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Thiazolides as Novel Antiviral Agents: I. Inhibition of Hepatitis B Virus Replication

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Abstract

We report the syntheses and activities of a wide range of thiazolides [viz. 2-hydroxyaroyl-*N*-(thiazol-2-yl)amides] against hepatitis B virus replication, with QSAR analysis of our results. The prototypical thiazolide, nitazoxanide [2-hydroxybenzoyl-*N*-(5-nitrothiazol-2-yl)amide; NTZ] **1** is a *broad spectrum* antiinfective agent, effective against anaerobic bacteria, viruses and parasites. By contrast, 2-hydroxybenzoyl-*N*-(5-chlorothiazol-2-yl)amide **3** is a novel, potent and selective inhibitor of hepatitis B replication (EC_{50} = 0.33 μm) but is inactive against anaerobes. Several 4′- and 5′-substituted thiazolides show good activity against HBV; by contrast, some related salicyloylanilides show a narrower spectrum of activity. The ADME properties of **3** are similar to **1**, viz. the *O*-acetate is an effective prodrug and the *O*-aryl glucuronide is a major metabolite. The QSAR study shows a good correlation of observed EC_{90} s for intracellular virions with thiazolide structural parameters. Finally we discuss the mechanism of action of thiazolides in relation to the present results.

Introduction

Hepatitis B Virus

It is currently estimated that two billion people worldwide have been affected by hepatitis B virus (HBV) and that of these around 360 million are chronically affected.[1] The so-called Dane particle, observed in the blood of patients with hepatitis, was identified as the viral

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Supporting Information Available: Spectroscopic data for compounds **3-6**, **9-13**, **18-21**, **26-27**, **31**, **32**, **33** and **35-37** along with C, H and N analyses for selected examples. This material is available free of charge via the Internet at http://pubs.acs.org.

agent in 1970.[2] The virion is characterized by the surface antigen HBsAg, originally known as the 'Australia antigen', and related to HBV by the pioneering research of Blumberg.[3] While vaccination is often effective as a preventative measure,[4] treatment of patients with chronic HBV requires chemotherapy. Since HBV, like HIV, replicates via reverse transcriptase through an RNA template, and HBV is common in patients who develop AIDS, structurally related nucleoside analogues may have valuable activity against both these viruses: the HBV polymerase and the HIV-1 reverse transcriptase have significant homology.[5]

Indeed, the earliest chemotherapeutic agents for HBV were viral DNA polymerase inhibitors such as lamivudine, adefovir and entecavir. All these agents were effective, with sub-micromolar anti-HBV IC_{50} values, but as usual with such agents resistant strains quickly appeared.[6-8] HBV displays a particularly fast mutation rate (10^{10-11} point mutations per day in individuals with active replication)[9] and additionally cross-resistance has been noted between chemical agents from different structural groups.

Among other approaches, agents which target the encapsidation step (viz. assembly of viral RNA, polymerase and core into the nucleocapsid prior to replication) have been developed, notably heteroaryldihydropyrimidines[10] and phenopropenamides.[11] Since the HBsAg is a heavily glycosylated protein, inhibitors of the *N*-glycosylation pathway have also been studied[12] and found effective both *in vitro* and in an experimental *in vivo* infection model.[13] Finally, other nucleic acid-based approaches have been studied, notably antisense nuceotides[14] and gene therapy employing short interfering RNA (siRNA), which has shown promising results in model infection.[15] In summarizing new approaches to HBV therapy, including immune modulation, Loomba and Liang[16] commented that 'an improved understanding of virus-host interactions' was a key need in the development of new therapies. Indeed there is a clear need for effective new small molecule drugs as anti-HBV agents, ideally ones which would act through a mechanism not leading to rapid generation of resistant viral mutants. We now report that a series of thiazolide analogues [2-hydroxyaroyl-*N*-(thiazol-2-yl)amides] appear to fulfil these requirements.

Thiazolides

The aminothiazole derivative nitazoxanide **1** (Figure 1) was first developed as an antiparasistic agent, particularly indicated against *Cryptosporidium parvum*[17] and has been marketed in the USA since 2002. Indeed **1** is a broad-spectrum antiinfective agent, also active against anaerobic bacteria[18] and as an antiprotozoal[19] and anthelmintic agent. It was in the late 1990s that the antiviral activity of nitazoxanide was first noted,[20] during its use in the treatment of AIDS patients who had developed cryptosporidiosis. In most of its indications, nitazoxanide **1** behaves as a prodrug for the free phenol, tizoxanide **2** which is the effective circulating drug *in vivo*.

There is strong evidence that **1** owes its activity against anaerobic bacteria and parasites to inhibition of the enzyme pyruvate:ferredoxin reductase (PFOR),[21] whereby production of acetyl CoA within the anaerobe is prevented. Nevertheless, the nitro group is *not* directly involved (e. g. by reduction), nor is it essential for broader antiinfective activity. For instance, halothiazolides are also effective against the parasites *S. neurona* and *C. parvum*.[17(b), 22] We used nitazoxanide as the starting-point for a programme of synthesis and evaluation of a wide range of thiazolide analogues, particularly as selective antiviral agents. We have already presented data showing that nitazoxanide and a few close analogues are effective inhibitors of HBV and in some cases of hepatitis C virus (HCV) replication in cell cultures.[23] Further testing has revealed that **1** is active against a range of both DNA and RNA viruses, and it has been evaluated in clinical trials.[24]

In this paper we present a full account of the synthesis and structure-activity relationships of a wide range of thiazolides, plus some related salicyloyl anilides, against HBV. We also present a quantitative structure-activity relationship (QSAR) study, showing that the activities of thiazolides against HBV demonstrate excellent correlation for intracellular virions. Our results have led to the identification of 2-hydroxybenzoyl-*N*-(5-chlorothiazol-2-yl)amide **3** as a potent, selective anti-HBV agent. The *O*-glucuronide of **3**, the primary *in vivo* metabolite of this analogue, has been independently synthesized and evaluated.

Chemistry

Coupling Methods

The synthesis of nitazoxanide **1** and analogues (Tables 1 and 2) without further aryl substitution is generally achieved straightforwardly from commercially available acetylsalicyloyl chloride (Scheme 1, X = H) or a substituted salicyloyl chloride. In the case of nitazoxanide **1** itself,[25] anhydrous coupling with 2-amino-5-nitrothiazole (Et_3N-THF) is necessary in view of the amine's low nucleophilicity. More nucleophilic 2-aminothiazoles, e.g. 5-Br and 5-Cl (both commercially available), can be successfully coupled in a two-phase system (CH_2Cl_2/ aq. $NaHCO_3$); analogues **3-13**, Table 1, were made using one or other of these procedures. The fluoro analogue **14** required the prior synthesis of 2-amino-5-fluorothiazole (see below), followed by acylation as above. When using the two-phase acylation method, bis-acylated products of type **15** may also be isolated: their amounts decrease with time, in keeping with their base instability: indeed, the imides have proved more base-labile than the phenolic acetates. The imides are inactive under the assay conditions described.

Similarly compounds **16-22** and **26-28**, Table 2, were obtainable by coupling of the appropriate 2-aminothiazole with the appropriate acid chloride; 2-amino-4-phenylthiazole is also commercially available. Analogue **23** (v. i.) was prepared directly from nitazoxanide, and special chemistry was used for the sulfone analogues **24** and **25**: see below for these analogues. Finally, the salicyloylanilides **30-37** (Table 3) were made by acylation of the appropriate aniline with acetylsalicyloyl chloride; compounds **35** and **37** were commercially available.

For aryl-substituted analogues where the acid chloride is not commercially available, e. g. 3-methylsalicylic acid, standard acid-chloride forming conditions may be used from the *O*-acetyl acids [$(COCl)_2$/ cat. DMF or $SOCl_2$/ pyridine]. In general, carbodiimide-based methods lead to very sluggish reactions even when HOBt and/or DMAP are added. However, both BrPyBOP[26] and BOP-Cl[27] gave reasonable yields with 2-amino-4-phenylthiazole, using NMM as base, though the reactions were slow (3-4 days at 20°C). Both 2-acetoxy-3-methylbenzoic acid (see analogues **19** and **20**, Table 2) and 4-acetoxybenzoic acid could be coupled in this way: in the case of 2-acetoxy-3-chlorobenzoic acid, a reasonable yield was obtained using 3eq. of BOP-Cl for an extended period. These conditions are less satisfactory for 2-amino-5-bromo- or 2-amino-5-chlorothiazole, however, with generally slow reactions resulting: in general the rapid acid chloride method is preferred here. Carpino's reagent HATU[28] gives a good yield when coupling 2-amino-5-chlorothiazole with 2-acetoxy-3-methylbenzoic acid, but gives a very sluggish coupling between 2-amino-4-phenylthiazole and the same acid. In the Experimental section we have only described the acid chloride methods in detail.

