Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Thiazolides as Novel Antiviral Agents. 2. Inhibition of Hepatitis C Virus Replication

Andrew V. Stachulski,*[,†] Chandrakala Pidathala,[†] Eleanor C. Row,[†] Raman Sharma,[†] Neil G. Berry,[†] Alexandre S. Lawrenson,[†] Shelley L. Moores,[†] Mazhar Iqbal,[†,#] Joanne Bentley,[†] Sarah A. Allman,[‡] Geoffrey Edwards,[¶] Alison Helm,[¶] Jennifer Hellier,[¶] Brent E. Korba,[§] J. Edward Semple,[∥] and Jean-Francois Rossignol[‡,∥,⊥]

[†]Robert Robinson Laboratories, Department of Chemistry, University of Liverpool, Liverpool L69 7ZD, U.K.

[‡]Department of Biochemistry, Institute of Glycobiology, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

[¶]Department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool, U.K.

[§]Department of Molecular Microbiology and Immunology, Georgetown University Medical Center, 3900 Reservoir Road, Washington, D.C. 20057-1440, United States

[∥]Romark Laboratories, L.C., Tampa, Florida 33607, United States

[⊥]Department of Medicine, Division of Gastroenterology and Hepatology, Stanford University Medical Center, Stanford, California 94305-5187, United States

Supporting Information

ABSTRACT: We report the activities of a number of thiazolides [2-hydroxyaroyl-*N*-(thiazol-2-yl)amides] against hepatitis C virus (HCV) genotypes IA and IB, using replicon assays. The structure−activity relationships (SARs) of thiazolides against HCV are less predictable than against hepatitis B virus (HBV), though an electron-withdrawing group at C(5′) generally correlates with potency. Among the related salicyloylanilides, the *m*-fluorophenyl analogue was most promising; niclosamide and close analogues suffered from very low solubility and bioavailability. Nitazoxanide (NTZ) **1** has performed well in clinical trials against HCV. We show here that the 5′-Cl analogue **4** has closely comparable in vitro activity and a good cell safety index. By use of support vector analysis, a quantitative structure−activity relationship (QSAR) model was obtained, showing good predictive models for cell safety. We conclude by updating the mode of action of the thiazolides and explain the candidate selection that has led to compound **4** entering preclinical development.

INTRODUCTION

Hepatitis C Virus (HCV). HCV is classed among the *Flaviviridae* as a single-strand RNA virus.[1] About 170 million people worldwide are chronically infected by HCV, which is transmitted principally through blood infection, and there is no vaccine available.[2] Initial symptoms on infection are typically very mild, and resulting characteristic liver infections (fibrosis, cirrhosis, cancer)[3] may not be observed for up to 30 years. Many genotypes of HCV have been recognized, with six main types and subdivisions of each recognized: genotypes IA/IB account for about 70% of all cases, and genotypes IA/IB and IIA/IIB combined account for over 90%.[4] Evaluation of anti-HCV agents therefore normally begins with genotypes IA/IB.

On the basis of the known sequence of the HCV genome, a number of therapeutic approaches to HCV treatment are possible.[1,5] The combination of (pegylated) interferon α (IFN-α) and ribavirin is still regarded as "standard of care" (SOC) even though a sustained virological response is only observed in 50−60% of patients, with genotype 1 infections being more difficult to treat, and important side effects are observed.[6,7] The mode of action of IFN-α−ribavirin is not wholly clear, but some kind of immunomodulatory effect does seem to be involved, and indeed a number of candidate anti-HCV therapies involve either new formulations of IFNs[8] or other immune stimulants. The activation of host pathways that induce endocellular type 1 IFNs is also a recognized result of HCV infection.[9]

There are currently numerous compounds in anti-HCV clinical trials. Most early drug discovery against HCV was directed toward either protease inhibitors, generally targeting the nonstructural protein NS3, or polymerase inhibitors, directed toward the NS5B RNA-dependent RNA polymerase.[2,5] In the former class, telaprevir[10,11] and boceprevir[12,13] have recently been approved as therapies for chronic HCV infection in combination with SOC. Among nucleoside-based polymerase inhibitors, 2′-modified nucleosides appear to be the most effective[14] and appear to confer a relatively high barrier to drug resistance. Non-nucleoside HCV NS5B inhibitors appear to generate resistance more readily but are generally more potent.[15] The HCV NS5A protein has also emerged as a promising antiviral target.[16]

Cyclosporin A, long known to be an effective immunosuppressant, is an effective HCV inhibitor per se, and one of its analogues, alisporivir (formerly known as DEBIO-025), an inhibitor of RNA polymerase acting on protein folding and

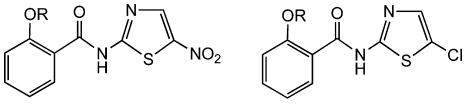
Received: September 21, 2011
Published: November 7, 2011

dx.doi.org/10.1021/jm201264t | J. Med. Chem. 2011, 54, 8670−8680

isomerization now undergoing phase III clinical trials, is a potent (equiactive) inhibitor that is not immunosuppressive.[17,18] Finally, it is worth noting that iminosugars have also been studied as anti-HCV agents,[19] using bovine viral diarrhea virus (BVDV) which acts as a reliable surrogate for HCV in tissue cell cultures. Formation and secretion of BVDV were both prevented by N-alkylated analogues of deoxynojirimycin.

Thiazolides and HCV. NTZ **1**, a broad spectrum thiazolide anti-infective, is licensed in the United States for the treatment of diarrhea. We have summarized the discovery and development of NTZ **1** (Chart 1), its effective circulating metabolite tizoxanide **2**, and analogous thiazolides, notably the 5′-chloro analogue **3**, as broad-spectrum antiviral agents effective against both DNA and RNA viruses including HBV in particular.[20−22] Current evidence, including resistance studies, indicates that **1** and **2** act via modulation of host cell processes[23,24] and not by directly inhibiting HCV polymerase, protease, or helicase enzymatic activities.[24]

Chart 1. Basic Thiazolide Structures 1−4



1 R = Ac **3** R = H

2 R = H **4** R = Ac

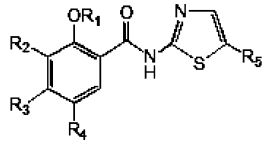
NTZ **1** is an effective anti-HCV agent either alone or in combination with SOC treatment (see above) or pegylated IFN only.[25,26] Thus, significant increases in both rapid (RVR) and sustained virologic response (SVR) were observed when using a triple therapy of **1**, pegylated IFN, and ribavirin compared to SOC alone.[25] Monotherapy with **1** (4 weeks) followed by pegylated IFN and **1** for 36 weeks, without ribavirin, gave an SVR in 80% of patients:[26] combinations of **1** with 2′C-methyl cytidine and telaprevir also displayed synergy. This offers promise for the treatment of patients unable to tolerate ribavirin. It is likely that these results will have to be compared to a new standard of care including a direct acting antiviral agent (DAA) such as telaprevir or boceprevir combined with pegylated IFN and ribavirin. Latest results concerning the mechanism of action of the thiazolides are summarized in the section Discussion below.

In this paper we shall present a full account of the activity of **1** and a number of thiazolide analogues, together with a smaller number of related salicyloylanilides, against HCV in cell culture. A recent review commented that "the search for more potent, singly effective and less toxic antiviral drugs is mandatory to improve clinical anti-HCV chemotherapies."[5] We believe that both NTZ/tizoxanide **1**/**2** and the newer thiazolides, notably the *O*-acetate **4** of the 5′-chloro analogue, offer considerable promise in this regard.

CHEMISTRY

A total of 72 derivatives of 2-amino-5-nitrothiazole were originally synthesized at the Radium Institute Department of Chemistry,[27] and 12 of them including NTZ (PH-5776),[28] were the most active compounds. Several years later the research was reinitiated at the University of Liverpool, U.K. and, with further assistance from Kalexsyn Inc., Kalamazoo, MI, 146 additional related chemical structures, with wider variation of the thiazole moiety, were prepared. In a previous paper[20] we gave general methods for the preparation of thiazolides, from the appropriate aminothiazole derivative and *O*-acetylsalicyloyl chloride. Compounds **3**−**35**, as given in Tables 1−3, were all

