilla luciferase activities were measured in the lysates using the Dual-Luciferase reporter assay system kit (Promega) according to the manufacturer’s instructions. Luciferase activity was determined in a MicroLumatPlus LB96V luminometer (Berthold Technologies, Bad Wildbad, Germany). For the library screen and the validation of the screening results, we seeded 9 × 10^4 cells into 15-cm dishes and added a transfection mixture containing 90 μg of pGL3–8×ARE-mGclm reporter plasmid, 171 μl of polyethyleneimine, and 4.2 ml of (w/v) NaCl. Because all cells were transfected in the same plate with the same reporter plasmid, co-transfection with a Renilla luciferase plasmid was not performed in this case. Luciferase activity was measured by adding luciferase assay reagent consisting of 20 mM Tricine, 2.67 mM MgSO4, 0.1 mM EDTA, 33.3 mM dithiothreitol, 0.27 mM coenzyme A, 0.53 mM adenosine triphosphate (all from Sigma), and 0.47 μM luciferin potassium salt (Synchem, Kassel, Germany), pH 7.8.

Screening of a Chemical Library—The LOPAC library (Sigma), containing 1280 pharmacologically active compounds, was used for the screen. All chemicals of this library were arranged in 96-well plates, dissolved in DMSO, and have a concentration of 10 mM. To generate 1 mM stock plates, aliquots of the compounds were diluted with DMSO in fresh 96-well tissue culture plates. HaCaT cells were transiently transfected with the reporter vector pGL3–8×ARE-mGclm (see above). Cells were trypsinized 24 h after transfection and seeded into 96-well plates (25,000 cells/well). 24 h later, the medium was replaced by fresh growth medium, and the cells were treated for 24 h with 10 μM of the chemicals of the library. Afterward, cells were washed with PBS and lysed in passive lysis buffer (Promega), and luciferase activity was measured as described above using the luciferase assay reagent.

Screening Compound Library—For virtual screening, we compiled a collection of 1,033,267 compounds from selected commercial vendors as follows: Specs Natural Products v08/2010 and Specs Screening Collection v08/2010 (Specs, Delft, The Netherlands); InterBioScreen Natural Compound Library v08/2010 and InterBioScreen Synthetic Compound Collection v08/2010 (InterBioScreen, Moscow, Russia); Asinex Gold v08/2010, Asinex Platinum v08/2010, and Asinex Synergy v08/2010 (Asinex, Moscow, Russia). All compounds were pre-processed using the “wash” function (i.e. deprotonation of strong acids and protonation of strong bases) in the Molecular Operating Environment software (The Chemical Computing Group Inc., Montreal, Canada). For each compound, a single heuristic three-dimensional conformation was generated with CORINA 3.46 (Molecular Networks, Erlangen, Germany) for autocorrelation (LIQUID) and graph (PoliMorph) descriptor calculation. For ligand-based virtual screening, we computed similarity values between queries and all screening pool compounds. We considered pool compounds as virtual hits if they were retrieved among the top 1% of most similar compounds for both descriptors and among the top 100 compounds using the added rank of both methods for sorting. From the resulting virtual hit lists, we manually selected compounds for bioactivity determination.

Quantitative Real Time RT-PCR (qRT-PCR)—RNA was isolated using the RNEasy mini kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. cDNA was generated using the iScript™ cDNA synthesis kit (Bio-Rad). Relative gene expression was determined using the LightCycler 480 SYBR Green system (Roche Applied Science). The following set of primers were used: mGclm-for 5′-AACAAGAAACATCGGCTCTC-3′ and mGclm-rev 5′-CTGAGCTCTGGAAGAAC-3′; mGclm-for 5′-TCCCCATGCAAGAAATGAGAT-3′ and mGclm-rev 5′-AGCTGTGAACCTCAAGGAC-3′; mNqo1-for 5′-CTGGCCCATTTTACAGAGAGC-3′ and mNqo1-rev 5′-GTTCGAGGTGTTTTTCTACTG-3′; and mNqo1-rev 5′-AGCTGTGAACCTCAAGGAC-3′ and mNqo1-rev 5′-CTGAGCTCTGGAAGAAC-3′. Each sample was analyzed in duplicate, and the amplification of cDNA of the housekeeping genes mRps29 (murine) or hRPL27 (human) was used for normalization.

MTT and LDH Assays—Human foreskin keratinocytes (HK) were seeded in 24-well plates (45,000 cells/well) and cultured overnight. Cells were treated for 24 h with different concentrations of test compounds (1–100 μM). For the MTT assay, the medium was supplemented with 100 μl of MTT solution (1 mg/ml in sterile PBS) (Sigma). After 2 h of incubation, the supernatant was aspirated, and the cells were lysed in 200 μl of 40 mM HCl/isoproprop alcohol for 10 min at RT. The reaction was stopped by addition of an equal volume of water. The absorbance was measured at 590 nm. To measure LDH release, the CytoTox 96® nonradioactive cytotoxicity assay kit (Promega) was used according to the manufacturer’s protocol.