Other Thiazoles

Earlier results suggested that the nitro group was essential for activity against anaerobes, via inhibition of PFOR (see above), but not for antiviral activity. It was therefore of interest to study the reduction of nitazoxanide **1**, Scheme 2. This was achieved using Raney Ni/H_2, as

reported for 2-acetamido-5-nitrothiazole.[29] The free 5-amino compound could not be isolated, but by performing the reaction in Ac_2O the diacetate **37** resulted in high yield; mild base-catalysed deprotection afforded the free phenol **23**. This compound was antivirally inactive.

To prepare the 5′-F analogue (Scheme 2), 2-acetamidothiazole **38** was reacted with excess of SelectFluor[30] in MeCN: optimum reaction (ca. 40% conversion to **39**) was achieved after about 3h, longer reaction leading to decomposition. Hydrolysis of the acetyl group afforded free amine **40**. After we had used this procedure, a report appeared confirming our result.[31] These authors, however, found that on a larger scale treatment of Boc-2-aminothiazole with two equivalents of Bu^tLi, followed by addition of *N*-fluorobenzenesulfonimide, then acidolysis with HCl, was a more reliable route to **40**. Coupling of **40** with acetylsalicyloyl chloride followed by standard deprotection gave the 5′-F analogue **14**.

Syntheses of the 4′- and 5′-sulfone analogues led to some interesting chemistry, Scheme 3. Heating the 5′-Br intermediate **41** with sodium sulfinate under CuI catalysis in DMF afforded a sulfone product in 37% yield. However, this product proved to be the 4′-sulfone **24** (concomitant deacetylation occurs) and *not* the 5′- isomer.[32] Several variations of this procedure, however, failed to repeat the result: complex mixtures of products resulted. Subsequent rigorous comparison of **24** and the 5′-sulfone **25**, prepared unambiguously as described below, using HPLC and $^1H/^{13}C$ NMR studies confirmed that they were indeed isomeric products. Their physical properties are almost identical but there are small characteristic differences in their ^{13}C NMR shifts and HPLC retention times.

A reasonable mechanism for the sulfinate displacement reaction involves addition at C(4′), followed by a hydride shift and loss of Br^-. In fact, the 5′-sulfone may be unambiguously prepared by displacement of Br^- from 2-amino-5-bromothiazole **42** using MeSNa, Scheme 3, giving 2-amino-5-methylthiothiazole **43**. Reaction of **43** with acetylsalicyloyl chloride, followed by peracid oxidation of the resulting 5′-SMe thiazolide **44**, then deprotection, afforded **25**. Rather surprisingly, both these analogues were inactive against HBV (the 5′-methylthio thiazolide intermediate was also inactive) though as we have reported[23] the 4′-sulfone **24** is a potent inhibitor of HCV.

To prepare the 5′-cyano- and 5-methoxycarbonyl analogues **26** and **27**, methyl 3-cyano- **45** and methyl 3-methoxyacrylate **46** (Scheme 4) were treated with NBS and the crude bromo products condensed directly with thiourea. After purification, the 2-amino-5- substituted thiazole products **47** and **48** were condensed with acetylsalicyloyl chloride to give **26** and **27** after deacetylation. Recently both **47** and **48** have become commercially available. A similar Hantzsch synthesis *via* the appropriate α-bromoaldehyde (Scheme 4) was used for the synthesis of the isopropyl and 4-chlorophenyl analogues **17** and **18** via the appropriate thiazoles **49** and **50**.

Further prodrugs

Although in general the *O*-acetyl derivatives serve as efficient prodrugs for the corresponding free phenols, we studied a range of other prodrugs made by acylation of tizoxanide **2**. The *O*-ethyl carbonate **29**, Table 2, is given as an example. A variety of other sterically bulkier prodrugs (not shown) were less effective in cell culture; nevertheless their potential utility in animal PK and *in vivo* studies is being pursued.

Deacetylation

In general, free phenols are used in the biological assays for hepatitis B although the *O*-acetates are equiactive. The acetates of the precursors may be cleaved using acid or base catalysis: we have generally employed mild heating in aq. HCl, as given in Scheme 1 but aq.

ammonia is also effective, as for the sulfone analogue **25**. Traditional Zemplen deprotection using catalytic methoxide is rather inefficient, probably because of the highly acidic NH proton.

Finally, the 5-trifluoromethyl analogue **28** was prepared (Scheme 5) from the αβ-unsaturated sulfone **51** which was itself obtained by a known procedure.[33] Base-catalysed epoxidation of **51**, using MCPBA- K_2CO_3 in MeCN rather than Bu^nLi-TBHP in THF as described, followed by treatment of the epoxide **52** with thiourea, afforded the desired 2-amino-5-trifluoromethylthiazole **53** in good yield.[34] Without further purification, **53** was coupled with acetylsalicyloyl chloride; the intermediate was deacetylated as described above (acid conditions) to afford the desired analogue **28**.

Glucuronide Metabolite

Once it was established that the *O*-aryl glucuronide was a general *in vivo* metabolite for this class, as already known for tizoxanide itself, it was important to prepare a reference sample of the glucuronide of the lead candidate **3**. The protected glucuronide of salicylic acid **54** prepared by us before[35] was condensed with 2-amino-5-chlorothiazole using EDCI and DMAP as catalyst, Scheme 6, giving a reasonable yield of the protected glucuronide **55**. It is important not to use too strong a base: when Et_3N was employed instead of DMAP, significant amounts of the unsaturated glucuronide **56** were obtained. This elimination is well known in the literature, [36] but stronger bases than Et_3N have generally been used. Finally, hydrolysis of **55** led to the free glucuronide **57** which was isolated as its sodium salt.

Results: Antiviral Activity

In general terms, the best activity against HBV was observed for thiazolides with electron-withdrawing groups at C(5′), especially 5′-nitro and 5′-halo, and this data, obtained in Hep G2 (2.2.15 cells),[37] is summarized in Table 1. Here the therapeutic (selectivity) index, SI, is shown for each analogue as a ratio of the cytotoxicity (CC_{50}) to efficacy (EC_{90}: the drug concentration at which a 10-fold depression of intracellular HBV DNA was observed relative to the average levels in untreated cultures). Both intracellular HBV replication and extracellular virus production were measured, using the virion (VIR) and replication intermediate (RI) assays, respectively.

Considering first tizoxanide **2** and other nitro analogues, introduction of a methyl group at positions 3, 4 or 5 (not shown) or halogenation at these positions led to 5- to 10-fold loss of activity and selectivity, as typified in compounds **4** and **5**. Ortho-substitution as in **4** did not have such a drastic effect as in the antiparasitic screens, where as we have noted[38] activity against a *Neospora* sp. was virtually abolished by a 3-Me group. In the 5′-bromo series, a reasonable level of activity, though at a lower level than the nitro compounds, was observed in compounds **6** to **10**. Here again a loss of activity for the 3-Me analogue **7** was noted: the 4- and 5-Me compounds (not shown) were superior but offered no advantage over **6**. The 3- and 5-chloro analogues **8** and **10** were rather more potent than **6**, but there were concerns over their reduced SI; by contrast, fluorinated analogues typified by **9** were significantly less active and demonstrated low selectivity.

The 5′-chloro analogues **3** and **11-13** showed significantly better activity than the bromo compounds and retained high SI values (as noted above, the direct 5′-Br analogues of **12** and **13** are not shown but were less active). Here the loss of activity in the 3-methyl analogue was more severe: compound **11** was essentially inactive, but both the unsubstituted compound **3** and the 5-methyl analogue **12** exhibited sub-micromolar EC_{90} values; the 4-methyl compound **13** was less active than **3** or **12**. Nominally the activity of **12** was slightly superior to **3**, but it later proved to have an unfavourable metabolic/toxicity profile, probably

owing to CYP oxidation to a reactive *p*-quinonemethide: this is discussed in more detail in the next section. The *p*-cresol structural element is indeed now regarded as a 'structural alert'.[39] The 5′-fluoro analogue **14** was significantly less active than **2**, **3** or **6**, a trend also noted in the activities of halo-analogues against *C. parvum* .[17(b)]

We screened a large number of other thiazolides, and the most interesting of these in structure-activity terms are summarized in Table 2. As stated above, nitazoxanide **1** is a prodrug of tizoxanide **2** and their activities are virtually identical in this assay: this may not be true under the conditions of all antiviral assays, depending on the local availability of esterases in each case. From a range of other possible prodrugs, the ethyl carbonate **29** was the most effective though its SI value appears less than that of **1**. The unsubstituted thiazole **16**[40] showed complete loss of HBV activity, and nonpolar, electronically neutral alkyl and aryl groups at C(5′) were also inactive, compounds **17** and **18**.