Table 1. Activities of 5′-Nitro- and 5′-Halothiazolides against Hepatitis C Replication[a]



						primary assay genotype 1B				secondary assay genotype 1B				secondary assay genotype 1A			
compd	R_1	R_2	R_3	R_4	R_5	CC_{50}[b] (μM)	EC_{50}[c,d] (μM)	EC_{90}[d] (μM)	SI	CC_{50}[b] (μM)	EC_{50}[c,d] (μM)	EC_{90}[d] (μM)	SI	CC_{50}[b] (μM)	EC_{50}[c,d] (μM)	EC_{90}[d] (μM)	SI
1	Ac	H	H	H	NO_2	38.0	0.21	0.93	181.0	35.0	0.25	0.95	143.0	49.0	0.33	1.10	149.0
2	H	H	H	H	NO_2	15.0	0.15	0.81	100.0	18.0	0.15	0.85	124.0	14.0	0.25	1.00	56.0
3	H	H	H	H	Cl	15.0	10.0	10.0									
4	Ac	H	H	H	Cl	4.3	0.23	1.10	18.9	4.9	0.31	1.50	16.0	5.7	0.40	1.90	14.0
5	H	Me	H	H	NO_2	5.0	0.36	6.4	14.0	11.0	0.35	0.85	31.0	12.0	0.39	2.30	31.0
6	H	H	H	Cl	NO_2	>100.0	2.0	5.9	>50.0								
7	Ac	H	H	H	Br	15.0	3.8	11.0	4.0	12.0	4.3	20.0	3.0	21.0	3.3	10.0	6.0
8	H	H	H	H	Br	20.0	10.0	10.0		98.0	4.9	20.0	20.0	88.0	2.80	9.40	31.4
9	H	Me	H	H	Br	21.0	1.9	11.0	11.0	15.0	>10.0	>10.0		25.0	>10.0	>10.0	
10	Ac	H	H	Me	Br	12.0	4.2	13.0	29	10.0	3.0	7.8	3.3	11.0	3.0	8.6	3.7
11	H	Cl	H	H	Br	14.0	5.2	12.0	2.7	10.0	>10.0	>10.0		11.0	>10.0	>10.0	
12	Ac	Me	H	H	Cl	12.0	0.59	23.0	20.0	16.0	1.5	8.9	11.0	14.0	1.00	3.80	14.0
13	H	H	Me	H	Cl	30.0	>10.0	>10.0									
14	H	H	H	Me	Cl	2.3	>20.0	>20.0		20.0	10.0	>10.0		16.0	10.0	10.0	
15	H	H	H	H	F	24.0	>10.0	>10.0						2.9	>10.0	>10.0	

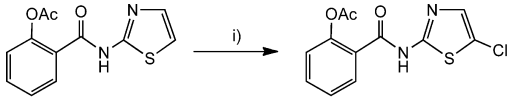
[a]Antiviral activity was assessed in a 3-day assay using the stably expressed HCV replicon cell lines AVA5 (for genotype 1B) and H/FL-Neo (for genotype 1A) as described previously.[21] [b]Drug concentration at which a 2-fold lower level of neutral red dye uptake is observed relative to average level in untreated cultures. [c]Determined by measuring intracellular HCV RNA concentration.[21] [d]The EC_{50} and EC_{90} values were determined in triplicate and with standard deviations of ±20% of the value quoted.

dx.doi.org/10.1021/jm201264t | J. Med. Chem. 2011, 54, 8670−8680

prepared by these methods. In this section we describe the synthesis of other 2-aminothiazoles not given earlier; the standard coupling method was then applied to each.

Although both 2-amino-5-bromo and 2-amino-5-chlorothiazole are commercially available as their hydrohalide salts, it is interesting that the halogen may be introduced via the known[29] unsubstituted thiazolide **36**. Thus, heating **36** with *N*-chlorosuccinimide (NCS) in MeCN at gentle reflux afforded **4** in 56% unoptimised yield (Scheme 1), identical to the

Scheme 1. Alternative Synthesis of the 5′-Chlorothiazolide 4[a]



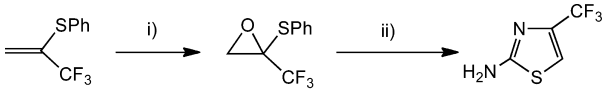
36 **4**

[a](i) *N*-chlorosuccinimide, MeCN, heat, 56%.

product of reaction of 2-amino-5-chlorothiazole and acetylsalicyloyl chloride. Clearly the additional electron-withdrawing effect of the acetate in **36** directs electrophilic substitution to the thiazole 5′-position with high selectivity.

Regarding the 4′/5′-trifluoromethyl analogues, we described the synthesis of the 5′-analogue previously.[20] 2-Amino-4-trifluoromethylthiazole was also prepared from the appropriate epoxysulfone as described by Viehe et al.[30] (Scheme 2).

Scheme 2. Synthesis of 2-Amino-4-trifluoromethylthiazole 39[a]



37 **38** **39**

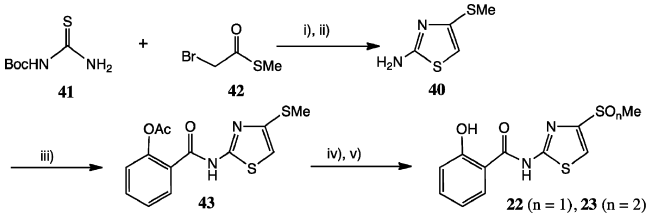
[a](i) MCPBA (3.5 equiv), CH_2Cl_2, reflux, 16 h, 50−60%; (ii) $(H_2N)_2C{=}S$, MeOH, reflux, 16 h, 91%.

Oxidation of α-(phenylthio)trifluoropropene **37**[31] with excess of *m*-chloroperbenzoic acid (MCPBA), followed by heating the resulting epoxysulfone **38** with thiourea in MeOH, afforded 2-amino-4-trifluoromethylthiazole **39** in good yield.

In a previous paper[20] we described the synthesis of a 4′-sulfonylthiazole as a fortuitous result of the displacement reaction between a 5′-bromo derivative and sodium sulfinate. It was difficult to reproduce this result; a much more reliable route to the 4′-sulfone, which also provided access to a sulfoxide analogue, proceeded via 2-amino-4-(methylthio)thiazole **40** (Scheme 3). It is known that the "halogen dance" of protected 2-amino-5-bromothiazoles[32] affords the isomeric 4-bromo compounds in good yield. This offered a reasonable route to **40**, but we found a simpler method via the known *N*-Boc thiourea **41**[33] that reacted with the methylthio ester **42**[34] of bromoacetic acid, followed by acidolysis, to afford **40** in good overall yield.

After standard acylation of **40** with acetylsalicyloyl chloride, controlled oxidation of the product **43** with Oxone followed by deprotection afforded the desired 4′-sulfoxide analogue (±)-**22**. Alternatively, oxidation of **43** with 2 equiv of MCPBA, then deprotection, afforded the 4′-sulfone analogue **23**.

Scheme 3. Syntheses of 4′-Sulfoxide and 4′-Sulfone Thiazolide Analogues 22, 23[a]



41 **42** **40** **43** **22** (n = 1), **23** (n = 2)

[a](i) EtOH, 0−20 °C, 50%; (ii) CF_3CO_2H, CH_2Cl_2, then 0.1 M HCl, then sat. aq $NaHCO_3$, 71%; (iii) acetylsalicyloyl chloride, ref 20, 47%; (iv) Oxone, aq MeOH, then aq HCl, THF, 47% overall; (v) MCPBA (2 equiv), CH_2Cl_2, then aq HCl, warm, 58% overall.

RESULTS

Antiviral Activity. Our antiviral activities were obtained by a 3-day assay in the stably expressing replicon cell lines AVA5 (for genotype 1B) and H/FL-Neo (for genotype 1A).[21] Although in general terms the SAR of the thiazolides against HCV roughly paralleled their activity against HBV, there were some significant differences. Especially, it was not irrelevant in many cases whether the *O*-acetate or the free phenol was screened, and the selectivity (safety) index was much lower with some compounds such that some derivatives showed potential cytotoxicity. Nevertheless, a number of compounds demonstrated good activity with EC_{50} < 1 μM combined with fully acceptable selectivity index (SI, the ratio CC_{50}/EC_{50}) and druglike physical properties.

Table 1 summarizes the activity of 5′-nitro- and 5′-halothiazolides beginning with NTZ **1** and tizoxanide **2**. Our primary assay was always against genotype 1B; compounds showing good efficacy were then further subjected to a secondary assay against the same genotype and primary assay against genotype 1A.

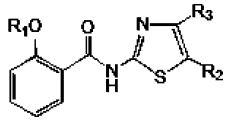
In the case of the 5′-nitro analogues, the SI was >100 for both **1** and **2**; their activities were the same to within experimental error in this assay. Methylation in the benzene ring led to a decrease in activity, least pronounced with the 3-Me compound **5**; the 4- and 5-Me analogues, not shown, were less active and the SI dropped sharply for these analogues.[20,35] The set of 5′-bromo analogues **7**−**11** showed moderate activity generally below the levels seen against HBV, again with no great difference between acetates and free phenols; the selectivity index was quite low, in the 3−10 range.

The 5′-chloro analogues were of considerable interest. Here the free phenol **3** was essentially inactive, in complete contrast to its activity against HBV, but the corresponding acetate **4** showed EC_{50} < 1 μM in all three assays together with a fully acceptable SI. Methylation of the benzene ring led to complete loss of activity for the 4- and 5-methyl compounds **13** and **14** (here, the *O*-acetates were also ineffective). The 3-methyl analogue **12** retained some activity with a reasonable SI. The 5′-fluoro compound **15** was inactive.