Mass Spectrometry Analysis of Cysteine Modification of Keap1—Recombinant human Keap1 protein (6 pmol) was incubated with a 5-fold molar excess of test compound in a total volume of 100 μl in 50 mM Tris-HCl, pH 8, 150 mM NaCl for 3 h
at room temperature. Subsequently, the protein was digested by addition of 10 ng of mass spectrometry grade trypsin (Pro-mega) and incubated for 16 h at 37 °C. Peptides were cleaned up using μC18-ZipTip columns (Millipore) and analyzed on an LTQ-Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an Eksigent-Nano HPLC system (Eksigent Technologies, Dublin, CA). Solvent composition at the two channels was 0.2% formic acid, 1% acetonitrile for channel A, and 0.2% formic acid, 80% acetonitrile for channel B. Peptides were loaded on a self-made tip column (75 μm × 80 mm) packed with reverse phase C18 material (AQ, 3 μm, 200 Å, Bischoff GmbH, Leonberg, Germany) and eluted with a flow rate of 200 nl/min by a gradient from 0 to 10% of B in 5 min, 47% B in 55 min, 97% B in 58 min. Full scan MS spectra (300–2000 m/z) were acquired with a resolution of 60,000 at 400 m/z after accumulation to a target value of 500,000. Collision-induced dissociation MS/MS spectra were recorded in a data-dependent manner in the ion trap from the three most intense signals above a threshold of 500, using a normalized collision energy of 35% and an activation time of 30 ms. Charge state screening was enabled, and singly charge states were rejected. Precursor masses already selected for MS/MS were excluded for further selection for 90 s, and the exclusion window was set to 20 ppm. The size of the exclusion list was set to a maximum of 500 entries. Peak lists were extracted from raw data files using Mascot Distiller (Matrix Science) and searched against a UniProtKB/Swiss-Prot database release 15.13 using the Mascot version 2.3 search engine with the following parameters: trypsin for enzyme specificity allowing up to one missed cleavage; oxidation, di- and trioxidation, and mass shifts at trioxidation corresponding to the test compound as variable modifications; parent mass error at 10 ppm; and fragment mass error at 0.8 Da.

Analysis of Intracellular ROS Levels—HFKs were seeded in 12-well plates and grown overnight. Medium was changed, and cells were incubated with 100 μM 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen) for 30 min in the CO2 incubator. The cells were washed once with HEPES-buffered salt solution (25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2,2 5 mM NaHCO3, 15 mM glucose, pH 7.4) and subsequently incubated in fresh medium with different concentrations of test compounds (1–100 μM). Fluorescence was measured after 0–24 h using a fluorescence multiwell plate reader (Spectramax M2, Molecular Devices, Ismaning, Germany) with excitation and emission wavelengths of 485 and 530 nm.

UVB Irradiation—HFKs were seeded in 12-well plates (90,000 cells/well) and grown overnight. After treating the cells for 1, 4, or 24 h with 5 μM SFN, 50 μM compound 1, 50 μM compound 2, or DMSO as control, the medium was replaced by fresh growth medium. Cells were then irradiated with 50 mJ/cm2 UVB using a Medisun FH-54 lamp (Schulze and Böhm, Huerth, Germany) equipped with six UVB-TL/12 bulbs (9 watts each; Philips, Amsterdam, Netherlands), which emit UVB light in the range of 280–315 nm with a peak emission at 312–315 nm. 24 h later MTT or LDH assays were performed.

Statistical Analysis—Statistical analysis was performed using the PRISM software (GraphPad Software Inc., La Jolla, CA). A two-way analysis of variance analysis with Bonferroni post-test to compare multiple groups was performed. *, p ≤ 0.05; **, p ≤ 0.005; ***, p ≤ 0.001.