The 4′-phenyl analogues **19**, **20** and **21** were of interest. With no further substitution in ring A, compound **19**, a modest level of activity was seen but with a low selectivity index. Introduction of a methyl or chloro group at R_2, however, led to a significant improvement in both potency and selectivity as in compounds **20** and **21**. It would appear nevertheless that these compounds are significantly less effective against *intracellular* HBV RNA replication intermediates. Thus the ratio of efficacy EC_{50}(VIR) : EC_{50}(RI) at about 1:35 is much less than that seen with the 5′-chloro analogues, ca. 1:3 (compare compound **3**, Table 1, with compound **20**, Table 2). Replacement of the 2-hydroxy group by 4-hydroxy as in **22** [41] led to complete loss of HBV activity.

A range of other heteroatom and electron-withdrawing C(5′) substituents was also screened, compounds **23** to **28**. The cyano and trifluoromethyl analogues **26** and **28** retained slight activity but the 5-methoxycarbonyl analogue **27** was inactive, as were the acetamido analogue **23** and sulfonyl analogues **24** and **25**.

Finally, we prepared and screened a set of salicyloyl anilides and the activities of the most interesting, compounds **30**-**37**, are summarized in Table 3. In general the activity profile was more restricted than the thiazolides, and among monosubstituted analogues only the 8-bromo **32** and 8-iodo **33** analogues showed useful activity; here the 8-nitro compound **34** was inactive. Solubility problems are probably responsible for the lack of activity of compound **31** (cf. acetate analog **30**, which has low potency) and in these analogues the EC_{50}s of the *O*-acetates are probably more reliable. Among other analogues, the mono- and bis-trifluoromethyl derivatives **35** and **36** showed moderate activity and the trisubstituted **37** showed good activity. From a practical point of view, however, these molecules are close in structure to niclosamide (Table 3), which is inactive against HBV and has poor aqueous solubility and oral absorption. Additionally the scope for finding new compounds is much less, e. g. the free phenol form of **31** is known.[42]

In summary, the 5′-chloro analogue **3** was selected for further evaluation in view of its good activity against both extra- and intracellular HBV replication, EC_{90}: EC_{50} of about 3:1 and its good selectivity index (viz. low cell toxicity).

Drug absorption, distribution, pharmacokinetics and metabolism

Nitazoxanide **1** and the thiazolides generally are rather insoluble compounds and the free phenolic forms are highly protein bound (v. i.). Nevertheless, their log P values (e. g. clogP=1.24 for **1**) and other physicochemical parameters, notably H-bond donors (2) and acceptors (7) are favourable for oral absorption according to well-known guidelines.[43, 44] Indeed, nitazoxanide is well absorbed from the gastrointestinal tract: [45, 46] when administered after food, the oral bioavailability rises to about 50%. This behaviour contrasts

with the poor absorption of the related series of *N*-salicyloylanilides, typified by the anthelmintic agent niclosamide (Table 3). In clinical trials vs. hepatitis C, nitazoxanide is generally administered as two 500 mg tablets per day: recent clinical trial experience has helped to define the optimum dosing schedule.[47] Nitazoxanide, tizoxanide and the newer thiazolides are strongly bound to plasma proteins, typically >95%, but as we have reported[23] the consequent raising of EC_{90} does not prevent an effective therapeutic concentration being achieved.

In ADME/Tox studies, compound **12** was not an inhibitor of 14 different CYP isozymes and showed complete stability in human blood and plasma after a one hour incubation period. However it was found to be >99% protein bound and was extensively metabolized by human liver microsomes and S9 fraction, with only 16% and 6% of the parent compound remaining after a 60 minute incubation period. In rat and dog PK studies, **12** is not orally absorbed. For this reason, compound **3** is the preferred new generation thiazolide as it shows no adverse effects against a panel of CYPs, nor does it show unfavourable drug-drug interactions. Full details of the pharmacokinetic study will be published elsewhere.

Deacetylation of **1** by blood plasma esterases is rapid ($t_{1/2}$ 6 min) and subsequently tizoxanide **2** is extensively metabolized in the liver as its *O*-arylglucuronide **58**,[35] Figure 2, which is excreted in both urine and bile. This behaviour is also general for the new thiazolides. We have reported that **58** retains some moderate activity against anaerobic bacteria but it is essentially devoid of antiviral activity: [35] as noted above, the glucuronide **57** of compound **3** has also been independently synthesized as a standard. Pharmacokinetic studies for the new generation thiazolides, including prodrugs, are under way and the results will be reported elsewhere.

Quantitative Structure Activity Relationships

Correlation of EC_{90} RI values (viz. inhibition of replication within cells)

Using the data in Tables 1 to 3, quantitative structure activity relationships for the drug concentration required to reduce intracellular HBV DNA by 90%, pEC_{90} RI, were constructed with the GA-MLR method using autoscaled and filtered subset of the descriptor set generated by the Pipeline Pilot, [48] DRAGON [49] and CDK [50] descriptor generation software as described in the Experimental Methods section.

Recent guidelines recommend that a training set for QSAR model development must contain at least 10 data points/compounds[51] and other sources strongly recommend that an external test set should contain a minimum of 5 data points/compounds.[52] As the EC_{90} RI dataset contained 11 compounds there were not a sufficient number data points to split into a training and test set and still have an adequate number for either, therefore internal leave-one-out, leave-many-out and bootstrap internal validations were performed on the constructed models.

The quality of each model was evaluated by calculation of the coefficient of determination (r^2), the adjusted coefficient of determination (r^2_{adj}), mean absolute error (MAE), standard deviation of regression, F-value, average fold error, leave-one-out r^2 (r^2_{LOO}), 10-fold leave-many-out r^2 (r^2_{LMO}) averaged over 1000 runs and bootstrap r^2 (r^2_{BS}) averaged over 5000 runs. Table 5 shows the contributing terms and performance statistics for pEC_{90} RI QSAR models generated with the three different fitness functions.

The QSAR models using r^2_{adj} and r^2_{adj} in leave-one-out validation as the fitness function yielded the same model as did the best model using average r^2 for bootstrap validation as the fitness function. This convergence of the descriptors that were selected by different fitness

functions empirically indicates that this model is likely to be the best available with respect to this dataset and set of descriptors. The descriptors within this model are IDDE, the mean information content on the distance degree equality,[53] BEHp7, the highest Eigen-value n. 7 of the Burden matrix weighted by atomic polarisabilities BCUT descriptors[54] and ATSc3, the Moreau-Broto autocorrelation descriptors using partial charges. [55] Figure 3 shows a graph for predicted pEC_{90} RI against experimentally determined pEC_{90} RI for model01A: it can be seen that there are no outliers as the residuals are of the same magnitude for all predicted data points.

The performance measures (Table 4) indicate a robust and predictive model with most measures falling within the recommended guidelines.[56] In particular r^2 is greater than 0.7, mean absolute error is less than 0.1, r^2_{LOO} is greater than 0.5 and the r^2_{BS} internal validation performance parameter is > 0.5 which indicates a robust model.

Aside from giving an empirical model of pEC_{90} RI activity these descriptors are not readily physically interpretable, therefore a correlation was sought with other descriptors that are more physically interpretable. Descriptors with > 0.75 correlation with the above three descriptors were identified (Table 2, supplementary information), showing the identity of the descriptors as well as their correlation coefficient. For the BEHp7 descriptor which is negatively correlated with the activity, there are a number of physically interpretable descriptors that are highly positively correlated to it; these are number of atoms, number of bonds, number of non-hydrogen atoms, number of non-hydrogen bonds, number of rotatable bonds, rotatable bond fraction. This indicates a small, rigid molecule is more likely to have activity.

Correlation of EC_{90} VIR values (viz. inhibition of virions released from the cell)

Quantitative structure activity relationships for the drug concentration required to reduce extracellular HBV DNA by 90%, pEC_{90} VIR, were constructed with the GA-MLR method using autoscaled and filtered subset of the descriptor set generated by the Pipeline Pilot, DRAGON and CDK descriptor generation software as defined in the Methods section.

In analogy to the EC_{90} RI dataset the EC_{90} VIR dataset contained 13 compounds: therefore there were insufficient data points to split into training and test sets and still have an adequate number for either. Hence internal leave-one-out, leave-many-out and bootstrap internal validations was performed on the constructed models. Table 5 shows the contributing terms and performance statistics for pEC_{90} VIR QSAR models generated with the three different subjective fitness functions. All QSAR models constructed for the pEC_{90} VIR end point fall short of thresholds recommended for the mean absolute error and internal validation r^2 statistics. The performance of these models indicates that a robust model could not be constructed for the pEC_{90} VIR end point.

In summary, our SAR data were substantiated by a QSAR study based on the activities against intracellular virions which showed an excellent correlation. In the future we will aim to employ the QSAR to inform the molecular design of novel derivatives.

Discussion and mechanism of action

In summary, we have synthesized a range of thiazolides [2-hydroxyaroyl-*N*-(thiazol-2-yl)amides] together with some related salicyloyl anilides and screened them for inhibition of HBV replication. A number of the thiazolides exhibited sub micromolar EC_{50} values against both extracellular HBV virions and intracellular replication intermediates; the salicyloyl anilides were generally less potent. In general thiazolides with an electron-withdrawing substituent at C(5′), especially nitro and chloro, were most potent in this assay; substitution

in the phenyl ring had less effect, with a 3-methyl group generally causing loss of potency. The introduction of a 3-chloro substituent improved potency at the cost of selectivity. Other electron-withdrawing 5′-substituents were significantly less effective, as were 5′-alkyl or aryl groups. Our results were substantiated by a QSAR study based on the activities against intracellular virions which showed an excellent correlation.