Of the other substituents (Table 2), the 4′-CF_3 analogue **16** displayed moderate activity in the primary screen but with a very low SI. Here the free phenol initially appeared very active, but this was not reproducible on further assay; the 5′- CF_3 analogue **17** was inactive. Both **16** and **17** had low aqueous solubility in addition to the potential cytotoxicity. For this virus, activity was not confined to electron-withdrawing 5′-substituents; the simple 5′-Me compound **18** displayed moderate

dx.doi.org/10.1021/jm201264t | J. Med. Chem. 2011, 54, 8670−8680

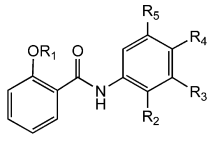
Table 2. Activities of Other 4′- and 5′-Substituted Thiazolides against HCV Replication[a]

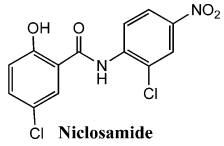


				primary assay genotype 1B				secondary assay genotype 1B				secondary assay genotype 1A			
compd	R_1	R_2	R_3	CC_{50} (μM)	EC_{50} (μM)	EC_{90} (μM)	SI	CC_{50} (μM)	EC_{50} (μM)	EC_{90} (μM)	SI	CC_{50} (μM)	EC_{50} (μM)	EC_{90} (μM)	SI
16	Ac	CF_3	H	3.7	4.3	8.2	0.9	1.7	>10.0	>10.0		14	>10.0	>10.0	
17	Ac	H	CF_3	2.8	>10.0	>10.0									
18	H	Me	H	45.0	4.2	14.0	11.0								
19	H	NHAc	H	11.0	>10.0	>10.0									
20	H	CO_2Et	H	14.0	>10.0	>10.0									
21	H	H	Ph	26.0	3.5	10.0	7.4	14.0	>10.0	>10.0		29.0	>10.0	>10.0	
22	H	H	SOMe	63.0	2.00	4.60	32.0								
23[b]	H	H	SO_2Me	85	0.50	1.70	170								
24[c]	H	SO_2Me	H	43.0	1.5	5.2	29.0								
25	H	Br	Me	15.0	>10.0	>10.0									
26	H	Br	Ph	16.0	2.2	7.0	7.3	100.0	>10.0	>10.0		62.0	>10.0	>10.0	
27	H	CN	H	15.0	37	12.0	4.1	15.0	>10.0	>10.0		11.0	>10.0	>10.0	

[a]All footnotes to Table 1 apply. [b]Variable results, see text; average values over five determinations are shown. [c]Variable results, average over two determinations. EC_{50} >10.0 μM in retest.

Table 3. Activities of Various Salicyloylanilides against HCV Replication: Primary Genotype 1B Assay Only[a]





Niclosamide

						primary assay genotype 1B			
compd	R_1	R_2	R_3	R_4	R_5	CC_{50} (μM)	EC_{50} (μM)	EC_{90} (μM)	SI
28	Ac	H	F	H	H	>100.0	3.8	11.0	26.0
29	H	H	F	H	H	3.4	0.13	0.83	26
30	H	Cl	H	H	H	72.0	3.80	21.0	19
31[b]	Ac	H	H	Br	H	0.6	0.45	3.40	1.3
32	H	Cl	H	H	CF_3	0.33	0.01	0.03	65.2
33	H	H	CF_3	H	CF_3	3.70	3.50	8.10	1.1
34	Ac	H	H	CO_2Me	H	4.4	0.06	0.46	76
35	H	H	H	SO_2Me	H	>100.0	>10.0	>10.0	
niclosamide						10.0	0.16	0.70	16.2

[a]All footnotes to Table 1 apply. [b]Variable results, see text; average values from over two determinations are shown.

activity. Among other 5′-substituents, the behavior of nitrile as a halogen isostere was again borne out by the moderate activity of **27**, but acetamido **19** and alkoxycarbonyl **20** analogues were inactive. Against HBV, 4′-phenyl analogues showed interesting activity, but here the activity of **21** was only slight and analogues of **21** with a 3-Me or Cl substituent in the benzene ring (not shown) were less active, in contrast to their good activity vs HBV.[20]

We initially reported that the 5′-methylsulfone derivative **24** had very good activity against HCV,[21] but this compound was actually the 4′-isomer **23** resulting from an adventitious rearrangement commencing from a 5′-bromo precursor.[20] Both sulfone analogues **23** and **24** were later made by unambiguous syntheses (see above and ref 20), and neither compound gave consistently submicromolar EC_{50} values in our assay. In fact both compounds gave variable results on further evaluation, the values in Table 2 being averaged over several assays, and the nominally more active 4′-sulfone **23** did not confirm its activity in the secondary assay (not shown). We have no simple explanation for this variability, but it does not appear to be due to solubility issues. Indeed the Na salt of **24** showed no better activity or consistency. By contrast, the 4′-sulfoxide **22** showed reproducible, though moderate, activity.

Finally, two 4′,5′-disubstituted analogues are shown. The 4′-Me, 5′-Br analogue **25** was essentially inactive. The 4′-Ph, 5′-Br analogue **26** showed moderate activity in the primary screen, but (unlike the 5′-Br analogues in Table 1) this was not confirmed on secondary assay.

In Table 3 we show a number of salicyloyl anilides that were similarly assayed in our primary screen. Some of these show prima facie interesting activity, but the generally poor physical properties of this series (generally very low aqueous solubility), combined with lower scope for novelty compared to the thiazolides, make them less attractive for drug development. The known anthelmintic agent niclosamide is added to Table 3 and makes a good reference standard. Indeed niclosamide

dx.doi.org/10.1021/jm201264t | J. Med. Chem. 2011, 54, 8670–8680

Table 4. GA-MLR QSAR Models and Their Performance Statistics for the Primary Assay Genotype 1B CC_{50}, Primary Assay 1B EC_{50}, Secondary Assay CC_{50}, and Secondary Assay Genotypes 1B and 1A CC_{50}[a]

	primary assay genotype 1B		secondary assay	
	CC_{50}	EC_{50}	genotype 1B CC_{50}	genotype 1A CC_{50}
$+r^2$	0.805	0.766	0.807	0.770
r^2_{LOO}	0.679	0.595	0.630	0.624
r^2_{BS}	0.593	0.499	0.509	0.481
F	21.727	12.294	15.372	13.3813
descriptor	BLI	ATSC2p	nX	SpMax_B(s)
	GATS8m	P_VSA_i_3	JGI9	RDF040v
	P_VSA_MR_2	G2u	RDF090m	G1v
	RDF030p	R5s+		

[a]Abbreviations specific to this table: BLI, Kier benzene-likeliness index topological indices; GATS8m, Geary autocorrelation of lag 8 weighted by mass 2D autocorrelations; P_VSA_MR_2, P_VSA-like on molar refractivity, bin 2 P_VSA-like descriptors; RDF030p, radial distribution function-030/weighted by polarizability; ATSC2p, centered Broto−Moreau autocorrelation of lag 2 weighted by polarizability 2D autocorrelations; P_VSA_i_3, P_VSA-like on ionization potential, bin 3 P_VSA-like descriptors; G2u, second component symmetry directional WHIM index/unweighted WHIM descriptors; R5s+, R maximal autocorrelation of lag 5/weighted by I-state GETAWAY descriptors; nX, number of halogen atoms constitutional indices; JGI9, mean topological charge index of order 9 2D autocorrelations; RDF090m, radial distribution function-090/weighted by mass; SpMax_B(s), leading eigenvalue from Burden matrix weighted by I-State 2D matrix-based descriptors; RDF040v, radial distribution function-040/weighted by van der Waals volume; G1v, first component symmetry directional WHIM index/weighted by van der Waals volume WHIM descriptors. Further details of QSAR descriptors are given in Todeschini, R.; Consonni, V. *Molecular Descriptors for Chemoinformatics*; Wiley-VCH: Weinheim, Germany, 2009.

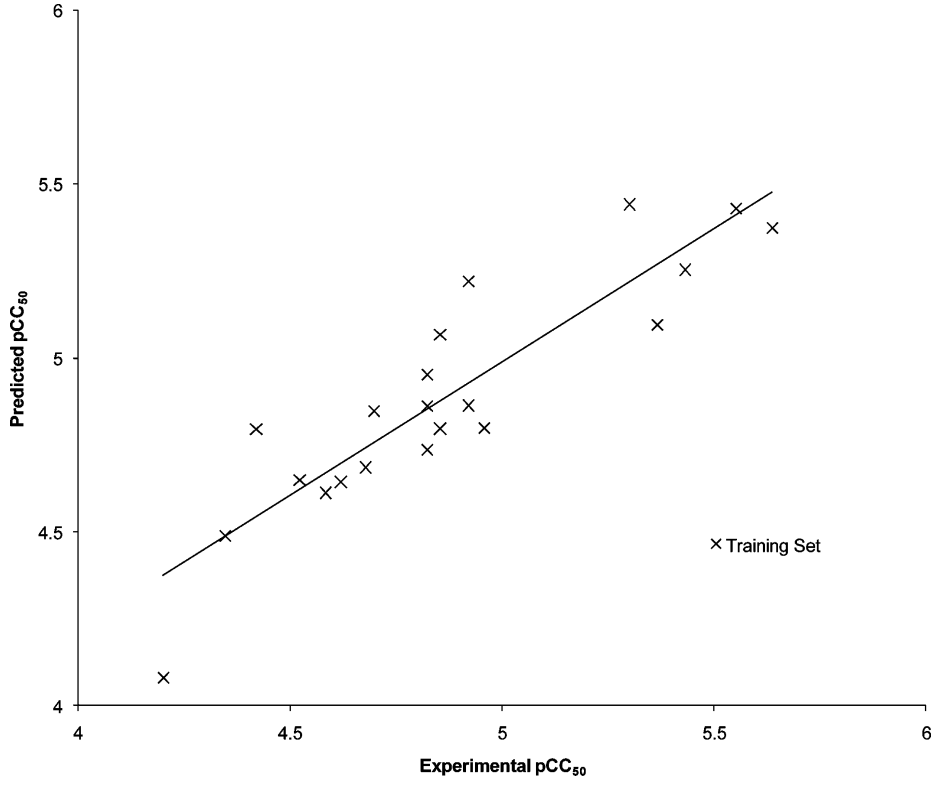


Figure 1. GA-MLR regression model for the primary assay genotype 1B CC_{50}.