RESULTS

Screening of a Chemical Library for New Nrf2 Activators—To identify novel Nrf2 activators in keratinocytes, we performed a chemical library screen. For this purpose, we first tested the efficiency of several AREs (supplemental Table S1) to activate a luciferase reporter gene with a minimal promoter in the human HaCaT keratinocyte cell line. The ARE from the murine (m) Gclm gene was identified as particularly potent (data not shown) and was therefore chosen for further studies. Because of the high homology between murine and human Nrf2 (5), most mouse AREs, including the mGclm ARE, can be used to study Nrf2 activity in human cells. First, we generated a multimter with eight copies of the ARE (pGL3–8×ARE-mGclm), which further enhanced the efficiency in the activation of a luciferase reporter gene compared with the monomeric ARE (supplemental Fig. S1). This reporter gene construct was subsequently used to screen the Library of Pharmacologically Active Compounds (LOPAC1280TM, Sigma), which comprises 1280 bioactive, well characterized compounds that act via 56 classes of pharmacological targets. HaCaT keratinocytes were transiently transfected with pGL3–8×ARE-mGclm and treated for 24 h with the compounds from the library (assay concentration 10 μM each). The solvent DMSO was used as a negative control, and the known Nrf2 activators tBHQ and SFN served as positive controls. Three independent screening rounds were conducted. A 2-fold increase in luciferase activity was set as a threshold for a positive hit. Forty five hit compounds were discovered in at least one screening experiment. Six of them were positive in each screen, and seven were positive in two screens. These 13 putative Nrf2-activating substances were chosen for further analysis (compounds 1–13; Table 1). The 32 hit compounds appearing in only one of the three screening runs (supplemental Table S2) were not further investigated.

Three of the 13 hit compounds had been previously described as activators of Nrf2. These included iodoacetamide (24), parthenolide (25), and quercetin dehydrate (26). In addition, 3,4-dichloroisocoumarin is structurally related to the known Nrf2 activator coumarin (27), and tBHQ is a derivative of the aromatic compound hydroquinone (28), which we identified in our screen. The identification of these known Nrf2 activators corroborates the efficiency and suitability of the approach.

Validation of the Screening Results Using HaCaT Kera
tinocytes—To validate the eight novel potential Nrf2 activators (hit compounds), we extended the reporter assays with HaCaT cells using different incubation times (5, 10, or 24 h) and increasing concentrations of the compounds (0.1, 10, 25, and 50 μM). Furthermore, we tested the hit compounds on cells transfected with the pGL3-promoter vector (without ARE) to determine whether the activation is indeed ARE-dependent. None of the eight compounds activated the reporter after transfection with this control vector, neither after 5 or 10 h (data not shown) nor after 24 h (Fig. 1A). By contrast, luciferase activity was strongly enhanced in cells transfected with the pGL3–8×ARE-mGclm vector within 10 h (results not shown) and 24 h after
### TABLE 1

List of compounds that activated the luciferase reporter gene in at least two screening experiments

Additional information on the structure, targets, and mechanisms of action of these compounds are provided in the description of the LOPAC library (Sigma) (M2 receptor is the muscarinic acetylcholine receptor M2; NO is nitric oxide; FXR is farnesoid X receptor; IκBα is nuclear factor of κ light polypeptide gene enhancer in B-cell inhibitor α; D1/2 is dopamine receptor D1/D2; and PDE is phosphodiesterase).

| No. | Compound | Structure | Main target according to LOPAC library | Action       | Described as Nrf2 activator | Discovered in x screening experiments |
|-----|----------|-----------|----------------------------------------|--------------|-----------------------------|-------------------------------------|
| 1   | 2-Chloro-5-nitro-N-phenyl-benzamide (GW9862) | ![Structure](image1) | PPARγ | Inhibitor | no | 3x |
| 2   | Arecaidine propargyl ester hydrobromide | ![Structure](image2) | M2 receptors | Agonist | no | 3x |
| 3   | 4-Phenyl-3-furoxan-carbonitrile | ![Structure](image3) | NO Donor | no | 3x |
| 4   | (Z)-Gugglesterone | ![Structure](image4) | FXR | Antagonist | no | 3x |
| 5   | Bay 11-7085 | ![Structure](image5) | IκBα | Inhibitor | no | 3x |
| 6   | SKF 83959 hydrobromide | ![Structure](image6) | D1 | Agonist | no | 2x |
| 7   | Spiperone hydrochloride | ![Structure](image7) | D2 | Antagonist | no | 2x |
| 8   | Tosyl-L-phenyl-alanine chloromethyl ketone | ![Structure](image8) | Chymotrypsin α | Inhibitor | no | 2x |
| 9   | Iodoacetamide | ![Structure](image9) | Cysteine peptidases | Inhibitor | yes²¹ | 3x |
| 10  | Parthenolide | ![Structure](image10) | IκBα | Inhibitor | yes²⁴ | 2x |
| 11  | Quercetin dihydrate | ![Structure](image11) | PDE | Inhibitor | yes²⁶ | 2x |
| 12  | 3,4-Dichloroisocoumarin | ![Structure](image12) | Serine protease | Inhibitor | shown for coumarin²⁷ | 2x |
| 13  | Hydroquinone | ![Structure](image13) | Arachidonate 12-lipoxygenase | Inhibitor | shown for TBHQ²⁸ | 2x |
addition of the compounds (Fig. 1B), and the activation occurred in a dose-dependent manner. The strongest induction (5.5-fold) was achieved with 2-chloro-5-nitro-N-phenylbenzamide (compound 1), followed by arecaidine propargyl ester hydrobromide (compound 2) and 4-phenyl-3-furoxan-carbonitrile (compound 3). (Z)-Guggulsterone (compound 4), Bay 11-7085 (compound 5), SKF 83959 hydrobromide (compound 6), spiperone hydrochloride (compound 7), and N-p-tosyl-L-phenylalanine chloromethyl ketone (compound 8) caused a 2–3.5-fold increase in luciferase activity. For compounds 3–5 and 8, a dramatic reduction of reporter gene expression was observed with increasing concentrations, most likely reflecting cytotoxicity. Therefore, we used the concentration that induced the maximal activation for all further experiments.