Analogues with a 4′-phenyl group were of interest. Although the activity of the unsubstituted aryl analogue was modest, here the introduction of a 3-methyl or 3-chloro substituent dramatically improved activity, the likely drawback being a significant increase in log P value for this series. Finally a number of salicyloyl anilides, analogues of niclosamide, were screened. In general these compounds were less active, though it is interesting that here again analogues with electron-withdrawing substituents were most effective, particularly the *p*-iodo compound. The known poor oral absorption of this class of drug, in addition to the reduced scope for novelty, makes them less attractive for development than the thiazolides.

From the analogues described, 2-hydroxybenzoyl-*N*-(5-chlorothiazol-2-yl)amide **3** was selected for further development: in activity terms, this was very similar to the 5-methyl analogue **12**, but the latter proved to have an unfavourable metabolic profile. After scale-up, the pharmacokinetic behaviour of the two chloro analogues, as their acetate pro-drugs, will be determined in both rats and dogs and compared to that of nitazoxanide **1**. The results of these studies will be reported in due course.

The mechanism of action of the thiazolides is still under active investigation, but over the last few years some important findings have appeared. The inhibition of PFOR as a mechanism of action against anaerobes alluded to above[21] is postulated to be due to mimicry of TPP anion by the anion of nitazoxanide, without involving direct redox reactions of the nitro group. This does not explain, however, why other thiazolides with an acidic NH [viz. in particular, those with strong electron-withdrawing groups at C(5)] apparently lack significant anaerobic activity. A very recent publication[57] reported the activity of a number of analogues of **1**, including some other heterocycles, against anaerobic bacteria: here again activity was essentially confined to nitro analogues, including a dinitrothiophene amide. Also, it has been shown by affinity chromatography that **1** inhibits the action of a nitroreductase in *Giardia lamblia*.[58]

Turning to antiviral activity, studies in rotavirus have shown that nitazoxanide is cytoprotective,[24(a)] that is, it acts at a post-entry level, as shown specifically in MDCK cells infected with influenza A virus.[59] Thus when infected cells were treated with nitazoxanide between 0 and 6 h post-infection, viral replication was effectively inhibited and a single administration remained effective for up to 48h. Pretreatment of cells with **1** *prior* to infection, however, was not cytoprotective. In the same study, it was shown that **1** effects post-translational modification of haemagglutinin (HA), specifically by inhibition of the maturation of HA glycoprotein at a stage before resistance to endoglycosidase H digestion. However, it must be noted that, in the same studies **1** was not found to be an inhibitor of cellular glycosidation pathways.[59]

In the case of HBV, it is known that **1** induces reductions in key HBV proteins, especially the surface antigen HBsAg, also HBeAg and HBcAg,[23] and in view of the observation with HA it is probably significant that all these proteins are heavily glycosylated. However, there is no effect of **1** on levels of HBV RNA transcription, consistent with a post-translational mechanism. The difference in mechanism is valuable in that **1** has been shown to be synergistic with lamivudine and adefovir against HBV, and indeed it maintains equivalent activity against HBV strains resistant to those agents.[23]

Recent studies including clinical trial results have shown that **1** is active against different genotypes of the Hepatitis C virus (HCV), and is also synergistic with interferon (with and without ribavirin), telaprevir (an HCV protease inhibitor) and 2′C-methylcytidine (an HCV RNA polymerase inhibitor).[47, 60] In the most recent trials, Phase II clinical studies carried out in 511 experienced and naïve patients with chronic hepatitis C Genotype 1 (240 subjects) and 4 (271 subjects) combined with pegylated interferon-alpha 2a (Pegasys ®) with or without ribavirin showed that NTZ **1** added a 40-50% increase in the SVR rate of the standard of care combining pegylated interferon-alpha 2a and ribavirin.[61]

Here too, equivalent activity is maintained by **1** against representative drug-resistant strains of HCV.[60] HCV replicon-containing cell lines resistant to **1** have been isolated, but the resistance phenotype was not found to be transferred to naive cells by HCV replicons isolated from these cell lines, indicating that a cellular mechanism is most likely responsible for this property. [62] In other studies in HCV containing cell lines, **1** was observed to enhance the phosphorylation of eukaryotic initiation factor-2 α (eIF2α).[63] In the same report, **1** was found capable of enhancing the activity of PKR in enzymatic assays, a protein kinase activated by double-stranded RNA that is part of innate cell defense mechanisms and activates eIF2α via phosphorylation.[63] The weight of evidence therefore supports a mechanism involving stimulation of the host cell immune system. Most recently, it was discovered that in peripheral blood mononuclear cells from human volunteers thiazolides showed potent immuno-modulating effect of both the innate and adaptive immune systems.[64]

In summary, it may be said that the mechanism of antiinfective action of nitazoxanide and other thiazolides is complex, and most likely involves more than one pathway: the complete elucidation of the mechanism is a work in progress. In the case of antiviral activity, an increasing body of evidence is consistent with a post-translational, host cell-mediated effect which may either stimulate innate cell defence processes or inhibit maturation of key viral proteins. What is beyond doubt is that the thiazolides are highly effective antiviral agents both *in vitro* and *in vivo*, as we have demonstrated here for HBV, and in further publications we shall report on their structure-activity properties against other important viruses.

Experimental Section

Chemical Procedures

Organic extracts were washed finally with satd. aq. 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, ^1H and ^{13}C NMR spectra were recorded on $CDCl_3$ solutions using either Bruker 250 or 400 MHz (100 MHz for 13C) 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 analysed 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.

Antiviral assays were performed as described previously:[37, 65] in summary, confluent cultures of the chronically HBV-producing cell line, 2.2.15, were maintained on 96-well flat-bottomed tissue culture plates (confluence in this culture system is required for active, high levels of HBV replication equivalent to that observed in chronically-infected individuals[37, 65]). Cultures were treated with nine consecutive daily doses of the test compounds. HBV DNA levels were assessed by quantitative blot hybridization 24 hr. after

the last treatment. Cytotoxicity was assessed by uptake of neutral red dye 24 hr. following the last treatment.

General procedures for acid chloride couplings and *O*-acetate deprotections

Acetylsalicyloyl chloride was used directly; substituted versions were made from the *O*-acetates as indicated below.

1. Two-phase method—To a stirred solution of a protected salicylic acid (1 mmol) in dry Et_2O (10 mL) at 0° C was added pyridine (1.2 mmol) followed by dropwise addition of thionyl chloride (1.2 mmol). Stirring was continued at 0° C for 4 hrs, then the white precipitate formed was filtered off and the filtrate was concentrated under vacuum to give the acid chloride as an oil which was used without further purification. The appropriate 2-aminothiazole (1 mmol) was added to a vigorously stirred two-phase mixture of $NaHCO_3$ (3 mmol for a free base, 4 mmol for an HCl or HBr salt) in H_2O (3 mL per mmol) and EtOAc (3 mL per mmol). A solution of the above acid chloride in EtOAc (2 ml per mmol) was then added with vigorous stirring. The reaction mixture was stirred at 20°C for 12 h. The layers were separated and the aqueous layer was extracted once with EtOAc. The combined organic extracts were washed with 0.5 N HCl (2x), followed by brine. The organic layer was dried and concentrated under vacuum to give a pale yellow solid which was chromatographed on silica, eluting with EtOAc-hexane mixtures; in favourable cases trituration of crude product with Et_2O followed by drying the resulting solid gave the *O*-acetyl intermediate directly. This material in conc. was heated in conc. aq. HCl (3ml per mmol) at 50° C for 24 hrs. The reaction mixture was cooled to ambient temp., then filtered and the solid washed with H_2O (dist.) until the washings were at neutral pH. The solid was dried under vacuum to give the product. On a small scale it was more efficient to extract the final product into EtOAc, followed by washing with H_2O (3x) and brine, then drying and evaporation. Chromatography of the final product, if necessary, was again carried out using EtOAc-hexane mixtures.

2-Hydroxy-3-methylbenzoyl-*N*-(5-bromothiazol-2-yl) amide (7) (75 mg, 66%): Mp 187-188°C; Anal. ($C_{11}H_9BrN_2O_2S$) C, H, N; ^{1}H NMR [400 MHz, $(CD_3)_2SO$] 2.21 (3H, s, CH_3Ar), 6.86 (1H, d, J = 7.7 Hz, ArH), 7.39 (1 H, d, J = 7.2 Hz, ArH), 7.73 (1 H, s, 4′-H) and 7.95 (1 H, d, J = 7.8 Hz, ArH); ^{13}C NMR [100 MHz$(CD_3)_2SO$] 15.6, 101.9, 114.3, 118.8, 126.2, 126.6, 135.7, 136.3, 158.4, 159.9 and 168.8; MS (CI) m/z 313 and 315 (M^+ for ^{79}Br, ^{81}Br respectively); HRMS, found, m/z 312.9641, $C_{11}H_{10}BrN_2O_2S$ (MH^+ for ^{79}Br) requires m/z, 312.9646.