shows potent activity in the primary assay, but concerns over cytotoxicity and its notoriously low aqueous solubility make it unattractive as an antiviral lead. Simple *m*- and *p*-nitrophenyl analogues (not shown), however, did not display consistent activity. By contrast, the known[36] *m*-fluoro compound **29** was consistently active in the primary assay and with a good SI, although the absolute CC_{50} was quite low. In this series, the *O*-acetate **28** performed less well, although with a higher CC_{50}.

Other halo analogues with *o*, *m*, or *p*-Cl/Br were generally less active with rather low SI values and did not give consistent assays. In fact the *o*-Cl analogue **30** showed a relatively good SI but lost activity compared to positional isomers. The *p*-Br derivative **31** is typical of the other *m*,*p* halo analogues; the activity was not reproducible, and cytotoxicity was observed. Compounds **32** and **33** are known.[20] **32** appeared very active in the primary assay, but this was not borne out in a secondary assay (not shown) and the very low CC_{50} value was a concern. The bis-CF_3 analogue **33** was appreciably less active; the CF_3 compounds suffered from particularly low aqueous solubility. On the other hand, the 4-methylcarboxylate **34** was appreciably

dx.doi.org/10.1021/jm201264t | J. Med. Chem. 2011, 54, 8670–8680

more water-soluble, and the SI appeared to be fully acceptable; nevertheless, its apparent activity was not borne out in the secondary assay. In this series, sulfone analogues, of which **35** is shown here, were quite inactive.

Quantitative Structure–Activity Relationships. In order to assist in analyzing and interpreting the SAR associated with the above compounds, QSAR models were developed for some of the biological end points/activities against HCV replication. In order to assess the predictive ability of models developed, it is required that a training set should comprise a minimum of 10 compounds[37] and an external test set a minimum of 5 compounds.[38] As such, sufficient quantitative data to satisfy this exist for only four of the assays: primary assay genotype 1B CC_{50} (26 data points); primary assay genotype 1B EC_{50} (20 data points); secondary assay genotype 1B CC_{50} (15 data points); secondary assay genotype 1A CC_{50} (16 data points).

Initially models were constructed using the genetic algorithm-multiple linear regression (GA-MLR) machine learning method[39] for each of the four assays using an autoscaled and objectively filtered subset of the descriptors generated by known software for molecular descriptors (DRAGON, version 6)[40] (for further computational details see Experimental Section). The internal quality of each model was evaluated by considering statistical measures[41] including coefficient of determination (r^2), leave-one-out cross-validated r^2 (r^2_{LOO}), bootstrap r^2 (r^2_{BS}) (averaged over 100 runs), and F statistic. Table 4 shows the statistics for the models found for each of the four assays each displaying extremely good performance measures. Thus, a set of internally statistically valid QSAR models was identified and displayed a very good correlation between measured and predicted activity, as illustrated in Figure 1 for the primary assay genotype 1B, CC_{50}. The molecular descriptors used within each model were all significant with $|t| \geq 2$; however, with one exception they are challenging to interpret with respect to aiding further manual molecular design. The one interpretable descriptor selected in the secondary 1B CC_{50} assay was nX (number of halogens in the model). For the other descriptors in the models a correlation with physically interpretable descriptors was sought: descriptors with a correlation of ≥0.75 were identified. For the primary 1B EC_{50} assay a high correlation (>0.75) was discovered between one descriptor in the model (ATSC2p negative coefficient with respect to activity), nH (number of H atoms), and N% (percentage of N atoms). Additionally another descriptor selected in the model (R5s+, negative coefficient with respect to activity) was negatively correlated with nH. For the secondary 1A CC_{50} results the SpMax_B(s) has a positive coefficient with activity and was positively correlated with nF (number of F atoms).

The models all perform very well internally; however, external validation, where the performance of a model is tested against molecules not used to develop the model, is the only truly predictive test for a QSAR model.[42] Two methods of selecting a training set and test set compounds were explored: the computer adjunct data evaluator X (CADEX) algorithm[43] and activity binning. In the activity binning protocol each molecule was assigned to a specific bin according to its activity, and the median molecule from each bin was selected for the test set. The performance of the QSAR on the external test sets was examined with reference to[44] coefficient of determination $r^2 \geq 0.6$, leave-one-out cross validated $r^2(q^2) \geq 0.5$; $|r_0^2 - r_0'^2| < 0.3$, where r_0^2 is the correlation coefficient between predicted and observed activities of the line of best fit passing through the origin and where $r_0'^2$ is the correlation coefficient between the observed and predicted activities of the line of best fit through the origin; $(r^2 - r_0^2)/r^2 < 0.1$; $0.85 \leq k \leq 1.15$, where k is the gradient of the line of best fit passing through the origin when predicted activities are plotted against observed. Disappointingly, the models developed using the GA-MLR protocol failed to produce any models that were valid on the external test sets created using either CADEX or activity binning methods.

With the failure of a linear QSAR methodology (GA-MLR), a nonlinear technique was explored to derive valid models for the data sets. Support vector machine (SVM) learning has previously proven to be useful for addressing a wide range of classification and regression problems.[45] SVM regression models using the radial basis function (RBF) kernel were developed using a grid search algorithm with 10-fold cross-validation in order to search for optimium kernel parameters (ε, γ). When the CADEX algorithm was used to split the data sets into training and test sets, SVM failed to produce any good models. However, when activity binning was used, good models were identified. Table 5 illustrates the good SVM QSAR models using activity binning for each of the four assays. A statistically signicant model, both internally and externally, was found for only the primary assay genotype 1B, CC_{50} data set (the largest data set). The performance of this model internally and externally is excellent. Figure 2 represents the plot of the training and test sets for this model and indicates the good correlation. We propose that this model will be useful for compound selection and design, as it can predict the cell safety indices of future molecules.

Table 5. Best SVM Regression Models and Their Performance Statistics for the Primary Assay Genotype 1B CC_{50}, Primary Assay 1B EC_{50}, Secondary Assay CC_{50}, and Secondary Assay Genotypes 1B and 1A CC_{50}

	primary assay genotype 1B		secondary assay	
	CC_{50}	EC_{50}	genotype 1B CC_{50}	genotype 1A CC_{50}
r^2	0.8052	0.420	0.0109	0.5218
q^2	0.561	0.396	−0.0329	0.1051
$\lvert r_0^2 - r_0'^2 \rvert$	0.0001	0.0001	0	0
$(r^2 - r_0^2)/r^2$	0.000103	0.000104	0	0
k	1.0073	0.987	1.0096	1.0176

DISCUSSION

HCV Activity and Mode of Action. From the above results, eight thiazolides analogues showed $IC_{50} < 5$ μM against both genotypes 1A and 1B. The structure–activity profile is more restricted than against HBV, but the fact that such levels of activity are seen against both DNA and RNA viruses is significant, as is the demonstration of activity of **1** and **2** against HCV strains resistant to NS5B-targeting and NS3-targeting antiviral compounds.[21] In pharmacokinetic terms, the log P values of thiazolides are within the limits for expected oral absorption, and although they are typically strongly serum bound, this does not prevent an effective therapeutic concentration being obtained.[20,21]

It is well-known that the high rate of HCV replication and its error-prone RNA leads to a high risk of generating resistant strains. In the case of telaprevir, the mutations conferring

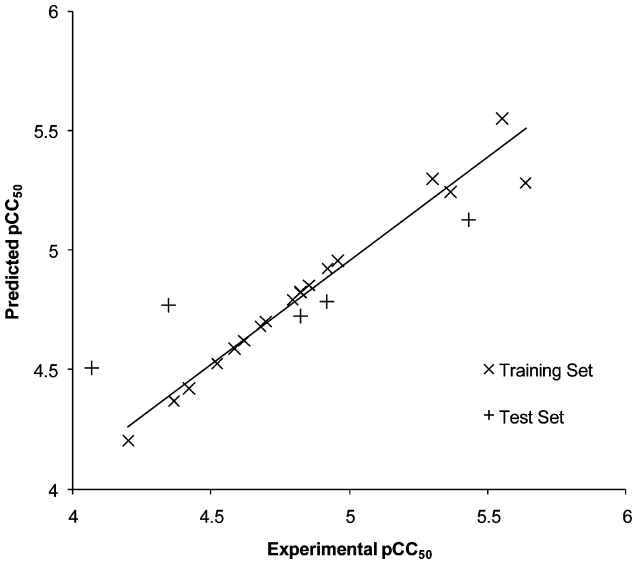


Figure 2. SVM regression model for the primary assay genotype 1B CC_{50} (C = 1, ε = 64, γ =1).