To determine whether the hit compounds also activate gene expression via other AREs, we performed reporter assays with HaCaT cells transfected with the pGL3–1/H11003 ARE-rNqo1 plasmid that contains the ARE and some additional flanking sequences from the promoter region of the rat Nqo1 gene. Indeed, all hit compounds also activated gene expression via this ARE in a dose-dependent manner (supplemental Fig. S2), and compound 1 was again identified as the most potent inducer. Therefore, it seems likely that the compounds mediate gene expression via AREs and not via gene-specific flanking sequences.

**Hit Compounds Induce the Expression of Established Nrf2 Target Genes in HaCaT Cells**—We next determined whether the hit compounds induce the expression of the known Nrf2 target genes GCLC, GCLM, and NQO1 with most of the hit compounds. The time course of induction was target gene-dependent. Compounds 1–5 and to a lesser extent compound 8 induced the expression of all target genes, whereas only two of these genes were induced by compounds 6 and 7, and their effect was very mild. Taken together, six of the eight hit compounds efficiently induced the expression of Nrf2 target genes in HaCaT cells.

**Hit Compounds Act via Nrf2**—To determine whether the activation of the previously tested cytoprotective genes by the hit compounds requires Nrf2, we tested their regulation in spontaneously immortalized keratinocytes isolated from Nrf2 knock-out mice and their wild-type littermates. Lack of Nrf2 expression in cells from the knock-out mice was verified by qRT-PCR (data not shown).

Compounds 1, 2, 4, and 8 indeed induced the expression of Gclc, Gclm, and Nqo1 in cells from wild-type mice but not in cells from Nrf2 knock-out mice (Fig. 3, A–C). The only exception was compound 3, which also induced a mild up-regulation of Gclm expression, indicating that this gene can also be regulated by other transcription factors in response to compound 3. Surprisingly, compound 5 was not able to induce the expression of Nrf2 target genes in murine keratinocytes. Similar to the results obtained with HaCaT cells, compounds 6 and 7 did not or only mildly activate Nrf2 target gene expression in murine keratinocytes. Therefore, these compounds were not further characterized.

**FIGURE 1. Dose-dependent activation of luciferase activity by eight hit compounds.** A and B, HaCaT cells were transfected with the pGL3 promoter vector or with the pGL3–8×ARE-mGclm reporter plasmid and treated for 24 h with different concentrations of the hit compounds as indicated or with 50 μM tBHQ or 5 μM SFN. Luciferase activities were determined in triplicate and normalized to luciferase activities seen in DMSO-treated cells. Bars represent mean of three independent experiments ± S.D.
Identification of Additional Nrf2 Activators by Virtual Screening Based on the Hit Compounds—To identify additional and possibly improved Nrf2 activators and to determine structural features that are important for Nrf2 activation, we selected six of the hit compounds (1–5, and 8) as queries for ligand-based virtual screening experiments (similarity searching) using the computational methods LIQUID (30) and PoLiMorph (31). We compiled a database comprising 1,033,267 commercially available compounds, which was screened using a consensus scoring scheme as described previously (32). We selected in total 20 compounds for in vitro testing (Table 2) from the six resulting hit lists. At least two compounds were chosen for each query. Their capability to activate Nrf2 in HaCaT cells using reporter gene assays with the pGL3–8/H11003 ARE-mGclm construct was tested at three different concentrations (1, 10, and 100 μM). Six out of 20 test compounds (Table 2, highlighted in gray) increased reporter gene activity in three independent experiments by more than 1.5-fold. These included three analogues of compound 1 (1a–1c), a bioisoster of compound 2 (2a), and two analogues of compound 5 (5a and 5b) (supplemental Table S3).

Subsequently, we tested the ability of these six new compounds to induce the expression of the Nrf2 target gene Gclc in murine keratinocytes from wild-type mice (Fig. 4A). Compounds 1c, 2a, and 5b did not up-regulate Gclc on the mRNA level (data not shown). However, compound 1b and in particular compound 1a caused a stronger increase in the expression of Gclc on the mRNA level compared with the original compound 1. Furthermore, treatment with compound 5a resulted in a higher up-regulation of this Nrf2 target gene compared with the hit compound 5, although the overall activity of both compounds was low compared with the other more active compounds. None of these compounds enhanced the expression of Gclc in keratinocytes lacking Nrf2.