2. Anhydrous conditions—Either the free amine was used directly, or the HBr or HCl salt of the appropriate 2-aminothiazole was partitioned between dil. aq. NaOH and EtOAc, then the organic layer was separated, dried and evaporated to dryness. A solution of the thiazole (1 mmol) in THF (3 mL) was added to a stirred solution of salicyloyl chloride (1 eq.) in THF (3 mL per mmol) at 0°C before the addition of Et_3N (1 eq.). The solution was stirred at room temperature until reaction was complete, then the reaction mixture was poured into water and extracted with ethyl acetate (2 x). The organic layer was washed with 1M HCl, water, dried and evaporated. The product was purified by column chromatography to give the intermediate *O*-acetate, which was hydrolysed as above to deliver the product.

2-Hydroxybenzoyl-*N*-(5-chlorothiazol-2-yl) amide (3): Mp 227-228°C (dec.); ^{1}H NMR [500 MHz, $(CD_3)_2SO$] 7.00 (1 H, t, ArH), 7.04 (1 H, d, ArH), 7.48 (1 H, t, 4-H), 7.60 (1 H, s, 4′-H) and 7.96 (1 H, d, 6-H); ^{13}C NMR [125 MHz, $(CD_3)_2SO$] 116.5, 117.1, 118.5, 119.7, 130.3, 134.6, 135.4, 155.8, 157.3 and 164.7; m/z (ES +ve ion mode) 277 (MNa^+, 100%); Found: m/z, 276.9806; $C_{10}H_7{}^{35}ClN_2O_2SNa$ requires m/z, 276.9809.

3-Chloro-2-hydroxybenzoyl-*N*-(5-bromothiazol-2-yl)amide (8) (0.116g, 48%): Mp 200°C. Found: m/z, 332.90930. $C_{10}H_7BrClN_2O_2S$ (MH^+) requires m/z, 332.91003. ^1H NMR [400 MHz, $(CD_3)_2SO$] 7.02 (1 H, t, J = 7.9 Hz, ArH), 7.69 (1 H, dd, J = 7.9 and 1.4 Hz, ArH), 7.84 (1 H, s, 4′-H) and 8.02 (1 H, dd, J = 7.9 and 1.4 Hz, ArH); m/z (CI, NH_3) 333 (MH^+, 35%).

2-Hydroxy-5-methylbenzoyl-*N*-(5-chlorothiazol-2-yl) amide (12): Mp 212-213°C; Anal. ($C_{11}H9ClN_2O_2S$) C, H, N; ^1H NMR [$(CD_3)_2SO$] 2.27 (3 H, s, CH_3Ar), 6.94 (1 H, d, J = 8.3 Hz, ArH), 7.29 (1 H, d, J = 8.1 Hz, ArH), 7.59 (1 H, s, 4′-H) and 7.78 (1 H, s, ArH); ^{13}C NMR [$(CD_3)_2SO$] 19.9, 99.1, 115.7, 117.0, 118.5, 128.5, 130.1, 135.3, 135.7, 155.0 and 164.1; MS (CI) m/z 269, 271 (MH^+ for ^{35}Cl, ^{37}Cl respectively); HRMS, found, m/z 269.0149, $C_{11}H_{10}ClN_2O_2S$ (MH^+ for ^{35}Cl) requires m/z, 269.0151.

2-Hydroxy-3-methylbenzoyl-*N*-(4-phenylthiazol-2-yl)amide (20): Mp 180°C; ^1H NMR (400 MHz, $CDCl_3$) 7.94 (1H, d, *J* = 8.1, ArH), 7.83 (2H, d, *J* = 8.5, 2 × ArH), 7.52-7.41 (4H, m, ArH), 7.2 (1H, s, ArH), 6.69 (1H, t, *J* = 7.7, ArH), 2.31 (3H, s, CH_3); ^{13}C NMR [125 MHz, ($CDCl_3$] 15.8, 108.4, 112.0, 118.7, 123.2, 126.0, 128.2, 128.8, 134.1, 136.4, 150.2, 157.2, 160.4 and 167.7; *m/z* (CI) 311 (35%, $[M+H]^+$); (ES +ve ion mode) 333 (MNa^+, 100%); Found: m/z, 333.0659; $C_{17}H_{14}N_2O_2SNa$ requires m/z, 333.0668.

See Supporting Information for the characterisation of all other analogues made using either of the above methods. The thiazole precursors of compounds **17** and **18** are known.[66, 67]

Acetate deprotection, basic conditions. Mild base hydrolysis using aq. ammonia is also used for deacetylation, as in the following example.

2-Hydroxybenzoyl-*N*-(5-acetamidothiazol-2-yl)amide (23): To a solution of nitazoxanide **1** (615 mg, 2.0 mmol) in acetic anhydride (~70 cm^3), Raney Ni was added with vigorous stirring. The reaction mixture was evacuated and twice refilled with hydrogen, then allowed to stir at room temperature under the atmosphere of hydrogen until the theoretical volume (~135 cm^3, 6.0 mmol) was consumed after 30 minutes. The reaction mixture was stirred for a few minutes under atmosphere to allow the escape of hydrogen present in flask. The solution was filtered through a sinter followed by the evaporation of solvent *in vacuo*. The crude product was re-dissolved in EtOAc (50 cm^3) and washed with excess of satd. aq. $NaHCO_3$. The organic fraction was again evaporated and the crude product was dissolved in acetone (10 cm^3) and stirred for two hours with 1N HCl (5 cm^3) to hydrolyse over-acetylated material. The reaction was neutralized with satd. aq. $NaHCO_3$ and extracted with EtOAc (4 × 20 cm^3). The combined organic fractions were evaporated and the *O*-acetate of **23** was purified through flash column chromatography as white amorphous solid (548 mg, 86%), mp 176.5°C, ^1H NMR (400 MHz, $CDCl_3$) 2.08 (3H, s, CH_3), 2.25 (3H, s, CH_3), 7.16 (1H, s, CH), 7.26 (1H, dd, *J* 0.6, 8.1 Hz, ArH), 7.39 (1H, td, *J* 1.0, 7.6 Hz, ArH), 7.60 (1H, td, *J* 1.6, 8.0 Hz, ArH), 7.75 (1H, dd, *J* 1.6, 7.6 Hz, ArH), 11.0 (1H, s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) 21.1, 22.6, 123.7, 126.1, 127.3, 130.0, 131.0, 132.7, 148.8, 166.8, 169.1, 169.4, 184.5, 201.8; m/z EI $[M + Na]^+$ 342 $[(M + Na)^+$, 100%]; Found: m/z, 342.0524; $C_{14}H_{13}N_3O_4NaS$ requires 342.0538. To a solution of this *O*-acetate (319 mg, 1.0 mmol) in acetone (5 cm^3), 20 cm^3 of aq. NH_3 was added, then the mixture was stirred overnight at room temperature, followed by evaporation. The crude mixture was dissolved in EtOAc (20 mL) and was washed with 1M HCl. The desired product **23** was purified through flash column chromatography as a white solid (0.25g, 90%); ^1H NMR (400 MHz, $CDCl_3$) 2.10 (3H, s, CH_3), 6.98 (1H, t, *J* 7.0 Hz, ArH), 7.03 (1H, d, *J* 8.0 Hz, ArH), 7.18 (1H, s, ArH), 7.45 (1H, td, *J* 1.75, 7.0 Hz, ArH), 8.01 (1H, dd, *J* 1.75, 8.0 Hz, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) 22.7, 117.3, 117.5, 119.8, 130.5, 130.7, 134.5, 158.2, 167.0; m/z CI $[M + H]^+$ 278, [278, 100%]; Found: m/z, 278.05983; $C_{12}H_{12}N_3O_3S$ requires 278.05994.

Ethyl 2-[(5-nitro-1,3-thiazol-2-yl)carbamoyl]phenyl carbonate (29): A solution of ethyl chloroformate (0.114 g, 1.11 mmol) in dry THF (3 mL) was added to a bright yellow solution of tizoxanide **2** (0.250 g, 0.942 mmol) and Et_3N (0.118 g, 1.16 mmol) in dry THF (6 mL), yielding a white precipitate. The reaction was stirred at 20°C for 6 hours, then concentrated *in vacuo* and the residue was partitioned between water and dichloromethane. The organic layer was washed with water (2x), then with brine, dried and concentrated to give crude **14** (0.343 g, >100%) as a yellow solid. The crude product was purified by chromatography using a gradient of 0- 50% ethyl acetate in hexane. Appropriate fractions were combined and evaporated to give **29** (0.254 g, 80%) as a tan solid, mp 159-161 °C; HPLC area purity 100% (conditions C). ^{1}H NMR [400 MHz, $(CD_3)_2SO$] δ 1.25 (t, *J* = 7 Hz, 3H), 4.22 (q, *J* = 7 Hz, 2H), 7.43 (dd, *J* = 1, 8 Hz, 1H), 7.48 (td, *J* = 1, 8 Hz, 1H), 7.71 (td, *J* = 2, 8 Hz, 1H), 7.88 (dd, *J* = 2, 8 Hz, 1H), 8.71 (s, 1H) and 13.69 (br s, 1H); MS (ES +ve mode) *m/z* 360.0 $(M+Na)^+$, 338.0 $(M+H)^+$ and (ES −ve mode) *m/z* 336.1 $(M-H)^-$.