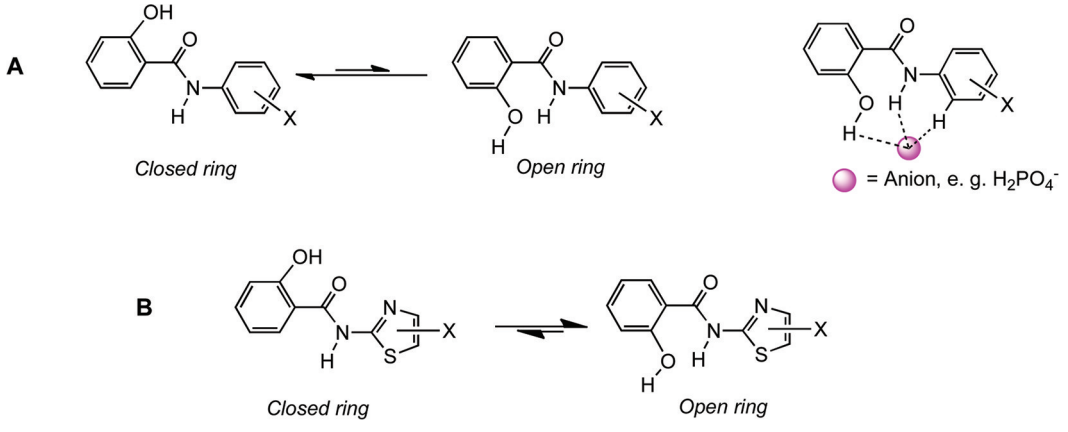
resistant phenotypes have been studied in detail[46] although these strains were still sensitive to IFα-2a as well as **1** and **2**.[23] In addition, **1** and **2** exhibit strongly synergistic interactions with telaprevir in cell culture assays.[23] When Huh-7 cells containing HCV replicons (genotype 1B) were subjected to passage with the antibiotic Geneticin and increasing concentrations of **1** or **2**,[23,24,47] the resulting cell lines exhibited increases of up to 36× in the EC_{50} values of **1** and **2**, but HCV replicons isolated from these cell lines had no more ability to confer resistance than replicons "naive" to **1** or **2**.[23] These HCV replicons had unaltered susceptibility toward a nucleotide inhibitor, 2′-C-methylcytidine, and were more susceptible to IFα-2b. Full HCV genome sequencing failed to identify mutations that could confer resistance.[24] Furthermore, versions of the resistant cell lines lacking HCV replicons were able to confer the resistant phenotype onto wild-type HCV replicons that were introduced via transfection; i.e., resistance was a host cell property.[24] These results are consistent with a cell-mediated effect by **1** and **2** reported previously.[26]

Studies to identify the mechanism of action of thiazolides are underway but have not yet yielded a definitive target pathway; nevertheless, the broad spectrum of antiviral activity against rotavirus and influenza A viruses[48] is again consistent with a host target for these compounds. Additionally, alterations of glycosylation patterns for specific rotavirus and influenza virus envelope proteins have been reported.[48] Nitazoxanide has also been shown to enhance the phosphorylation of a key mediator of the host unfolded protein response, eukaryotic initiation factor-2α, in HCV-replicon containing cells.[49]

It is intriguing to speculate what structural features might contribute to the inhibitory action of the thiazolides. Although, as we noted above, salicyloyl anilides were generally of less interest than the thiazolides as anti-HCV agents (narrower spectrum, poorer reproducibility), the two classes share a salicyloyl residue in common, and as pointed out by a recent publication,[50] such amides may adopt two distinct conformations (Scheme 4). It was suggested that while the closed ring (equilibrium A) with H-bonding between phenolic OH and the amide carbonyl oxygen is normally preferred, in the presence of an anion such as phosphate favorable interactions with OH, NH, and aryl CH bonds were all facilitated in the open ring. Clearly the thiazolide (equilibrium B) has no H atom corresponding to the aryl CH in the anilide, but for both equilibria the acidity of the NH is dependent on the substituent X: a strongly electron-withdrawing X will enhance the NH acidity. Salicyloyl anilides are indeed known as good anion receptors and inhibitors of tyrosine kinase, possibly by competition with ATP for the binding site.[51]

In summary, we have demonstrated the efficacy of a number of thiazolides as inhibitors of HCV replication. Our biological data were subjected to a rigorous 3D-QSAR analysis, which showed an excellent correlation of observed cell safety data against physicochemical parameters using both internal and external test sets. The parental compounds **1** and **2** have a high barrier to resistance in cell culture and clinical trials, are equally effective against HCV mutants resistant to anti-HCV DAAs, and act in synergy with several DAAs targeting various HCV processes.[21,23,24] A direct immunomodulatory effect of nitazoxanide has lately been demonstrated and is observed in both innate and adaptive immune systems with a subsequent up-regulation of the interferon-stimulated genes pathways and their known antireplication activity as seen in hepatitis C with protein kinase R and eIF-2α for instance.[52] These properties support the continued development of this class of compounds as potential antiviral agents.

Scheme 4. Open and Closed Conformations of Salicyloyl Anilides (A) and Thiazolides (B), after Reference 42



dx.doi.org/10.1021/jm201264t | J. Med. Chem. 2011, 54, 8670–8680

Candidate Selection. Of the compounds discussed above, three thiazolides underwent preclinical development involving subacute toxicity studies in rats and dogs along with their toxicokinetic/pharmacokinetic profiles. While their structures were closely related to nitazoxanide **1** and their metabolism similarly involved deacetylation of the parent compound followed by glucuronidation, the outcomes for the three differed greatly. Thus, the 5′-bromothiazolide derivative **9** was considerably more toxic in rats and dogs than nitazoxanide, with maximum single oral tolerated dose in dogs below 25 mg/kg, for example. By comparison, the *O*-acetate of 5-Me, 5′-chlorothiazolide **14** (this compound was very active against HBV[20]), showed an acceptable toxicity profile but was surprisingly rapidly metabolized. A single 1 mg/kg intravenous dose of **14** given to dogs showed an undetectable level of its deacetyl derivative 1 h after injection and of its glucurono-conjugate after only 2 h. Finally, the 5′-chloro compound **4** showed a favorable toxicity and pharmacokinetic profile and is undergoing phase 1 clinical trials as the first of the second generation of thiazolides. Full details of these results will be published elsewhere.

EXPERIMENTAL SECTION

Chemical Procedures. Organic extracts were washed finally with saturated aqueous NaCl and dried over anhydrous Na_2SO_4 prior to rotary evaporation at <30 °C. Analytical thin-layer chromatography was performed using Merck Kieselgel 60 F 254 silica plates. Preparative column chromatography was performed on Merck 938S silica gel. Unless otherwise stated, ^{1}H and ^{13}C NMR spectra were recorded on $CDCl_3$ solutions using either Bruker 250 or 400 MHz (100 MHz for ^{13}C) or Varian 500 MHz instruments with tetramethylsilane as internal standard. Both low- and high-resolution mass spectra were obtained by direct injection of sample solutions into a Micromass LCT mass spectrometer operated in the electrospray mode, +ve or −ve ion as indicated. CI mass spectra (NH_3) were obtained on a Fisons Instruments Trio 1000. All compounds tested were analyzed by HPLC using an Agilent 1100 system, eluting with a variable percentage of MeCN in water containing 0.1% CF_3CO_2H, and were of at least 97% peak area purity. HPLC data were collected on Agilent 1100HPLC systems with the following columns and conditions: condition A, Agilent Zorbax C8 75 mm × 4.6 mm, 5 μm column (part no. 993967-906) maintained at 30 °C; solvent A, water (0.1% TFA); solvent B, acetonitrile (0.07% TFA); gradient, 5 min 95% A to 95% B; 2 min hold; then recycle; UV detection at 210 and 250 nm. Condition B: Agilent Zorbax Eclipse XDB-C18 50 mm × 4.6 mm, 1.8 μm column (part no. 927975-902) maintained at 30 °C; solvent A, water (0.1% TFA); solvent B, acetonitrile (0.07% TFA); gradient, 5 min 95% A to 95% B; 1 min hold; 1 min recycle; 30 s hold; UV detection at 210 and 254 nm with no reference. Condtion C: Agilent Zorbax C8 150 mm × 4.6 mm, 5 μm column maintained at 30 °C; solvent A, water (0.1% TFA); solvent B, acetonitrile (0.07% TFA); gradient, 10 min 95% A to 95% B; 2 min hold; then recycle; UV detection at 210 and 254 nm with no reference.