For the further characterization of the most interesting hit compounds (1c, 2a, and 5b), in particular with regard to their potential future in vivo application, we used primary HFK. In general, the increase in the mRNA levels of the target gene GCLC was lower than in immortalized murine keratinocytes. Nevertheless, compounds 1c, 1a, and 1b as well as 2 enhanced the expression of this gene in HFKs. Remarkably, compounds 1, 1a, and 1b were even more potent than SFN in this assay. Because compounds 5 and 5a had
Compounds 1 and 1a Specifically Activate ARE Reporter Genes—We first determined the specificity of compounds 1 and 1a with regard to reporter gene activation. For this purpose, we tested if these compounds also activate reporter genes with response elements for unrelated transcription factors (NF-κB-binding site, serum-response element, or cAMP-response element). Although these reporter genes were efficiently activated by TNF-α, serum, or forskolin, respectively, compounds 1 and 1a, sulforaphane, and tBHQ did not activate these reporter genes at different concentrations (supplemental Fig. S3). This result demonstrates the specificity of these compounds for AREs.

Compounds 1 and 1a Activate Nrf2 in a PPARγ-independent Manner—Compound 1 (2-chloro-5-nitro-N-phenyl-benzamide; GW9662) is a potent (IC_{50} in the nanomolar range) and irreversible antagonist of PPARγ (33), a nuclear receptor tran-

### Table 2

| Hit compounds | Predicted Nrf2 activators |
|---------------|---------------------------|
| ![Structure1](image1) | ![Structure1a](image1a) | ![Structure1b](image1b) | ![Structure1c](image1c) | ![Structure1d](image1d) | ![Structure1e](image1e) |
| ![Structure2](image2) | ![Structure2a](image2a) | ![Structure2b](image2b) | ![Structure2c](image2c) | ![Structure2d](image2d) |
| ![Structure3](image3) | ![Structure3a](image3a) | ![Structure3b](image3b) |
| ![Structure4](image4) | ![Structure4a](image4a) | ![Structure4b](image4b) |
| ![Structure5](image5) | ![Structure5a](image5a) | ![Structure5b](image5b) | ![Structure5c](image5c) | ![Structure5d](image5d) |
| ![Structure8](image8) | ![Structure8a](image8a) | ![Structure8b](image8b) | ![Structure8c](image8c) |
Keap1 (39), which could also be modified by iodoacetamide (see above). These findings strongly suggest that compound 1a activates Nrf2 via Keap1 but not via PPARγ.

Compounds 1 and 1a Reveal Only Low Toxicity for Primary Human Keratinocytes—We next analyzed the toxicity of compounds 1 and 1a for primary human keratinocytes.Remarkably, MTT assays revealed a significantly lower toxicity of these compounds at higher concentrations compared with SFN and tBHQ (Fig. 6A). This was confirmed by analysis of LDH activity in the cell supernatant (Fig. 6B). The presence of this cytosolic enzyme in the medium reflects cell lysis. The discrepancy in the result obtained with SFN in the MTT and LDH assays may result from an interference of SFN with the LDH activity assay as suggested by the increase in the levels of β-actin in the cell supernatant at high concentrations of SFN (supplemental Fig. 6A).

To determine the effect of compounds 1 and 1a on ROS production, keratinocytes were treated with 2′,7′-dichlorodihydrofluorescein diacetate and subsequently analyzed for DCF fluorescence. Only a moderate ROS production was seen with both compounds, which was comparable with the production induced by SFN (Fig. 6C). By contrast, ROS production was much higher in tBHQ-treated cells. The functionality of the assay was confirmed by analysis of cells treated with glucose oxidase, which results in continuous production of hydrogen peroxide (supplemental Fig. 6B).

Compounds 1 and 1a Protect Keratinocytes from UVB-induced Apoptosis—Because SFN protects keratinocytes in vitro and in vivo against UVB damage (18, 40), we tested if com-
pounds 1 and 1a have a similar photoprotective potential. For this purpose, HFK were treated with Nrf2 activators for different times (1, 4, or 20 h) prior to irradiation with a physiological dose of UVB (50 mJ/cm²). Remarkably, already a 1-hour incubation with compounds 1 or 1a or with SFN reduced the LDH release compared with DMSO-treated cells. The reduction was even stronger after a 20-h pretreatment, and cells treated with compounds 1 or 1a were more protected compared with SFN-treated cells (Fig. 7A). The UVB-protective effect of both compounds was verified by MTT assay, where pretreatment with these compounds enhanced the viability of UVB-irradiated keratinocytes (Fig. 7B). These findings reveal that the novel Nrf2 activators identified in this study have a potent cytoprotective potential.