Methyl 1-[2-*N*-(5-Chlorothiazol-2-yl)carboxamido]phenyl-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate (55): Methyl 1-(2-carboxyphenyl)-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate **54**[35] (0.45 g, 1 mmol) was dissolved with 2-amino-5-chlorothiazole, hydrochloride (0.17 g, 1 mmol), DMAP (0.12 g, 1 mmol) and anhydrous 1-hydroxybenzotriazole (0.14 g, 1 mmol) in anhydrous CH_2Cl_2 (5 mL). The clear solution was stirred and cooled to 0°C, then *N*-methylmorpholine (0.11 mL, 1 mmol) and EDCI (0.19 g, 1 mmol) were added. The pale yellow solution was stored at 0°C for 72 h, then diluted with EtOAc (30 mL) and washed with 5% aq. citric acid (25 mL), backwashing with EtOAc, then the combined organic phases were washed with satd. aq. $NaHCO_3$, water and brine, dried and evaporated to crude product which was purified by chromatography, eluting first with a gradient of 30-100% EtOAc in hexane, then with 10% MeOH-$CHCl_3$. Appropriate fractions were combined and evaporated to give reasonably pure product as a pale yellow solid (0.31g, 54%) which was recrystallised from CH_2Cl_2-EtOH-hexane to give **55** as off-white crystals (0.169 g, homogeneous by TLC); concentration gave a second crop (0.032g) also of good purity; mp 245-246.5°C dec.; ^{1}H NMR [500 MHz, $(CD_3)_2SO$] 1.91, 1.95, 2.03 (9 H, 3s, 3×CH_3CO), 3.66 (3 H, s, CH_3O), 4.77 (1 H, d, J = 10.0 Hz, 5′-H), 5.00-5.10 (2 H, m) and 5.48 (1 H, m, 2′-H + 3′-H + 4′-H), 5.66 (1 H, d, J = 7.9 Hz, 1′-H), 7.18-7.25 (2 H, m, ArH), 7.50-7.60 (2 H, m, ArH), 7.58 (1 H, s, thiazole 4-H) and 12.6 (1 H, br s, NH); ^{13}C NMR [125 MHz, $(CD_3)_2SO$] 20.2, 20.3 (x2), 52.6, 68.9, 69.9, 70.9 (x2), 97.7, 115.4, 118.2, 122.9, 124.8, 128.9, 132.3, 135.7, 153.4, 155.7, 164.8, 167.0, 168.6 and 169.3(x2); m/z (ES +ve mode) 593 (MNa+, 100%); Found: m/z, 593.0603. $C_{23}H_{23}ClN_2O_{11}S$ Na requires m/z, 593.0603.

Any traces of elimination by-product **56** were distinguished by: ^{1}H NMR [400 MHz, $CDCl_3$] 2.02, 2.13 (6 H, 2s, 2×CH_3CO), 3.76 (3 H, s, CH_3O), 5.40 (1 H, m), 5.44 (1 H, m), 6.10 (1 H, brs), 6.42 (1 H, m), 7.26 (1 H, s, thiazole 4-H), 7.32, 7.46, 7.62 and 8.13 (4 H, 4m, ArH). See also reference 33.

1-[2-*N*-(5-Chlorothiazol-2-yl)carboxamido]phenyl-β-D-glucopyranosiduronic Acid (57): The ester **55** (0.159 g, 0.28 mmol) suspended in MeOH (1.5 mL) was stirred at 0°C with 2.5 M NaOH (0.56 mL, added dropwise). After 2 h, allowing the reaction to regain ambient temperature, reaction appeared complete by TLC and glacial AcOH was added dropwise to achieve a pH of 5.6, then EtOH (4 mL) was added and the mixture cooled to 0°C. The resulting light beige solid was filtered, washed with ether and dried to give substantially pure product (0.113 g, 90%); analytical material was obtained by chromatography on Lichroprep (Merck), eluting with a gradient of 0-60% MeCN in H_2O. Appropriate fractions were combined and evaporated to give off-white solid which was triturated with a few drops of moist EtOH and excess ether to afford the product, which was filtered, washed with ether and dried to give highly pure **57** (0.066g) as Na salt; ^{1}H NMR

[400 MHz, $(CD_3)_2SO + D_2O$] 3.25-3.40 (3 H, m, 2′-H + 3′-H + 4′-H), 3.65 (1 H, m, 5′-H), 5.15 (1 H, m, 1′-H), 7.22 (1 H, m, ArH), 7.41 (1 h, m, ArH), 7.59 (1 H, s, thiazole 4′-H), 7.61 (1 H, m, Ar H) and 7.84 (1 H, m, ArH); ^{13}C NMR[125 MHz, $(CD_3)_2SO + D_2O$] 71.7, 73.3, 74.4, 76.1, 101.4, 116.6, 118.5, 121.6, 122.7, 130.8, 134.0, 135.9, 155.3, 155.6, 163.7 and 171.1; m/z (ES +ve mode) 452 (100%, M^+ for Na salt); HPLC analytical purity 98.4% at 280 nm (C_{18} reverse-phase column, MeCN-H_2O); m/z (ES +ve mode) 475 (100%); Found: m/z, 474.9945. $C_{16}H_{14}ClN_2O_8SNa_2$ requires m/z, 474.9949.

Quantitative Structure Activity Relationship Methods

In order to assist in analysing and interpreting the structure-activity relationships (SAR) associated with the 5′-nitro and 5′-halothiazolide compounds quantitative structure activity relationship (QSAR) models were developed for the some of the biological endpoints/ activities against HBV replication.

QSAR models were developed and validated for data concerning the drug concentration required to reduce intracellular HBV DNA by 90% (EC_{90} RI) and the drug concentration required to reduce extracellular HBV DNA by 90% (EC_{90} VIR). These data were chosen for their amenability towards modelling in terms of range of values and distribution of data (*vide infra*). The EC_{90} RI dataset contains 11 compounds and the EC90 VIR dataset contains 13 compounds (see Tables 4 and 5). The range for the range the EC_{90} RI is from 1.20 to 22.00 M whilst the EC_{90} VIR data spans from 0.58 to 12.0 M; both datasets are spread relatively evenly. The biological end points for these assays are reported in M; for the development of QSAR models, however, the activities were converted to pEC_{90} values in M ($pEC_{90} = -\log_{10}(EC_{90}/10^6)$). These end-points offer the greatest range of activity of the biological end points for datasets containing low-micromolar or sub-micromolar activities. Given the above characteristics these datasets provide were subject to QSAR analysis.

In total 685 0, 1 and 2-dimensional molecular descriptors/properties were calculated for the set of compounds using Pipeline Pilot Student Edition,[48] DRAGON Web Version 3.0 [49] and CDK.[50]

The combined descriptor set of 685 variables was autoscaled and filtered using two objective selection methods. Firstly descriptors that had the same value for 80% of the dataset were removed as these contained minimal information: this left 554 and 557 descriptors for the EC_{90} RI and EC_{90} VIR datasets respectively. Secondly the CHORCHOP procedure[68 (a), (b)] was used to eliminate one of a pair of descriptors that exhibited very high inter-correlation ($r > 0.99$). The procedure removed the descriptor whose distribution deviated the most from normal (as defined by maximum kurtosis 100). This left 37 descriptors for the EC_{90} RI dataset and 46 descriptors for the EC_{90} VIR dataset.