Biological Methods. Antiviral assays were performed as described previously.[21,53] In summary, a 3-day assay was performed in the stably expressing replicon cell line AVA5 (subgenomic CON1, genotype 1B) maintained as subconfluent cultures on 96-well plates.[54] Antiviral activity was determined by blot hybridization analysis of intracellular HCV RNA (normalized to the level of cellular B-actin RNA in each culture sample). Cytotoxicity was assessed by neutral red dye uptake parallel cultures seeded and treated in the same manner as for the antiviral analyses. Another HCV replicon, H/FL-Neo, a genotype 1A full-length construct, was used for additional assays.[55]

2-Acetoxybenzoyl-*N*-(5-chlorothiazol-2-yl)amide (4). A mixture of compound **36**[29] (0.26 g, 1 mmol) and *N*-chlorosuccinimide (0.14 g, 1.05 mmol) was stirred and heated at gentle reflux in MeCN (5 mL) for 3.25 h, when no starting material was detectable. The solution was cooled, diluted with EtOAc (20 mL), and washed with saturated aqueous $NaHCO_3$, water, and 5% aqueous $Na_2S_2O_3$. Evaporation followed by recrystallization from EtOAc−hexane gave in two crops the title compound **4** (0.165 g, 56%), which was identical with material prepared by reaction of acetylsalicyloyl chloride with 2-amino-5-chlorothiazole·HCl under our standard two-phase conditions (68%).[20] Mp 142.5−144 °C (from EtOAc−hexane); ^{1}H NMR (400 MHz, $CDCl_3$) 2.37 (3 H, s, CH_3CO), 6.79 (1 H, s, 4′-H), 7.28 (1 H, m, ArH), 7.41 (1 H, m, ArH), 7.66 (1 H, m, ArH), 7.88 (1 H, m, ArH), and 11.60 (1 H, br s, NH); ^{13}C NMR (125 MHz, $CDCl_3$) 21.0, 121.0, 123.7, 125.6, 126.4, 129.9, 133.4, 134.5, 148.6, 156.6, 163.3, and 168.7. *m*/*z* (ES +ve ion mode) 319 (MNa^+, 100%). Found: *m*/*z*, 318.9903; $C_{12}H_9ClN_2O_3SNa$ requires *m*/*z*, 318.9915.

2-Acetoxybenzoyl-*N*-(thiazol-2-yl)amide (36). On a 5 mM scale, 2-aminothiazole was reacted with acetylsalicyloyl chloride under our standard two-phase conditions.[20] After recrystallization from aqueous EtOH the product **36** was obtained in 76% yield. Mp 140−142 °C (lit.[29(c)] mp 140−142 °C); ^{1}H NMR (500 MHz, $CDCl_3$) 2.38 (3 H, s, CH_3CO), 6.94, 7.00 (2 H, 2 d, *J* = 4 Hz, 4′-H and 5′-H), 7.27 (1 H, d, ArH), 7.40 (1 H, t, ArH), 7.62 (1 H, t, ArH), and 7.92 (1 H, d, ArH). *m*/*z* (ES +ve ion mode) 263 (MH^+, 100%). Found: *m*/*z*, 263.0486. $C_{12}H_{11}N_2O_3S$ requires *m*/*z*, 263.0485.

Other new compounds are summarized in the Supporting Information or were described with characterization in a preceding paper.[20] Compound **34** was known as the free phenol.[50] We prepared and screened the *O*-acetate (see Supporting Information).

Quantitative Structure−Activity Relationship Methods. QSAR models were developed for data concerning the drug concentration required to elicit a response against four assays: primary assay genotype 1B CC_{50} (26 data points); primary assay genotype 1B EC_{50} (20 data points); secondary assay genotype 1B CC_{50} (15 data points); secondary assay genotype 1A CC_{50} (16 data points). These data sets were chosen for their amenability toward modeling in terms of the number of data points, allowing splitting into sufficiently large training and test sets. Also, these data sets displayed the greatest range of evenly distributed values containing low micromolar or submicromolar activities. The IC_{50} values were converted into pCC_{50} or pEC_{50} values using the equation $-\log_{10}(CC_{50} \times 10^{-6})$ or $-\log_{10}(EC_{50} \times 10^{-6})$. Structures of the molecules were generated using Spartan08[56] and energy minimized using the MMFF94 force field. In total 946 zero-, one-, and two-dimensional molecular descriptors/properties were calculated for the set of compounds using DRAGON 6.[40] The descriptor set of 946 variables was autoscaled[57] and filtered using two objective selection methods. First, descriptors that had the same value for 80% of the data set were removed, as they contained minimal information. Second, the CORCHOP (data-handling) procedure[58] was used to eliminate one of a pair of descriptors that exhibited very high intercorrelation (r > 0.99). The procedure removed the descriptor whose distribution deviated the most from normal (as defined by maximum kurtosis 100). The multiple linear regression machine learning method coupled with genetic algorithm subjective descriptor selection (GA-MLR) as implemented in the Pharmacokinetics in Silico (PHAKISO)[39] program was used to relate the activities (Y) of a set compounds to a combination of their molecular descriptors (X) using a linear equation. The genetic algorithm was set to have population size 50, replacement rate 0.6, crossover rate 1.0, and maximum number of generations 100. The maximum number of descriptors allowed was varied so that the molecule/descriptor ratio was ∼5 to minimize the occurrence of chance correlations.[59]

In order to split the data sets, three algorithms were used: sphere exclusion, CADEX (the CADEX algorithm[43] as implemented in PHAKISO), and activity binning. Activity binning was performed in Excel using an in-house protocol. Each molecule within the various assays was assigned to a specific activity bin, and the central molecule from each bin was selected for the test set. In cases where the bin contained an even number of molecules the highest median was selected, with this trend performed across all other even numbered bins. This was repeated but with the lower median compounds

selected, and from QSARs developed for both test splitting patterns, the best model was used.

The Konstanz information miner (KNIME)[60] was used to develop SVM regression models for the four biological end points. The RBF kernel was applied using a GridSearch node available in the KNIME Knowledge Flow extension.[61] The grid search algorithm found the SVM model with RBF kernel parameters ε and γ that gave the highest correlation coefficient in 10-fold cross-validation. The ε parameter was initially altered in a range of 2^{-10} to 2^{1}, and the γ parameter was from 2^{-15} to 2^{3}, in line with recommedations in the literature.[62] Both parameters were altered as powers of 2, and the grid search was allowed to extend three times. The C parameter for the RBF was manually scanned from 1 to 1000 in factors of 10 initially and then in half order of magnitude once a coarse optimum region of parameter space was identified.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis and characterization of compounds **6**, **7**, **10**, **12**, **16**, **17**, **26**, **28**−**31**, **34**, **35**, **40**, and **43**−**45**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +44 (0)151 794 3482. Fax: +44 (0)151 794 3588. E-mail: andrew.stachulski@bioch.ox.ac.uk.

Present Address

[#]Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan.

■ ACKNOWLEDGMENTS

We are grateful to Romark Laboratories for funding this work at the University of Liverpool, U.K. (2003−2007) and the University of Oxford, U.K. (from 2009). HCV analyses were supported by NIAID Contract NO1-AI-30046 to the Georgetown University Medical Center. We also thank K. Farrar and S. Uhm (Georgetown University Medical Center) for technical assistance with the HCV analyses and Kalexsyn Inc., Kalamazoo, MI, for the syntheses of certain compounds.

■ ABBREVIATIONS USED

HCV, hepatitis C virus; SAR, structure−activity relationship; HBV, hepatitis B virus; NTZ, nitazoxanide; QSAR, quantitative structure−activity relationship; IFN-α, interferon α; SOC, standard of care; NS, nonstructural (typically of viral proteins); BVDV, bovine viral diarrhea virus; R(S)VR, rapid (sustained) virological response; DAA, directly acting antiviral agent; NCS, *N*-chlorosuccinimide; MCPBA, *m*-chloroperbenzoic acid; AVA5, a replicon cell line used in HCV assay; SI, selectivity index; GA-MLR, genetic algorithm-multiple linear regression; DRAGON, software for molecular descriptor calculation; CADEX, computer adjunct data evaluator X; SVM, support vector machine; RBF, radial basis function; KNIME, Konstanz information miner; PHAKISO, pharmacokinetics in silico; CORCHOP, a computer data-handling iteration

■ REFERENCES

(1) Wagner, E. K.; Hewlett, M. J. *Basic Virology*, 2nd ed.; Blackwell Publishing: Malden, MA, 2004; pp 242−243.

(2) Neyts, J. Selective inhibitors of hepatitis C virus replication. *Antiviral Res.* **2006**, *71*, 363−371.

(3) (a) Tong, M. J.; El-Ferra, N. S.; Reikes, A. R.; Co, R. L. Clinical outcomes after transfusion-associated hepatitis C. *N. Engl. J. Med.* **1995**, *332*, 1463−1466. (b) El-Serag, H. B.; Mason, A. Rising incidence of hepatocellular carcinoma in the United States. *N. Engl. J. Med.* **1999**, *34*, 745−750. (c) Poynard, T.; Ratziu, V.; Benhamou, Y.; Opolon, P.; Cacoub, P.; Bedossa, P. Natural history of HCV infection. *Best Pract. Res., Clin. Gastroenterol.* **2000**, *14*, 211−228.