DISCUSSION

Activation of the cytoprotective transcription factor Nrf2 is a promising strategy for protection of cells under stress conditions. Nrf2 activation can be achieved by various low molecular weight compounds, of which some are in clinical trials for cancer prevention (41). Recently, novel Nrf2 activators have been identified by screening of chemical libraries in cell culture assays (42–44). Of particular interest is a screen that used the LOPAC library (published by C. Klaassen in PubChem BioAssay, ID 624149). Surprisingly, there was only one overlap between this screen and our library screen: hydroquinone, a previously identified Nrf2 activator (see “Results”). By contrast, the new compounds that we identified are not included in the positive hits of this screen. These differences may result from the use of different cell lines (transformed breast cancer cell line versus nontransformed keratinocyte cell line). Importantly, none of the previously performed chemical library screens had been combined with a virtual screening approach. The latter represents an efficient strategy to identify novel compounds exhibiting a desired biochemical activity (45). In addition, UV-protective functions of these compounds have not been demonstrated. The possibility of using Nrf2 activators for cell pro-
tection under stress conditions is particularly promising for the skin, as demonstrated by the beneficial effect of sulforaphane for the treatment of skin blistering or protection from UV damage (40, 46, 47). However, sulforaphane has various Nrf2-independent effects (19, 20) and also induces pathological abnormalities in the skin upon long term application (21). Therefore, identification of novel and specific Nrf2 activators with low toxicity is highly desirable. Here, we used a combination of chemical library screening and virtual screening to identify improved Nrf2 activators in keratinocytes, which revealed low toxicity for keratinocytes combined with a potent cytoprotective potential.

The chemical library screen identified six novel Nrf2 activators in keratinocytes. Compound 4 ((Z)-guggulsterone) is a plant sterol for which multiple targets have been described, including several nuclear hormone receptors as well as 1αB kinase (48, 49). Because of this broad activity spectrum, we decided not to further characterize this compound with regard to Nrf2 activation, in particular because none of the analogues revealed Nrf2-activating capacity. This was also the case for compound 3 (4-phenyl-3-furoxan-carbonitrile), which releases nitric oxide (NO) under the action of thiol co-factors, such as cysteines (50). NO was shown to cause (S)-nitrosation of cysteine residues in Keap1 and thereby activate Nrf2 (51, 52). Because of the cytotoxicity of NO, which was also reflected in the experiments with compound 3, the latter was also not further investigated. Obvious toxicity at higher concentrations was also observed for compound 8 (tosyl-1-phenyl-alanine chloromethyl ketone), and none of its analogues was able to activate Nrf2. Therefore, we decided to focus on the remaining three compounds. Among them, compound 1 and its derivatives appeared most interesting for the following reasons: (i) compound 1 caused the strongest activation of the ARE reporter genes and of the endogenous Nrf2 target genes; (ii) a class of structural analogues was functionally equivalent with regard to Nrf2 activation; (iii) neither compound 1 nor its derivatives revealed obvious toxicity in the reporter assays; and (iv) they are structurally unrelated to previously published Nrf2 activators. The difference between compounds 1 and 1a is a chinol ine versus a phenyl group. Para-substituted nitrobenzene derivatives of 1 were active, whereas meta-substituents were not tolerated. Similarly, derivatives 5a and 5b indicate a critical region in the scaffold. This points to preliminary structure-activity relationships that could be explored in future structure-based hit-to-lead optimization. The successful scaffold hop (53) from compound 2 to compound 2a is the most surprising finding among the newly found actives. The quinuclidine 2a features the tertiary nitrogen and a potential hydrogen bond acceptor of the template 2, but it offers alternative scaffold architecture. This example represents a pair of isofunctional chemotypes, with 2a being a second promising candidate, in addition to compound 1a, for further chemical exploration.

The published target of compound 1 is PPARγ (33), which is irreversibly inhibited. However, it seems unlikely that inhibition of PPARγ is important for the effect on Nrf2, because a structurally unrelated PPARγ inhibitor did not activate Nrf2 target genes and because PPARγ activation also had no effect. Rather, it seems likely that compound 1 and its derivatives act via Keap1 due to the presence of reactive cysteines. Consistent with this hypothesis, mass spectrometry analysis of PPARγ modified by compound 1a had identified the cysteine residue 285 in the ligand-binding site of PPARγ as the site of covalent modification (33). Using mass spectrometry, we indeed identified direct binding of compound 1a to the highly reactive cysteine residue 368 in Keap1, suggesting that compound 1 and its analogues interact in a similar manner with Keap1 cysteine residues and thus activate Nrf2.