The multiple linear regression machine learning method coupled with genetic algorithm subjective descriptor selection (GA-MLR) as implemented in the PHAKISO program was used to relate the activities (Y) of a set compounds to their molecular descriptors (X) using a linear equation.[69, 70]

The genetic algorithm was set to have population size 50, replacement rate 0.6, cross-over rate 1.0 and maximum number of generations 100. The maximum number of descriptors allowed was set to 3, in order to follow the recommended 5:1 to ratio of number of descriptors to molecule so minimise the occurrence of chance correlations. The subjective fitness function for descriptor selection in this case was chosen to be either the highest adjusted r^2 for the training set, leave-one-out validation or bootstrap validation (averaged of 1000 repeats each validation). For the bootstrap method a number of descriptor selection runs were performed and the best model of the multiple runs selected. QSAR models were

developed using all three adjusted R^2 criteria as a subjective fitness function with the model that gave the best performance for training set and internal validation tests was selected for further analysis if minimum performance threshold are met.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

BOP-Cl	bis(2-oxo-3-oxazolidinyl)phosphonic chloride
GA-MLR	genetic algorithm and multiple linear regression
HATU	*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*’,*N*’-tetramethyluronium hexafluorophosphate
HBsAg	hepatitis B surface antigen
NMM	*N*-methylmorpholine
NTZ	nitazoxanide [2-hydroxybenzoyl-*N*-(5-nitrothiazol-2-yl)amide]
PFOR	pyruvate ferredoxin reductase
PyBroP®	bromotripyrrolidinophosphonium hexafluorophosphate
QSAR	quantitative structure-activity relationship(s)
SelectFluor®	1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis (tetrafluoroborate)

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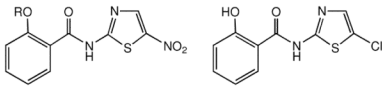
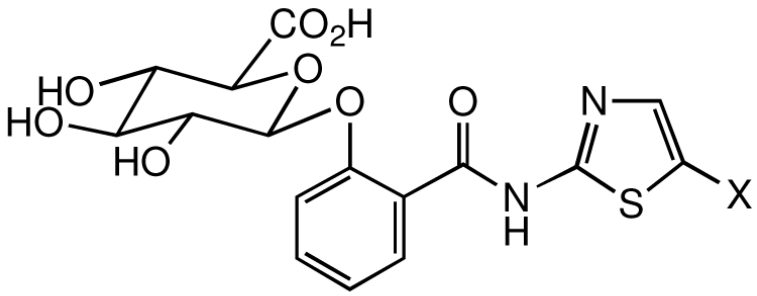


1 R = Ac, **2** R = H **3**

Figure 1.
Basic thiazolide structures **1-3**.



57 X = Cl

58 X = NO_2

Figure 2.
Thiazolide *O*-glucuronides.

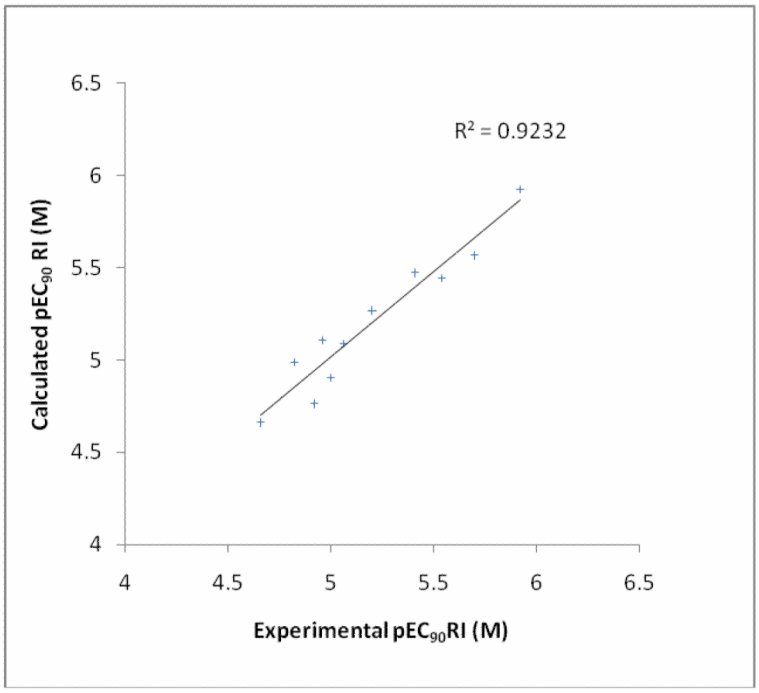
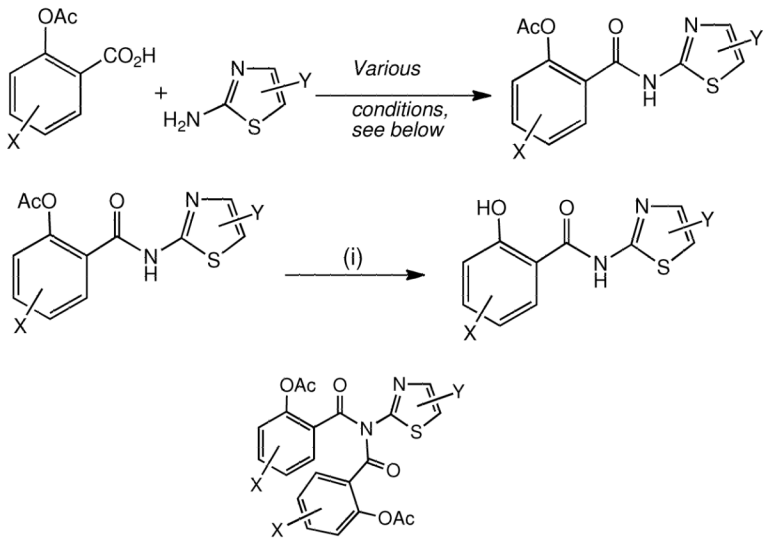


Figure 3.
Predicted vs Experimental pEC_{90} RI as predicted by the QSAR model01A for pEC_{90} RI.

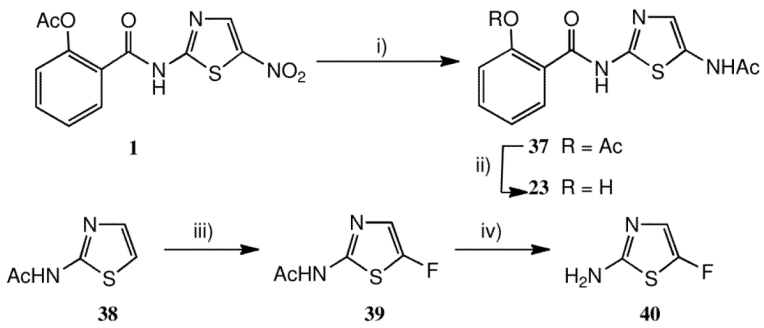


15

Scheme 1.

[a] General synthetic procedures for thiazolides.

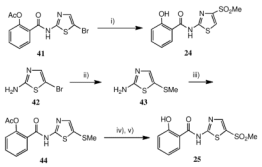
[a] i) *Coupling step*: Carboxylic acid reacted with $SOCl_2$/ pyridine or $(COCl)_2$/ DMF, DCM, then *either* add acid Cl to amine in aq. $NaHCO_3$− organic solvent with stirring, *or* add to amine in dry THF with Et_3N: *or* BrPyBOP or BOP-Cl, NMM, CH_2Cl_2; *deprotection*: aq. HCl, 60°C or aq. NH_3.



Scheme 2.

[a] Syntheses of 5′-acetamido and 5′-fluorothiazolides

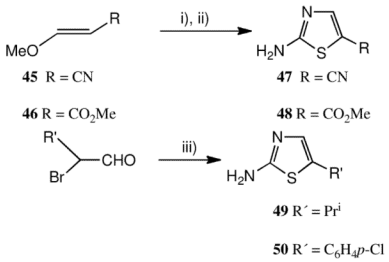
[a] i) H_2-Raney Ni, Ac_2O; ii) aq. NH_3; iii) SelectFluor, MeCN, heat; iv) aq. NaOH/EtOH.



Scheme 3.

[a] Syntheses of 4′-and 5′- methanesulfonylthiazolides

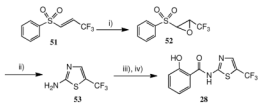
[a] i) $MeSO^2Na$, CuI, DMF, heat; ii) MeSNa, EtOH; iii) acetylsalicyloyl chloride; iv) 3-$ClC_6H_4CO_3H$ (2 eq.); v) aq. NH^3.



Scheme 4.

[a] Syntheses of 5′- aryl, alkyl, cyano and methoxycarbonylthiazolides.

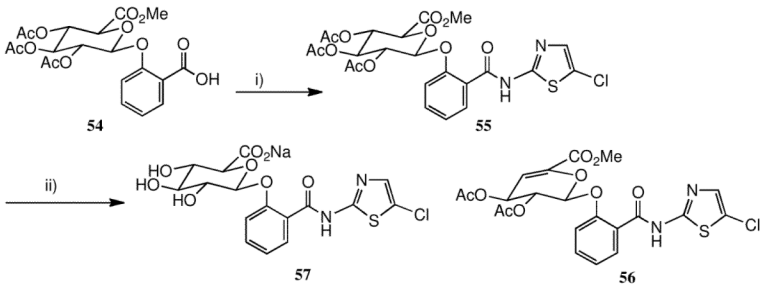
[a] i) *N*-bromosuccinimide, MeOH; ii), iii) $(H_2N)_2C{=}S$.



Scheme 5.