(4) Ohno, T.; Mizokami, M.; Wu, R.-R.; Saleh, M. G.; Ohba, K.; Orito, E.; Mukaide, M.; Williams, R.; Lau, J. Y. N. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J. Clin. Microbiol.* **1997**, *35*, 201−207.

(5) Sakamoto, N.; Watanabe, M. New therapeutic approaches to hepatitis C virus. *J. Gastroenterol.* **2009**, *44*, 643−649.

(6) Fried, M. W.; Shiffman, M. L.; Reddy, K. R.; Smith, C.; Marinos, G.; Goncales, F. L. Jr.; Haussinger, D.; Diago, M.; Carosi, G.; Dhumeaux, D.; Craxi, A.; Lin, A.; Hoffman, J.; Yu, J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.* **2002**, *347*, 975−982.

(7) Manns, M. P.; McHutchinson, J. G.; Gordon, S. C.; Rustgi, V. K.; Shiffman, M.; Reindollar, R.; Goodman, Z. D.; Koury, K.; Ling, M.; Albrecht, J. K. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet* **2001**, *358*, 958−965.

(8) Rustgi, V. K. Albinterferon alfa-2b, a novel fusion protein of human albumin and human interferon alfa-2b, for chronic hepatitis C. *Curr. Med. Res. Opin.* **2009**, *25*, 991−1002.

(9) Asselah, T.; Bieche, I.; Sabbagh, A.; Bedossa, P.; Moreau, R.; Valla, D.; Vidaud, M.; Marcellin, P. Gene expression and hepatitis C virus infection. *Gut* **2009**, *58*, 846−858.

(10) Reesink, H. W.; Zeuzem, S.; Weegink, C. J.; Forestier, N.; van Vliet, A.; van de Wetering de Rooij, J.; McNair, L.; Purdy, S.; Kauffman, R.; Alam, J.; Jansem, P. L. M. Rapid decline of viral RNA in hepatitis C patients treated with VX-950; a phase Ib, placebo-controlled, randomized study. *Gastroenterology* **2006**, *131*, 997−1002.

(11) Znabet, A.; Polka, M. M.; Janssen, E.; de Kanter, F. J. J.; Turner, N. J.; Orru, R. V. A.; Ruitjer, E. A highly efficient synthesis of telaprevir by strategic use of biocatalysis and multicomponent reactions. *Chem. Commun.* **2010**, *46*, 7918−7920.

(12) Tong, X.; Chase, R.; Skelton, A.; Chen, T.; Wright-Minogue, J.; Malcolm, B. A. Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034. *Antiviral Res.* **2006**, *70*, 28−38.

(13) Poordad, F.; McCone, J. Jr.; Bacon, B. R.; Bruno, S.; Manns, M. P.; Sulkowski, M. S.; Jacobson, I. M.; Reddy, K. R.; Goodman, Z. D.; Boparai, N; DiNubile, M. J.; Sniukiene, V.; Brass, C. A.; Albrecht, J. K.; Bronowicki, J. P. SPRINT-2 investigators. Boceprevir for untreated chronic HCV genotype 1 infection. *N. Engl. J. Med.* **2011**, *364*, 1195−1206.

(14) Carroll, S. S.; Tomassini, J. E.; Bosserman, M.; Getty, K.; Stahlhut, M. W.; Eldrup, A. B.; Bhat, B.; Hall, D.; Simcoe, A. L.; LaFemina, R.; Rutkowski, C. A.; Wolanski, B.; Yang, Z. C.; Migliaccio, G.; De Francesco, R.; Kuo, L. C.; MacCoss, M.; Olsen, D. B. Inhibition of hepatitis C virus RNA replication by 2′-modified nucleoside analogues. *J. Biol. Chem.* **2003**, *278*, 11979−11984.

(15) McCown, M. F.; Rajyaguru, S.; Le Pogam, S.; Ali, S.; Jiang, W. R.; Kang, H.; Symons, J.; Cammack, N.; Najera, I. The hepatitis C virus replicon presents a higher barrier to resistance to nucleoside analogues than to nonnucleoside analogues or protease inhibitors. *Antimicrob. Agents Chemother.* **2008**, *52*, 1604−1612.

(16) Asselah, T. NS5A inhibitors: a new breakthrough for the treatment of chronic hepatitis C. *J. Hepatol.* **2011**, *54*, 1069−1072.

(17) Watashi, K.; Hijikata, M.; Hosaka, M.; Yamaji, M.; Shimotohno, K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* **2003**, *38*, 1282−1288.

(18) (a) Paeshuyse, J.; Kaul, A.; De Clercq, E.; Rosenwirth, R.; Dumont, K.J.-M.; Scalfaro, P.; Bartenschlager, R.; Neyts, J. The non-immunosuppresive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication in vitro. *Hepatology* **2006**, *53*, 761−770. (b) Scribner, A.; Houck, D.; Huang, Z.; Mosier, S.; Peel, M.; Scorneaux, B. Synthesis and biological evaluation of [D-lysine][8]