A remarkable feature of these new Nrf2 activators is the low toxicity for cultured keratinocytes, and both components were superior in this respect to SFN and tBHQ. Consistent with this finding, the capacity of compounds 1 and 1a to induce the production of ROS was lower compared with tBHQ but similar to SFN. Despite this reduced toxicity, the UVB-protective effect of our new compounds was similar or even higher compared with SFN. These findings strongly suggest that compounds 1 and 1a are interesting lead compounds for the development of drugs for skin protection under stress conditions. However, this will require establishment of an efficient in vivo delivery strategy. Thus, upon topical application of the compounds to mouse skin, we only found a minor Nrf2-activating activity of compounds 1 and 1a in preliminary experiments, although SFN induced Nrf2 target gene expression under the same conditions (data not shown). This is most likely due to inefficient penetration of compounds 1 and 1a through the epidermal barrier. Therefore, it will be important in the future to improve the topical delivery, either through chemical modification of the compounds or through use of alternative delivery vehicles. In addition, the consequences of long term in vivo application of the compounds need to be determined, in particular with regard to potential skin sensitization. Despite these open issues, our results demonstrate that library screening combined with ligand-based virtual screening is a potent strategy to identify novel Nrf2 activators. The latter are starting points for hit-to-lead optimization aiming at the development of drugs that can be used for skin protection in vivo.

Acknowledgments—We thank Dr. Claudia Defila for providing the immortalized keratinocytes from Nrf2 knock-out and wild-type mice; Dr. Yet Wai Kan (University of California, San Francisco) for Nrf2 knock-out mice; Dr. Petra Boukamp (German Cancer Research Center Heidelberg) for HaCaT keratinocytes; Drs. Walter Wahl and Liliane Michalik (University of Lausanne) for helpful suggestions with the PPAR experiments; and Drs. Yong Chen and Martin Schulz (Institute of Pharmaceutical Sciences, ETH Zurich) for helpful advice with the library screen.

REFERENCES
1. Bickers, D. R., and Athar, M. (2006) Oxidative stress in the pathogenesis of skin disease. J. Invest. Dermatol. 126, 2565–2575
2. Schäfer, M., and Werner, S. (2008) Oxidative stress in normal and impaired wound repair. Pharmacol. Res. 58, 165–171
3. Braun, S., Hanselmann, C., Gassmann, M. G., auf dem Keller, U., Born-Berclez, C., Chan, K., Kan, Y. W., and Werner, S. (2002) Nrf2 transcription factor, a novel target of keratinocyte growth factor action that regulates gene expression and inflammation in the healing skin wound. Mol. Cell. Biol. 22, 5492–5505
Nrf2 Activators in Keratinocytes

4. Motohashi, H., O’Connor, T., Katsuoka, F., Engel, J. D., and Yamamoto, M. (2002) Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. Gene 294, 1–12

5. Sykiotis, G. P., and Bohmhan, D. (2010) Stress-activated cap’n’collar transcription factors in aging and human disease. Sci. Signal. 3, re3

6. Holland, R., and Fishbein, J. C. (2010) Chemistry of the cysteine sensors in Kelch-like ECH-associated protein 1. Antioxid. Redox. Signal. 13, 1749–1761

7. Tong, K. I., Kobayashi, A., Katsuoka, F., and Yamamoto, M. (2006) Two-site substrate recognition model for the Keap1-Nrf2 system. A hinge and latch mechanism. Biol. Chem. 387, 1311–1320

8. Itoh, K., Tong, K. L., and Yamamoto, M. (2004) Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radic. Biol. Med. 36, 1208–1213

9. Durczelewski, M., Beyer, T. A., Johnson, D. A., Johnson, J. A., Werner, S., and on dem Keller, U. (2007) Electrochemical chemicals but not UV irradiation or reactive oxygen species activate Nrf2 in keratinocytes in vitro and in vivo. J. Invest. Dermatol. 127, 646–653

10. Rushmore, T. H., Morton, M. R., and Pickett, C. B. (1991) The antioxidant-responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J. Biol. Chem. 266, 11632–11639

11. Yu, X., and Kensler, T. (2005) Nrf2 as a target for cancer chemoprevention. Mutat. Res. 591, 93–102

12. on dem Keller, U., Huber, M., Beyer, T. A., Künim, A., Siemes, C., Braun, S., Bugnon, P., Mitropoulos, V., Johnson, D. A., Johnson, J. A., Hohl, D., and Werner, S. (2006) Nrf2 transcription factors in keratinocytes are essential for skin tumor prevention but not for wound healing. Mol. Cell. Biol. 26, 3773–3784

13. Xu, C., Huang, M. T., Shen, G., Yuan, X., Lin, W., Khor, T. O., Conney, A. H., and Kong, A. N. (2006) Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. Cancer Res. 66, 8293–8296