[a] Synthesis of 5′-trifluoromethylthiazolide **28**.

a) i) MCPBA, K_2CO_3, CH_3CN, RT to 40 ^{0}C; ii) $NH_2C(=S)NH_2$ (2 equiv.) DMF, 75 ^{0}C; iii) Acetylsalicyloyl chloride, Et_3N, CH_2Cl_2; iv) HCl, H_2O, THF, 50 ^{0}C



Scheme 6.

[a] Synthesis of the *O*-glucuronide of **3**.

[a] i) 2-amino-5-chlorothiazole hydrochloride, NMM, EDCI, DMAP, HOBt, CH_2Cl_2; ii) NaOH, aq. MeOH, then pH 6.

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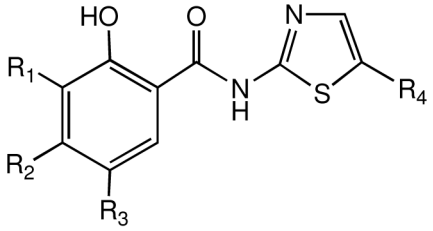
Table 1

Activities of 5′-nitro and 5′-halothiazolides against HBV replication.



Compound	R_1	R_2	R_3	R_4	CC_{50}[a] µM	EC_{50}[b] (VIR) µM	EC_{50}[c] (RI) µM	EC_{90}[d] (VIR) µM	EC_{90}[e] (RI)µM	SI[f] (VIR)	SI[g] (RI)
2	H	H	H	No_2	>100	0.15	0.46	0.58	1.20	>172	>83
3	H	H	H	Cl	38	0.33	1.00	0.89	2.90	43	13
4	Me	H	H	No_2	85	0.64	2.10	3.5	12.0	24	7.1
5	H	Me	H	No_2	>100	1.30	4.20	6.90	15.0	>14	>6.7
6	H	H	H	Br	>100	1.20	2.90	4.00	8.7	>25.0	>12.0
7	Me	H	H	Br	>100	3.50	7.60	9.0	22.0	>11	>4.5
8	Cl	H	H	Br	30.0	0.32	0.59	2.00	3.90	15	7.7
9	H	F	H	Br	12.0	2.10		5.60		2.1	
10	H	H	Cl	Br	45.0	0.21	3.50	1.10	11.0	41	4.1
11	Me	H	H	Cl	100	>10.0	>10.0	>10.0	>10.0	-	-
12	H	H	Me	Cl	100	0.33	0.90	0.83	2.0	>120	>51
13	H	Me	H	Cl	100	1.00	2.70	3.30	6.3	>30	>16
14	H	H	H	F	51	3.1	-	12.0	-	4.3	-

[a] Drug concentration at which a two-fold lower level of neutral red dye uptake is observed relative to average level in untreated cultures.[34]

[b] Drug concentration required to reduce extracellular (virion) HBV DNA by 50% relative to untreated cells. All EC_{50} and EC_{90} figures were determined in triplicate, with standard deviations ±20% of the figure quoted.

[c] Drug concentration required to reduce intracellular HBV DNA by 50%.

[d] Drug concentration required to reduce extracellular (virion) HBV DNA by 90%

[e] Drug concentration required to reduce intracellular HBV DNA by 90%

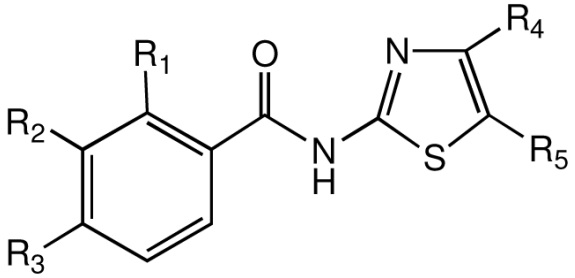
[f] Selectivity index: CC_{50}/EC_{90} for extracellular HBV

[g] Selectivity index: CC_{50}/EC_{90} for intracellular HBV.

J Med Chem. Author manuscript; available in PMC 2012 June 23.

Table 2

Activities of other thiazolides including prodrugs against HBV replication.

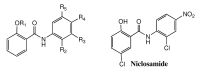


Compound	R_1	R_2	R_3	R_4	R_5	CC_{50}[a] µM	EC_{50} (VIR) µM	EC_{50} (RI) µM	EC_{90} (VIR) µM	EC_{90} (RI)µM	SI (VIR)	SI (RI)
1	AcO	H	H	H	NO_2	>100	0.12	0.59	0.83	2.10	>121	>48
16	OH	H	H	H	H	16.0	>10.0		>10.0			
17	OH	H	H	H	i-Pr	>100	>10.0	>10.0	>10.0	>10.0	-	-
18	OH	H	H	H	C_6H_4Cl	>100	>10.0	>10.0	>10.0	>10.0	-	-
19	OH	H	H	Ph	H	22.0	5.60		21.0		1.0	-
20	OH	Me	H	Ph	H	>100	0.22	7.80	0.73	30.0	>137	3.3
21	OH	Cl	H	Ph	H	>100	0.15		0.60		>166	
22	H	H	OH	H	NO_2	>100	>10.0		>10.0			
23	OH	H	H	H	NHAc	>100	>10.0	>10.0	>10.0	>10.0		
24	OH	H	H	SO_2Me	H	>100	>10.0		>10.0			
25	OH	H	H	H	SO_2Me	>100	>10.0	>10.0	>10.0			
26	OH	H	H	H	CN	>100	3.2	5.5	9.4	14.0	>11	>7.1
27	OH	H	H	H	CO_2Me	15.0	>10.0		>10.0			
28	OH	H	H	H	CF_3	>100	3.8	9.1	11.0	27.0	>9.1	>3.7
29	EtO_2CO	H	H	H	NO_2	9.9	0.21	0.54	0.69	1.80	13	5.5

[a] All abbreviations and definitions as in Table 1. See Table 1 for experimental details.

Table 3

Activities of salicoyl anilides against HBV replication.



Compound	R_1	R_2	R^3	R^4	R_5	CC_{50}[a] µM	EC_{50} (VIR) µM	EC_{50} (RI) µM	EC_{90} (VIR) µM	EC_{90} (RI)µM	SI (VIR)	SI (RI)
30	Ac	H	H	Cl	H	>100	3.50		9.7		11	
31	H	H	H	Cl	H	>100	>10		>10			
32	Ac	H	H	Br	H	>100	0.76	2.10	3.00	6.80	>33	>15
33	Ac	H	H	I	H	>100	0.20	0.73	1.20	3.30	>83	>30
34	H	H	H	NO_2	H	>100	>10		>10			
35	H	Cl	H	H	CF_3	>100	3.70		13.0			
36	Ac	H	CF_3	H	CF_3	>100	3.80		14.0		>7.1	
37	H	Me	H	NO_2	Cl	89.0	0.28	0.66	0.73	1.7	122	52
Niclosamide						>100	>10.0		>10.0			

[a] All abbreviations and definitions as in Table 1. See Table 1 for experimental details.

Table 4

QSAR models and their performance statistics for the pEC_{90} RI (M) end point, as determined by GA-MLR using different fitness functions for subjective descriptor selection.

Subjective Descriptor Selection Fitness Function			
	Model01A r^2_{adj}	**Model01B r^2_{adj} in LOO validation**	**Model01C r^2_{adj} in bootstrap validation**
Model activity (EC90 RI) =	21.31-1.85(IDDE)−3.30(BEHp7) −23.63(ATSc3)	21.31-1.85(IDDE)−3.30(BEHp7) −23.63(ATSc3)	21.31-1.85(IDDE)−3.30(BEHp7) −23.63(ATSc3)
r^2	0.923	0.923	0.923
r^2_{adj}	0.904	0.904	0.904
MAE	0.088	0.088	0.088
S.D of regression	0.131	0.131	0.131
F-value	28.046	28.046	28.046
Av. Fold error	1.013	1.013	1.013
r^2_{LOO}	0.767	0.767	0.767
r^2_{LMO}	0.763	0.763	0.763
r^2_{BS}	0.528	0.528	0.528

Table 5

QSAR models and their performance statistics for the pEC_{90} VIR (M) end point, as determined by GA-MLR using different fitness functions for subjective descriptor selection.

Subjective Descriptor Selection Fitness Function			
	Model02A r^2_{adj}	**Model02B r^2_{adj} in LOO validation**	**Model02C r^2_{adj} in bootstrap validation**
Model activity (EC_{90} RI) =	15.27-1.53(BEHe8) −70.97(BEHp7) +0.50(khs.sCl)	4.37+1.85(X5sol) −15.27(VC-5)	74.81− 63.96(MATS6m) +31.83(ATSc2)
r^2	0.721	0.415	0.551
r^2_{adj}	0.666	0.361	0.511
MAE	0.185	0.288	0.231
S.D of regression	0.273	0.376	0.329
F-value	7.762	3.540	6.153
Av. Fold error	1.035	1.053	1.043
r^2_{LOO}	0.443	0.125	0.281
r^2_{LMO}	0.436	0.109	0.270
r^2_{BS}	−1.200	−1516.856	0.041