cyclosporin A analogues as potential anti-HCV agents. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6542−6546.
(19) (a) Zitzmann, N.; Mehta, A. S.; Carrouee, S.; Butters, T. D.; Platt, F. M.; McCauley, J.; Blumberg, B. S.; Dwek, R. A.; Block, T. M. Imino sugars inhibit the formation and secretion of bovine viral diarrhea virus, a pestivirus model of hepatitis C virus: implications for the development of broad spectrum anti-hepatitis virus agents. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11878−11882. (b) Ouzounov, S.; Mehta, A.; Dwek, R. A.; Block, T. M.; Jordan, R. The combination of interferon alpha-2b and *n*-butyl deoxynojirimycin has a greater than additive antiviral effect upon production of infectious bovine viral diarrhea virus (BVDV) in vitro: implications for hepatitis C virus (HCV) therapy. *Antiviral Res.* **2008**, *55*, 425−435.
(20) Stachulski, A. V.; Pidathala, C.; Row, E. C.; Sharma, R.; Berry, N. G.; Iqbal, M.; Bentley, J.; Allman, S. A.; Edwards, G.; Helm, A.; Hellier, J.; Korba, B. E.; Semple, E. J.; Rossignol, J.-F. Thiazolides as novel antiviral agents: 1. Inhibition of hepatitis B virus replication. *J. Med. Chem.* **2011**, *54*, 4119−4132.
(21) Korba, B. E.; Montero, A. B.; Farrar, K.; Gaye, K.; Mukerjee, S.; Ayers, M. S.; Rossignol, J.-F. Nitazoxanide, tizoxanide and other thiazolides are potent inhibitors of hepatitis B virus and hepatitis C virus replication. *Antiviral Res.* **2008**, *77*, 56−63.
(22) Rossignol, J.-F. Thiazolides: a new class of antiviral drugs. *Expert Opin. Drug Metab. Toxicol.* **2009**, *5*, 667−674.
(23) Korba, B. E.; Elazar, M.; Lui, P.; Rossignol, J.-F.; Glenn, J. S. Potential for hepatitis C virus resistance to nitazoxanide or tizoxanide. *Antimicrob. Agents Chemother.* **2008**, *52*, 4069−4071.
(24) Yon, C.; Viswanathan, P.; Rossignol, J.-F.; Korba, B. E. Mutations in HCV non-structural genes do not contribute to resistance to nitazoxanide in replicon-containing cells. *Antiviral Res.* **2011**, *91*, 233−240.
(25) Rossignol, J.-F.; Elfert, A.; El-Gohary, Y.; Keeffe, E. B. Improved virologic response in chronic hepatitis C genotype 4 treated with nitazoxanide, peginterferon and ribavirin. *Gastroenterology* **2009**, *136*, 856−862.
(26) (a) Rossignol, J.-F.; Elferty, A.; Keeffe, E. B. Treatment of chronic hepatitis C using a 4-week lead-in with nitazoxanide before peginterferon plus nitazoxanide. *J. Clin. Gastroenterol.* **2010**, *44*, 504−509. (b) Korba, B. E.; Elazar, M.; Lui, P.; Glenn, J. S.; Rossignol, J.-F. Potential role for nitazoxanide in combination with STAT-C agents for the inhibition of HCV replication without the development of resistance. *Hepatology* **2008**, *48* (Suppl.), 356A.
(27) Cavier, R.; Gayral, Ph.; Guillaumel, J.; Clavel, J. M.; Demerseman, P.; Royer, R. Recherches sur les dérivés nitrés d'intérêt biologique. XVI. Relations entre structures et activités protozoocides antheminthiques et molluscicides dans la série du benzamido-2 nitro-5 thiazole. *Eur. J. Med. Chem.* **1978**, *13*, 539−543.
(28) Rossignol, J. F.; Cavier, R. New Derivatives of 2-Benzamido 5-Nitrothiazoles. U.S. Patent 3,950,351, April 13, 1976.
(29) (a) Luo, Q.-L.; Li, J.-Y.; Liu, Z.-Y; Chen, L.-L.; Li, J.; Ye, Q.-Z.; Nan, F.-J. Inhibitors of type I MetAPs containing pyridine-2-carboxylic acid thiazol-2-ylamide. Part I: SAR studies on the determination of the key scaffold. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 635−638. (b) Rossignol, J.-F.; Semple, J. E. Preparation of Thiazolylbenzamide Derivatives for Use as Antiviral Hepatitis Agents. U.S. Pat. Appl. Publ. 20090036467, Feb 5, 2009. (c) U.S. Patent 2401522, 1943; Sandoz.
(30) Laduron, F.; Janousek, Z.; Viehe, H. G. α- or β-Trifluoromethyl epoxysulfones: new C_3 reagents for heterocyclisation. *J. Fluorine Chem.* **1995**, *73*, 83−86.
(31) Feiring, A. E. 1-(Trifluoromethy1)ethenyl phenyl selenide and1-(trifluoromethy1)ethenyl phenyl sulfide. *J. Org. Chem.* **1980**, *45*, 1962−1964.
(32) Stanetty, P.; Schnurch, M.; Mereiter, K.; Mihovilovic, M. D. Investigations of the halogen dance reaction on N-substituted 2-thiazolamines. *J. Org. Chem.* **2005**, *70*, 567−574.
(33) Schiavi, B.; Ahond, A.; Poupat, C.; Potier, P. Preparation of *N-tert*-butoxycarbonylthiourea opens the way to protected 2-aminothiazoles. *Synth. Commun.* **2002**, *32*, 1671−1674.
(34) Praveen Rao, P. N.; Amini, M.; Li, H.; Habeeb, A. G.; Knaus, E. E. Design, synthesis, and biological evaluation of 6-substituted-3-(4-methanesulfonylphenyl)-4-phenylpyran-2-ones: a novel class of diarylheterocyclic selective cyclooxygenase-2 inhibitors. *J. Med. Chem.* **2003**, *46*, 4872−4882.
(35) For some parasites, a 3-methyl group may abolish thiazolide activity completely: Esposito, M.; Stettler, R.; Moores, S. L.; Pidathala, C.; Muller, N.; Berry, N. G.; Stachulski, A. V.; Rossignol, J.-F.; Hemphill, A. In vitro efficacy of nitazoxanide and other thiazolides against *Neospora caninum* tachyzoites reveals anti-parasitic activity independent of the nitro group. *Antimicrob. Agents Chemother.* **2005**, *49*, 3715−3723.
(36) Waisser, K.; Hladuvkova, J.; Kunes, J.; Kubicova, L.; Klimesova, V.; Karajannis, P.; Kaustova, J. Synthesis and antimycobacterial activity of salicylanilides substituted in position 5. *Chem. Pap.* **2001**, *55*, 121−129.
(37) Podlogar, B. L.; Muegge, I.; Brice, L. J. Computational methods to estimate drug development parameters. *Curr. Opin. Drug Discovery* **2001**, *4*, 102−109.
(38) Eriksson, L.; Jaworska, J.; Worth, A. P.; Cronin, M. T. D.; McDowell, R. M. M.; Gramatica, P. Methods for reliability and uncertainty assessment and for applicability evaluations of classification- and regression-based QSARs. *Environ. Health Perspect.* **2003**, *111*, 1361−1375.
(39) PHAKISO: Pharmacokinetics in Silico. http://www.phakiso.com/ (accessed Aug 19, 2011).
(40) *DRAGON* (Software for Molecular Descriptor Calculation), version 6.0; Talete srl: Milan, Italy, 2010; http://www.talete.mi.it/.
(41) *Guidance Document on The Validation of (Quantitative) Structure−Activity Relationships [(Q)SAR] Models*; OECD: Paris, France, 2007; http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono%282007%292&doclanguage=en
(42) Tropsha, A.; Gramatica, P.; Gombar, V. K. The importance of being earnest: validation is the absolute essential for successful application and interpretation of QSAR models. *QSAR Comb. Sci.* **2003**, *22*, 69−77.
(43) Kennard, R. W.; Stone, L. A. Computer aided design of experiments. *Technometric* **1969**, *11*, 137−148.
(44) Golbraikh, A.; Tropsha, A. Beware of q(2)! *J. Mol. Graphics* **2002**, *20*, 269−276.
(45) (a) Jorissen, R. N.; Gilson, M. K. Virtual screening of molecular databases using a support vector machine. *J. Chem. Inf. Model.* **2005**, *45*, 549−561. (b) Li, H.; Yap, C. W.; Xue, Y.; Li, Z. R.; Ung, C. Y.; Han, L. Y.; Chen, Y. Z. Statistical learning approach for predicting specific pharmacodynamic, pharmacokinetic or toxicological properties of pharmaceutical agents. *Drug Dev. Res.* **2006**, *66*, 245−259.
(46) Sarrazin, C.; Kieffer, T. L.; Bartels, D.; Hanzelka, B.; Muh, U.; Welker, M.; Wincheringer, D.; Zhou, Y.; Chu, H.-M.; Lin, C.; Weegink, C.; Reesink, H.; Zeuzem, S.; Kwong, A. D. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology* **2007**, *132*, 1767−1777.
(47) Korba, B. E.; Glenn, J. S.; Ayers, M. S.; Rossignol, J.-F. Studies of the potential for resistance to nitazoxanide or tizoxanide. *J. Hepatol.* **2008**, *48*, S11.
(48) Rossignol, J.-F.; La Frazia, S.; Chiappa, L.; Ciucci, A.; Santoro, M. G. Thiazolides, a new class of anti-influenza molecules targeting viral haemagglutinin at the post-translational level. *J. Biol. Chem.* **2009**, *284*, 29798−29808.
(49) Elazar, M.; Liu, M.; McKenna, S. A.; Liu, P.; Gehrig, E. A.; Puglisi, J. D.; Rossignol, J.-F.; Glenn, J. S. The anti-hepatitis C agent nitazoxanide induces phosphorylation of eukaryotic initiation factor 2 alpha via protein kinase activated by double-stranded RNA activation. *Gastroenterology* **2009**, *137*, 1827−1835.
(50) Guo, L.; Wang, Q.-L.; Jiang, Q.-Q.; Jiang, Q.-J.; Jiang, Y.-B. Anion-triggered substituent-dependent conformational switching of salicylanilides. New hints for understanding the inhibitory mechanism of salicylanilides. *J. Org. Chem.* **2007**, *72*, 9947−9953.

(51) Kamath, S.; Buolamwini, J. K. Targeting EGFR and HER-2 receptor tyrosine kinases for cancer drug discovery and development. *Med. Res. Rev.* **2006**, *26*, 569−594.
(52) Clerici, M.; Trabattoni, D.; Pacei, M.; Biasin, M.; Rossignol, J. F. The anti-infective nitazoxanide shows strong immumodulating effects. *J. Immunol.* **2011**, *186*, 155.21.
(53) Blight, K. J.; Kolykhalov, A. A.; Rice, C. M. Efficient initiation of HCV RNA replication in cell culture. *Science* **2000**, *290*, 1972−1974.
(54) Okuse, C.; Rinaudo, J. A.; Farrar, K.; Wells, F.; Korba, B. E. Enhancement of antiviral activity against hepatitic C virus in vitro by interferon combination therapy. *Antiviral Res.* **2005**, *65*, 23−34.
(55) Blight, K. J.; McKeating, J. A.; Marcotrigiano, J.; Rice, C. M. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. *J. Virol.* **2003**, *77*, 3181−3190.
(56) Wavefunction, Inc. http://www.wavefun.com/.
(57) Livingstone, D. J. *Practical Guide to Scientific Data Analysis*; Wiley-VCH: Chichester, U.K., 2009.
(58) Livingstone, D. J.; Rahr, E. Corchop, an interactive routine for the dimension reduction of large QSAR data sets. *Quant. Struct.−Act. Relat.* **1989**, *8*, 103−108.
(59) Topliss, J. G.; Edwards, R. P. Chance factors in studies of quantitative structure-activity relationships. *J. Med. Chem.* **1979**, *22*, 1238−1244.
(60) Berthold, M. R.; Cebron, N.; Dill, F.; Gabriel, T. R.; Kötter, T.; Meinl, T.; Ohl, P.; Thiel, K.; Wiswedel, B. KNIME, the Konstanz information miner: version 2.0 and beyond. *SIGKDD Explorations* **2009**, *11*, 26−31.
(61) Hall, M.; Frank, E.; Holmes, G.; Pfahringer, B.; Reutemann, P.; Witten, I. H. The WKEA Data Mining Software: An Update. *SIGKDD Explor.* **2009**, *11*, 10−18.
(62) Hsu, C.-W.; Chang, C.-C.; Lin, C.-J. *A Practical Guide to Support Vector Classification*; National Taiwan University: Taipei, Taiwan, 2010; http://www.csie.ntu.edu.tw/~cjlin/papers/guide/guide.pdf (accessed Aug 19, 2011).