14. Kensler, T. W., Chen, J. G., Egner, P. A., Fahey, J. W., Jacobson, L. P., Rushmore, T. H., Morton, M. R., and Pickett, C. B. (1991) The antioxidant response element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J. Biol. Chem. 266, 11632–11639

15. O’Dwyer, P. J., Szarka, C. E., Yao, K. S., Halbherr, T. C., Pfeiffer, G. R., O’Dwyer, P. J., Szarka, C. E., Yao, K. S., Halbherr, T. C., Pfeiffer, G. R., and Rushmore, T. H. (2005) The antioxidant-responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J. Biol. Chem. 266, 11632–11639

16. Rushmore, T. H., Morton, M. R., and Pickett, C. B. (1991) The antioxidant-responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J. Biol. Chem. 266, 11632–11639
(2010) Cancer chemopreventive mechanisms mediated through the Keap1-Nrf2 pathway. *Antioxid. Redox. Signal.* 13, 1713–1748
39. Hong, F., Freeman, M. L., and Liebler, D. C. (2005) Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. *Chem. Res. Toxicol.* 18, 1917–1926
40. Talalay, P., Fahey, J. W., Healy, Z. R., Wehage, S. L., Benedict, A. L., Min, C., and Dinkova-Kostova, A. T. (2007) Sulforaphane mobilizes cellular defenses that protect skin against damage by UV radiation. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17500–17505
41. Shapiro, T. A., Fahey, J. W., Dinkova-Kostova, A. T., Holtzclaw, W. D., Stephenson, K. K., Wade, K. L., Ye, L., and Talalay, P. (2006) Safety, tolerance, and metabolism of broccoli sprout glucosinolates and isothiocyanates. A clinical phase I study. *Nutr. Cancer* 55, 53–62
42. Zhu, M., Baek, H., Liu, R., Song, A., Lam, K., and Lau, D. (2009) LAS0811. From combinatorial chemistry to activation of antioxidant-response element. *J. Biomed. Biotechnol.* 2009, 420194
43. Hur, W., Sun, Z., Jiang, T., Mason, D. E., Peters, E. C., Zhang, D. D., Luesch, H., Schultz, P. G., and Gray, N. S. (2010) A small molecule inducer of the antioxidant-response element. *Chem. Biol.* 17, 537–547
44. Smirnova, N. A., Haskew-Layton, R. E., Basso, M., Hushpulian, D. M., Payappilly, J. B., Speer, R. E., Ahn, Y. H., Rakhman, I., Cole, P. A., Pinto, J. T., Ratner, R. R., and Gazaryan, I. G. (2011) Development of Neh2-luciferase reporter and its application for high throughput screening and real time monitoring of Nrf2 activators. *Chem. Biol.* 18, 752–765
45. Schneider, G. (2010) Virtual screening. An endless staircase? *Nat. Rev. Drug Discov.* 9, 273–276
46. Dinkova-Kostova, A. T., Holtzclaw, W. D., and Kessler, T. W. (2005) The role of Keap1 in cellular protective responses. *Chem. Res. Toxicol.* 18, 1779–1791
47. Kems, M. L., DePianto, D., Dinkova-Kostova, A. T., Talalay, P., and Couломbe, P. A. (2007) Reprogramming of keratin biosynthesis by sulforaphane restores skin integrity in epidermolysis bullosa simplex. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14460–14465
48. Wu, J., Xia, C., Meier, J., Li, S., Hu, X., and Lala, D. S. (2002) The hypolipidemic natural product guggulsterone acts as an antagonist of the bile acid receptor. *Mol. Endocrinol.* 16, 1590–1597
49. Shishodia, S., and Aggarwal, B. B. (2004) Guggulsterone inhibits NF-κB and IκB kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis. *J. Biol. Chem.* 279, 47148–47158
50. Medana, C., Ermondi, G., Fruttero, R., Di Stilo, A., Ferretti, C., and Gasco, A. (1994) Furoxans as nitric oxide donors. 4-Phenyl-3-furoxancarbonitrile. Thiol-mediated nitric oxide release and biological evaluation. *J. Med. Chem.* 37, 4412–4416
51. Li, C. Q., Kim, M. Y., Godoy, L. C., Thiantanawat, A., Trudel, L. J., and Wogan, G. N. (2009) Nitric oxide activation of Keap1/Nrf2 signaling in human colon carcinoma cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14547–14551
52. McMahon, M., Lamont, D. J., Beattie, K. A., and Hayes, J. D. (2010) Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18838–18843
53. Schneider, G., Neidhart, W., Giller, T., and Schmid, G. (1999) “Scaffold-hopping” by topological pharmacophore search. A contribution to virtual screening. *Angew. Chem. Int. Ed. Engl.* 38, 2894–2896